- Hogeboom, G. H., Schneider, W. C. & Pallade, G. E. (1948). *J. biol. Chem.* **172**, 619.
- Jedeikin, L. A. & Weinhouse, S. (1954). *Arch. Biochem. Biophys.* **50**, 134.
- Kates, M. (1955). *Canad. J. Biochem. Physiol.* **33**, 575.
- Kennedy, E. P. (1953). *J. biol. Chem.* **201**, 399.
- Kennedy, E. P. & Weiss, S. B. (1956). *J. biol. Chem.* **222**, 193.
- Kornberg, A. & Pricer, W. E., jun. (1953). *J. biol. Chem.* **204**, 329.
- Marinetti, G. V., Erbland, J. & Albrecht, M. (1957). *Fed. Proc.* **16**, 216.
- Plimmer, R. H. A. & Burch, W. J. N. (1937). *Biochem. J.* **31**, 398.
- Potter, V. R. & Elvehjem, C. A. (1936). *J. biol. Chem.* **114**, 495.
- Riley, R. F. (1944). *J. Amer. chem. Soc.* **66**, 512.
- Smith, S. W., Weiss, S. B. & Kennedy, E. P. (1957). *J. biol. Chem.* **228**, 915.
- Stadie, W. C. & Riggs, B. C. (1944). *J. biol. Chem.* **154**, 687.
- Stern, I. & Shapiro, B. (1953). *Brit. J. clin. Path.* **6**, 158.
- Szego, C. M. & White, A. (1949). *Endocrinology*, **44**, 150.
- Umbreit, W. W., Burris, R. H. & Stauffer, J. F. (1945). *Manometric Techniques*, 1st ed. Minneapolis: Burgess Publishing Co.
- Wilhelmi, A. E. (1955). In *The Hypophyseal Growth Hormone, Nature and Actions*, p. 59. Ed. by Smith, R. W., Gaebler, O. H. & Long, C. N. H. New York: McCraw-Hill Book Co. Inc.

Biochem. J. (1961) **80**, 34

The Demethylation of Griseofulvin by Fungi

By B. BOOTHROYD, EUNICE J. NAPIER AND G. A. SOMERFIELD
Glaxo Laboratories Limited, Sefton Park, Stoke Poges, Bucks.

(Received 18 November 1960)

As one method of obtaining strains of *Microsporum canis* resistant to griseofulvin [7-chloro-4:6:2'-trimethoxy-6'-methylgris-2'-en-3:4'-dione (I: R, R', R'', Me)] the organism was grown on agar containing griseofulvin at a low concentration. At first the hyphae exhibited typical curling and

alli and *Cercospora melonis* produced griseofulvic acid (I: R, R', Me; R'', H) (Grove, MacMillan Mulholland & Rogers, 1952), and 6-demethylgriseofulvin (I: R, H; R', R'', Me) (Barnes & Boothroyd, 1961) respectively.

distortion (Brian, 1949), but after several days the edges of the giant colony showed normal hyphal growth, owing to the destruction of griseofulvin by the fungus (Aytoun, Campbell, Napier & Seiler, 1960). Biological assay showed that griseofulvin had disappeared from the substrate, and paper chromatography detected the presence of another substance, a new compound that has been characterized as 7-chloro-4-hydroxy-6:2'-dimethoxy-6'-methylgris-2'-en-3:4'-dione (I: R, R'', Me; R', H) (4-demethylgriseofulvin).

In continuation of this work, many other fungi have been tested for their ability to metabolize griseofulvin. Some rapidly destroyed the griseofulvin; from two of them other griseofulvin metabolites were recovered and characterized. *Botrytis*

EXPERIMENTAL

Paper chromatography. The solvent system and methods used have been reported by Barnes & Boothroyd (1961). All the reported compounds gave blue fluorescent spots when paper chromatograms of these compounds were examined under ultraviolet light; R_f values are: griseofulvin, 0.90; 4-demethylgriseofulvin, 0.70; griseofulvic acid, 0.20; 6-demethylgriseofulvin, 0.15.

