The Metabolism of Griseofulvin in Mammals

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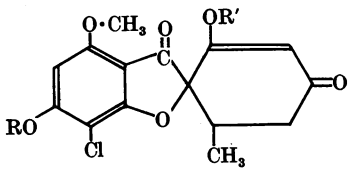
Griseofulvin [7-chloro-4:6:2′-trimethoxy-6′-methylgris-2′-en-3:4′-dione (I:R, R′, Me)], a product of *Penicillium griseofulvum* Dierckx, was first isolated by Oxford, Raistrick & Simonart (1939): its structure was first established by Grove, Ismay, MacMillan, Mulholland & Rogers (1951). This compound has fungistatic activity and was reported to control experimental ringworm in guinea pigs (Gentles, 1958) and in calves (Lauder & O'Sullivan, 1958) when administered orally.



(I)

Reports of its successful use in the treatment of human ringworm infections came from Williams, Marten & Sarkany (1958), Blank & Roth (1959) and Riehl (1959). During work on the distribution and excretion of [^{36}Cl]griseofulvin after oral administration, we found that a large amount of radioactive material was excreted in the urine of test animals, but that most of this was biologically inactive and only a very small percentage of it griseofulvin. The major metabolite present in the urine has been characterized as 7-chloro-6-hydroxy-4:2′-dimethoxy-6′-methylgris-2′-en-3:4′-dione (I:R, H; R′, Me) (6-demethylgriseofulvin).

EXPERIMENTAL

Material. [^{36}Cl]Griseofulvin was prepared by fermentation in a medium containing K^{36}Cl. Its specific activity was 2·36 μc/m-mole, and it gave 4240 counts/min./mg. on a thin-window Geiger–Müller counter. Samples were plated on 1 cm.^{2} planchets; as great accuracy was not essential, samples of extracts and column eluates were directly applied to the planchets, which were then dried in an oven before counting. All samples were prepared by plating less than 15 mg. of material; below this weight self-absorption errors were negligible. All results, unless otherwise stated, were obtained by this method, but have been expressed as percentages of the original activity.

Paper chromatography. The mobile solvent system used was the organic phase of benzene–*cyclo*hexane–methanol–water (5:5:6:4, by vol.) to which 0·5% of acetic acid had been added after separation. When run on Whatman no. 1 paper by descending chromatography, griseofulvin appeared as a bright fluorescent spot under ultraviolet light R_F 0·9, and the material subsequently shown to be 6-demethylgriseofulvin appeared as a darker-blue fluorescent spot, R_F 0·15. Chromatograms were run for 6 hr. at 24°.

Spectrometry. All ultraviolet spectra were determined in 80% ethanol to which was added 1% (v/v) of either 2N-HCl or 2N-NaOH. Infrared spectra were determined in $CHBr_3$, with a Perkin–Elmer model 21 instrument with a NaCl prism.

Extraction of urine

Rabbit. Two rabbits (total wt. 4 kg.) were each given a single oral dose of 500 mg. of [^{36}Cl]griseofulvin/kg., prepared by diluting our [^{36}Cl]griseofulvin 1 in 20 with non-radioactive griseofulvin, and their urine was collected. The urine excreted in the first 24 hr. after dosing (bulked volume 150 ml.) contained 11·2% of the radioactivity administered and the 24–48 hr. urine (310 ml.) contained 24%. The 24–48 hr. urine at pH 6·5 was exhaustively extracted with 300 ml. volumes of butan-1-ol, which removed 85% of the radioactive material. Subsequent paper chromatography of the aqueous residue showed no trace of 6-demethylgriseofulvin. A dark sticky solid (4·8 g.) was obtained on evaporation of the combined butan-1-ol extracts; this was extracted with $CHCl_3$, first by heating under a reflux condenser and then in a Soxhlet extractor. By these methods 92% of the radioactivity was taken into the $CHCl_3$, leaving behind about 3·8 g. of solid. The combined $CHCl_3$ extracts were concentrated to 5 ml. and run through a column of 30 g. of silica gel. (British Drug Houses Ltd.) prepared from a $CHCl_3$ slurry. The fractions from this column, which removed almost all the coloured impurities, were each checked for amount of radioactivity, and fractions 1 and 2, eluted from the column with 75 ml. and 50 ml. of $CHCl_3$ respectively, contained 70% of the input. The solid in these fractions was dissolved in a small volume of ethanol and precipitated as crystals by addition of benzene to the hot solution. These crystals (235 mg.) had approximately the same specific activity as the original [^{36}Cl]griseofulvin. Paper chromatography on further fractions from this column, eluted with ethanol–$CHCl_3$ (1:1, v/v) and finally with ethanol, showed that they contained small quantities of the same material, although they could not be crystallized. The crystalline material from the column was recrystallized from ethanol–2N-acetic acid (1:8, v/v) and then from ethyl acetate–light petroleum (b.p. 60–80°) until a constant m.p., 273–5° (decomp.) uncorr., was reached (Found: C, 57·0; H, 4·5; OMe, 18·4. $C_{16}H_{15}O_6Cl$ requires C, 56·7; H, 4·4; OMe, 18·3%.) Light-absorption max. in acid soln. 235·5 and 293·5 mμ