Biological assay. The griseofulvin content of the various substrates was determined by a serial dilution method with *Alternaria solani* as test organism. Griseofulvin solutions absorbed on filter paper were allowed to diffuse through a cellophan disk placed on the damp filter paper so as to come in contact with germinating *A. solani* spores. End-point concentrations caused the hyphae to exhibit regular curling without stunting.

Spectrophotometry. Ultraviolet spectra were determined in ethanol containing 1% (v/v) of either 2N-HCl or aq. 2N-NH₃ soln. Infrared spectra were determined in CHBr₃ unless otherwise stated, in a Perkin-Elmer model 21 instrument with a NaCl prism.

Materials. The [³⁵Cl]griseofulvin was obtained by fermentation as described in a previous paper (Barnes & Boothroyd, 1961), in which are also described methods of determining the amount of radioactive material. The [³⁵Cl]griseofulvin used had a specific activity of 1.48 μC/m-mole.

Organisms. The following organisms were used: *Alternaria solani*, Boyce-Thompson Institute; *Botrytis allii*, C.M.I. Culture no. 42078; *Cercospora melonis*, Cheshunt Experimental Station; *Microsporium canis*, isolated from human ringworm by Dr J. Gentles, University of Glasgow.

Methods of incubation. With a spore suspension prepared from Sabouraud's malt-agar slopes as inoculum, cultures of the various organisms were prepared in Glaxo mould-culture flasks containing Sabouraud's malt broth (200 ml.). This was prepared by dissolving maltose (40 g.), Evans peptone (10 g.) and Oxoid malt extract (24 g.) in distilled water (1 l.) and adjusting to pH 7. Slopes were prepared from this broth by adding agar (20 g.). After incubation at 27° for several days, the exact time depending upon the organism used, a thick-growing felt of mycelium covered the surface of the broth. The broth was then poured off; after the felts had been washed twice with sterile water, a sterile suspension of griseofulvin (200 ml.) was added to each flask. Two concentrations of griseofulvin suspension were used as a routine, 40 and 100 µg./ml. They were prepared by dissolving griseofulvin in a small volume of acetone and pouring this solution into a large volume of water. The 40 µg./ml. suspension contained a high proportion of the griseofulvin in solution (approx. 25 µg./ml.) and was used in initial tests for metabolism; the 100 µg./ml. suspensions were used for preparing the pure metabolites.

The griseofulvin suspensions were incubated with the different felts for several days, and breakdown of griseofulvin was followed by bioassay and paper chromatography in order to determine the optimum harvest time.

ISOLATION AND IDENTIFICATION OF METABOLITES

4-Demethylgriseofulvin. *Microsporium canis* felts were grown on Sabouraud's malt broth for 9 days before adding the griseofulvin substrate; incubation was then continued for 7 days before harvesting. At harvest the aqueous layer was decanted from the felts and clarified by filtration before extraction with CHCl_3 (2×0.33 vol.). Complete recovery by weight was obtained from 40 µg./ml. suspensions of griseofulvin, but only 60% when 100 µg./ml. suspensions were used. In these experiments the larger particles of griseofulvin were unchanged and were removed during the clarification.

The CHCl_3 extracts from several large-scale incubations were combined and evaporated to dryness, leaving a brown solid (0.27 g.). This material was purified by chromatography on acid alumina (15 g., Woelm) that had been deactivated to Brockmann grade 4 by treatment with water. The crude solid dissolved in benzene (5 ml.) was poured on the alumina column, prepared from a benzene slurry, and eluted with the same solvent (50 ml.). Griseofulvin (5 mg.) was eluted in this fraction. The eluting solvent was then changed to 2% of ethanol in benzene, and more material (162 mg.) was eluted. Further elution with higher concentrations of ethanol and finally pure ethanol gave only small amounts of amorphous solids,

which could not be purified. The solid from the main fraction was first crystallized by adding water to a hot solution of the material in 50% acetic acid and allowing this to cool. After several recrystallizations from a mixture of ethyl acetate and light petroleum (b.p. 60–80°) and finally of methanol and water, a constant m.p. 113–115° (uncorr.) was obtained. The melting point varied greatly with rate of heating; on rapid heating considerably higher values were observed. The figure quoted is that obtained by slow heating on a microscope hot-plate, taking the first breakdown of crystals to a viscous liquid as the melting point (Found: C, 54.3; H, 5.0; Cl, 10.1; OMe, 17.6. $\text{C}_{16}\text{H}_{15}\text{O}_6\text{Cl}\cdot\text{H}_2\text{O}$ requires C, 53.9; H, 4.8; Cl, 10.0; OMe, 17.4%); light-absorption max. for the hydrated crystals in acid soln., 236 and 290 mµ (log ϵ 4.39 and 4.42 respectively); in alkaline soln., 246, 290 and 368 mµ (log ϵ 4.42, 4.35 and 3.88 respectively). The pure material gave a green-brown colour with ethanolic FeCl_3 . Paper chromatograms of the material showed only one blue fluorescent spot corresponding to the one developing in the griseofulvin substrate on incubation with *Microsporium canis*.

The metabolite (5 mg.) was treated with a solution of diazomethane in ether. After 2 hr. the ether and excess of diazomethane were evaporated, and the residual solid was crystallized from ethanol. These crystals showed no depression of melting point when mixed with an authentic sample of griseofulvin. They also had the same ultraviolet-absorption spectrum and the same R_f on paper chromatography as griseofulvin.

Structure of 4-demethylgriseofulvin. Microanalysis, together with the evidence that diazomethane converted this material into griseofulvin, showed that demethylation of one of the three methoxy groups had taken place during its formation. Griseofulvic acid (I: R, R', Me; R'', H) (Grove *et al.* 1952) and 6-demethylgriseofulvin (I: R, H; R', R'', Me) (Barnes & Boothroyd, 1961) both had different melting points and ultraviolet and infrared spectra, so that 4-demethylgriseofulvin (I: R, R'', Me; R', H) is the only possible structure. Corroboration was obtained from infrared spectra in which the peak associated with the carbonyl group in the five-membered ring moved from 1705 cm^{-1} in the griseofulvin spectrum to 1690 cm^{-1} in that of the metabolite. This change of frequency is consistent with the presence of a bonded ketone in the new compound. Duncanson, Grove, MacMillan & Mulholland (1957), using related compounds, report a similar change of frequency (18 cm^{-1}) and also discuss changes in the ultraviolet spectra of these compounds caused by different pH values of the solvent. Reference to their work led to the structure 6-demethylgriseofulvin for the griseofulvin metabolite excreted in the urine (Barnes &

Boothroyd, 1961), since major changes in ultraviolet spectra between acidic and alkaline solution can be predicted for the *p*-hydroxy ketone structure present in this compound. Similarly, little change in the main *K* band would be expected for the *o*-hydroxy ketone structure present in this new griseofulvin metabolite, 4-demethylgriseofulvin.

Griseofulvic acid. *B. allii* felts were grown on Sabouraud's malt broth for 4 days before adding the griseofulvin substrate; incubation was then continued for 6 days before harvest: [^{35}Cl]griseofulvin was used in this experiment.

The solution (5 l., pH 6.3), decanted from the flasks at harvest, was acidified to pH 2.5 and extracted with CHCl_3 (3×1.6 l.). The combined CHCl_3 extracts were concentrated to 100 ml. and extracted with 2*N*-NaOH (3×25 ml.). Evaporation of the CHCl_3 phase produced a brown oil (40 mg.), which was shown by paper chromatography to contain griseofulvin. The combined alkaline extracts were acidified and extracted with CHCl_3 , and the CHCl_3 solution was evaporated to a brown solid (208 mg.). This was extracted with benzene, and the residue, a colourless solid (100 mg.), was crystallized from aqueous methanol to yield colourless needles, m.p. $254-256^\circ$ (decomp.) (uncorr.); $[\alpha]_D^{20} + 384^\circ$ ($c = 0.98$ as sodium salt in aqueous methanol); light-absorption max. in acid soln., 270 and 291 $m\mu$ (log ϵ 4.36 and 4.34 respectively), in alkaline soln. 290 $m\mu$ (log ϵ 4.69). Grove *et al.* (1952) report for griseofulvic acid m.p. $255-258^\circ$ (decomp.) and $[\alpha]_D^{20} + 399^\circ$ ($c = 1.004$ as sodium salt in aqueous methanol). A sample of griseofulvic acid prepared by their method gave identical ultraviolet and infrared spectra (Nujol mull) and had the same R_F (0.2) on paper chromatography as the crystalline material from *B. allii* incubation.