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(log ϵ 4·41 and 4·44 respectively); in alkaline soln. 249·5 and 330 mμ (log ϵ 4·42 and 4·58 respectively).

Rat. Rats, given oral doses of 100 mg. of [^{36}Cl]griseofulvin/kg., excreted in the urine 10·4% of the radioactivity administered in the first 24 hr. and 4·2% in the next 24 hr. The 0–24 hr. urine (300 ml. from 24 rats) adjusted to pH 5 with 0·1 N-HCl, was extracted with 3 × 0·33 vol. of $CHCl_3$. The combined extracts contained 65% of the original radioactivity of the urine, only 4% being present in the final extract. As in the extraction of rabbit urine, the $CHCl_3$ extract was purified on a silica-gel column, and a crystalline solid was obtained from the first two fractions. These crystals (15 mg.) had the same ultraviolet spectra and R_F as the crystalline material from rabbit urine and gave no depression of melting point when mixed with it. A portion of the $CHCl_3$ extracts was examined by paper chromatography. The spot corresponding to 6-demethylgriseofulvin, when cut out from the paper and counted, was equivalent to only about 45% of the original urine activity.

Human urine. One day's urine (pH 4·5, 1260 ml.) from a patient, taking a daily oral dose of 1 g. of griseofulvin, was extracted with 3 × 600 ml. of $CHCl_3$. These extracts were purified on a silica-gel column and crystallized as before; 105 mg. of crude crystals having the same properties as 6-demethylgriseofulvin were obtained.

To check that the 6-methylgriseofulvin did in fact appear free in the urine, samples of the fresh urine from the three species were chromatographed and gave a 6-demethylgriseofulvin spot which was radioactive. In all urine samples small amounts of griseofulvin were detected, but always less than 1% of the dose administered.

Demethylation of griseofulvin by rat-liver slices

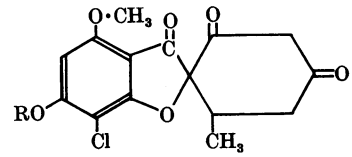
Rat-liver slices (3 g.) were incubated at 37° in 150 ml. of Krebs bicarbonate solution (6·9 g. of NaCl, 0·35 g. of KCl, 0·28 g. of anhydrous $CaCl_2$, 2·1 g. of $NaHCO_3$ and 0·16 g. of anhydrous KH_2PO_4 made up to 1 l. with water) containing 30 μg. of griseofulvin/ml.; $O_2 + CO_2$ (19:1) was bubbled through the solution continuously. After 4 hr. the mixture was filtered through cotton wool and the filtrate was acidified to pH 1·5 with 2 N-HCl. It was then extracted with 3 × 1 vol. of ethyl acetate, and the extract dried over anhydrous Na_2SO_4 and concentrated to 4 ml. When a portion of this was run on a paper chromatogram, spots corresponding to both griseofulvin and 6-demethylgriseofulvin were observed. The remainder of the material in the ethyl acetate was transferred to 80% ethanol and showed the same change in ultraviolet spectrum in acid and alkaline solution as 6-demethylgriseofulvin.

Preparation of griseofulvin from 6-demethylgriseofulvin

6-Demethylgriseofulvin (5 mg.) was treated with a solution of diazomethane in ether. After 2 hr. the ether and excess of diazomethane were evaporated off and the residual solid was recrystallized from ethanol. The crystals had the same R_F as griseofulvin and showed no depression of melting point when mixed with an authentic sample.