Preliminary work on the distribution of griseofulvin and its metabolites after incubation with *B. allii* felts showed that about 50 % of the radioactive material used was present in the decanted solution; the remainder extracted from the felts with acetone was shown to be unchanged griseofulvin. To check that no other major metabolites were present in the CHCl_3 extract, the benzene wash of the original solid (208 mg.) was evaporated to dryness (103 mg.). This solid was then purified by countercurrent distribution with benzene-methanol-0.002*N*-HCl (2:2:1, by vol.) as solvent system. After 50 transfers the distribution of radioactive material was determined by counting samples from each tube. Only one peak of radioactivity, in tubes 12-27, was observed; this contained 90 % of the input and from it only griseofulvic acid was isolated.

Griseofulvin incubated for 6 days with Sabouraud's medium alone was shown to be unchanged.

6-Demethylgriseofulvin. *C. melonis* felts were grown on Sabouraud's malt broth for 6 days before adding griseofulvin substrate; incubation was then continued for 2-3 weeks before harvest.

The solution (7.4 l.) decanted from the felts was adjusted to pH 2 and extracted with CHCl_3 (4×2 l.). The dried CHCl_3 extract was evaporated to a brown gum (242 mg.), which gave two blue fluorescent spots (R_F 0.15 and 0.90) on paper chromatography. The solid was dissolved in ethyl acetate (60 ml.) and extracted with *N*-NaOH (2×20 ml.); this alkaline solution was acidified and extracted with CHCl_3 ; on evaporation to dryness the extract gave a brown gum (82 mg.), which was purified by chromatography on Whatman 3MM paper with the usual solvent system, although running time was increased to 24 hr. The slow-running constituent appeared as a fluorescent band, which was detected under ultraviolet light. These strips of paper were eluted with acetone for 24 hr. Evaporation of the acetone left a colourless gum (52 mg.), which was purified by crystallization from benzene to give fine colourless needles (8 mg.) m.p. $276-278^\circ$, showing no depression of melting point with an authentic sample of 6-demethylgriseofulvin. Infrared and ultraviolet spectra were also identical with those of the authentic sample.

DISCUSSION

Metabolism of griseofulvin by fungi. During our work it has been demonstrated that the enzymic breakdown of griseofulvin by fungi can give rise to at least three different products, all of them monodemethylgriseofulvins. The amounts of these materials did not account for all the griseofulvin metabolized, but we could not find any other breakdown products among the metabolites of the three organisms used. These demethylated compounds could either be end products of the breakdown of griseofulvin or possibly the first stage of a more extensive degradation to less easily recognized materials. A similar hypothesis to the latter has already been proposed by Wright & Grove (1957) for the decomposition of griseofulvin by a *Pseudomonas* sp. In this case, prolonged incubation gave no recognizable products except chloride ions, suggesting rupture of the aromatic ring, but shorter incubation gave a product thought to be griseofulvic acid.

Wright & Grove (1957) also found that griseofulvin was destroyed by a fungus belonging to the Dematiaceae and present in the soil, but they characterized no breakdown products. Abbot & Grove (1959) reported the slow breakdown of griseofulvin by the two fungi, *B. allii* and *Mucor ramannianus*. Thorough investigation of the

products resulting from the incubation of griseofulvin with *Mucor ramannianus* yielded no recognizable material, although an unidentified phenolic substance, m.p. 142–145°, was isolated from the culture filtrate. From the evidence available, this phenolic substance does not appear to be one of the metabolic products discussed here.