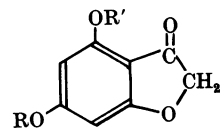
DISCUSSION

Structure of 6-demethylgriseofulvin (I: R, H; R′, Me). Microanalysis showed that this compound differed only by one carbon and two hydrogen atoms from griseofulvin; that a methyl group had been replaced by hydrogen at one of the methoxyl groups was confirmed by conversion of the material into griseofulvin by reaction with diazomethane. Of the three possible monodemethylgriseofulvins only griseofulvic acid (II: R, Me) was known (Grove, MacMillan, Mulholland & Rogers, 1952), and

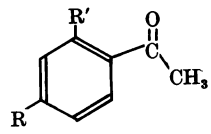


(II)

this material differed in m.p. and ultraviolet spectrum from the compound extracted from urine. Characterization of the excretion product has been obtained from spectroscopic evidence. Duncanson, Grove, MacMillan & Mulholland (1957) found that the methyl ether linkage in position 6 of griseofulvin was more labile to aqueous alkali than that in position 4. These workers found in the spectra of a series of hydroxycoumaranones (III) and Cram & Crantz (1950) found in a series of hydroxyacetophenones (IV) that in compounds with a hydroxyl



(III)



(IV)

group *para* to the carbonyl (III: R, H; R′, Me; IV: R, OH; R′, H) the K band of the anion occurs at a longer wavelength and is more intense than that of the undissociated molecule, whereas when the hydroxyl group is *ortho* to the carbonyl (III: R, Me; R′, H; IV: R, H; R′, OH) very little change in the K band takes place (Table 1). Duncanson *et al.* (1957) found that the infrared spectrum of 4-hydroxy-6-methoxycoumaranone (III: R, Me; R′, H) has a carbonyl frequency 18 cm.$^{-1}$ lower than that of 4:6-dimethoxycoumaranone (III: R, R′, Me), that of 6-hydroxy-4-methoxycoumaranone (III: R, H; R′, Me) being only 2 cm.$^{-1}$ lower. The carbonyl frequency for the metabolite of griseofulvin is only 1 cm.$^{-1}$ lower than that for griseofulvin. The spectra of the excretion product therefore are in accord with the structure 6-demethylgriseofulvin (I: R, H; R′, Me).

Metabolism of griseofulvin. From the results summarized in Table 2 it is clear that 6-demethylgriseofulvin is the major identified metabolite. It is possible that some of the material remaining in

Table 1. *Comparison of the ultraviolet spectra of model compounds and the urine metabolite*

Compound	Acid solution $\lambda_{max.}$ (mμ)	Acid solution log ε	Alkaline solution $\lambda_{max.}$ (mμ)	Alkaline solution log ε
(III): R, Me; R′, H	280	4·31	285	4·35
(III): R, H; R′, Me	284	4·35	318	4·53
(IV): R, H; R′, OH	252	3·97	257	3·76
(IV): R, OH; R′, H	277	4·16	325	4·36
(II): R, H	292	4·30	327	4·65
(I): R, Me; R′, Me	293	4·39	293	4·39
Urine metabolite	293·5	4·44	330	4·58

Table 2. *Summary of metabolism of griseofulvin*

Species	Dose (mg./kg. body wt.)	Period of urine collection (hr.)	Per cent of dose excreted in urine: Total	As 6-demethyl-griseofulvin	Unchanged
Rabbit	500	0–48	35·2	27·5	< 1
Rat	100	0–48	14·6	6·5	< 1
Man	17*	24	—	10·5	—

* This dose had been taken daily for 30 days.

the urine after exhaustive extraction with butanol or chloroform may be conjugated either as the glucuronide or sulphate of this metabolite.

The metabolic fate of some substituted aromatic alkyl ethers is known to involve a preliminary dealkylation. For example, substituted anisoles (Bray, Craddock & Thorpe, 1955) are converted into substituted phenols in the body. This work has been extended by Axelrod (1956), who has shown that the dealkylation taking place in the liver is caused by an enzyme system found in the microsomes of liver cells, and that for full activity this microsomal system requires the presence of reduced triphosphopyridine nucleotide and oxygen. That this cleavage takes place by an oxidative mechanism was demonstrated by the formation of formaldehyde. Of all the substituted anisoles studied in these experiments, the rate of dealkylation of *p*-methoxybenzaldehyde was among the greatest and of the three methoxybenzoic acids tested *p*-methoxybenzoic acid was metabolized about 20 times as fast as the *o*-isomer. These results help to confirm the structure proposed for the metabolite of griseofulvin. Previously, only quantitative work has been reported on the metabolism of griseofulvin (Bedford *et al.* 1960). These workers demonstrated rapid disappearance of griseofulvin from the blood which they suggested was due to distribution into the tissues followed by metabolic inactivation in the liver.

SUMMARY

1. The urine of animals given oral doses of griseofulvin contains little unchanged griseofulvin. A major metabolite has been characterized as 7-chloro-6-hydroxy-4:2′-dimethoxy-6′-methylgris-2′-en-3:4′-dione (6-demethylgriseofulvin).

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