SUMMARY

1. Incubation of griseofulvin with fungi gave rise to various demethylated metabolites. Thus, 7-chloro-4-hydroxy-6:2'-dimethoxy-6'-methylgris-2'-en-3:4'-dione (4-demethylgriseofulvin) was produced by *Microsporum canis*, griseofulvic acid (2'-demethylgriseofulvin) by *Botrytis allii* and 7-

chloro-6-hydroxy-4:2'-dimethoxy-6'-methylgris-2'-en-3:4'-dione (6-demethylgriseofulvin) by *Cerco-sporea melonis*.

REFERENCES

- Abbot, M. T. J. & Grove, J. F. (1959). *Exp. Cell Res.* **17**, 105.
 Aytoun, R. S. C., Campbell, A. H., Napier, E. J. & Seiler, D. A. L. (1960). *A.M.A. Arch. Derm.* **81**, 650.
 Barnes, M. J. & Boothroyd, B. (1961). *Biochem. J.* **78**, 41.
 Brian, P. W. (1949). *Ann. Bot., Lond.*, **13**, 59.
 Duncanson, L. A., Grove, J. F., MacMillan, J. & Mulholland T. P. C. (1957). *J. chem. Soc.* p. 3555.
 Grove, J. F., MacMillan, J., Mulholland, T. P. C. & Rogers, M. A. T. (1952). *J. chem. Soc.* p. 3949.
 Wright, J. M. & Grove, J. F. (1957). *Ann. appl. Bot.* **45**, 36.

Biochem. J. (1961) **80**, 37

The Effect of Glucose and Acetate on Milk Secretion by the Perfused Goat Udder

By D. C. HARDWICK, J. L. LINZELL AND SUSAN M. PRICE
Agricultural Research Council, Institute of Animal Physiology, Babraham, Cambridge

(Received 9 December 1960)

Acetate is now generally accepted as the major energy source for most ruminant tissues. A great deal of evidence, summarized by Folley (1956), suggests that acetate is more important than glucose in the metabolism of the ruminant mammary glands. Not only is acetate directly incorporated into milk fat, but the oxygen consumption of ruminant mammary slices taken during lactation is higher with acetate as substrate than with glucose (Folley & French, 1950). However, recent work in conscious lactating goats (Linzell, 1960) shows that the udder removes on average 10.3 g. of glucose, 3.2 g. of acetic acid, and at least 650 mg. of α -amino acid nitrogen from the blood passing through it for every 100 ml. of milk produced. Compared with the uptake in non-lactating glands, these figures represent a threefold increase in the rate of uptake for acetate but a tenfold increase for glucose. Of the glucose taken up by the lactating gland only 50% can be accounted for as lactose. These considerations suggest an important metabolic function for glucose, apart from its confirmed role in the formation of lactose.

We therefore felt that the influence of acetate and glucose upon mammary metabolism should be re-examined. It has not been possible to do this satisfactorily either in the whole animal or with

tissue slices. In the whole animal, glucose or acetate cannot be eliminated entirely, and, in the tissue slice, it is not easy to measure the amount and composition of milk secreted. These are both possible in an isolated perfused mammary gland and we have used the perfused goat's udder for this purpose. In our experiments the glands are removed under spinal anaesthesia with an anoxic period of only a few minutes and perfused with a simplified perfusate of known composition. With this preparation we have shown that for the first 2 hr. milk is formed from endogenous substrates, but after that it is only produced if a substrate mixture is run into the perfusate continuously at a rate appropriate to the amount of milk being formed. The mixture used contains glucose, acetate, amino acids and minerals in the proportions that are removed from the blood by the lactating goat's udder *in vivo*. In this paper we report the effects of varying the proportion of acetate and glucose in this mixture, leaving the other components constant.

METHODS

The method of perfusion (Hardwick & Linzell, 1960) included the modifications mentioned to maintain a high blood flow throughout perfusion and the incorporation of