MOLECULAR PHARMACOLOGY, 19:477–480

MOL PHARM

Comparison of the Effects of Griseofulvin and 3,5-Diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine on Ferrochelatase Activity in Chick Embryo Liver

SUSAN P. C. COLE, DAVID T. ZELT, AND GERALD S. MARKS

Department of Pharmacology, Queen's University, Kingston, Ontario, Canada K7L 3N6

Received July 29, 1980; Accepted January 27, 1981

SUMMARY

COLE, S. P. C., D. T. ZELT, AND G. S. MARKS. Comparison of the effects of griseofulvin and 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine on ferrochelatase activity in chick embryo liver. *Mol. Pharmacol.* **19**:477–480 (1981).

The effects of griseofulvin and 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine (DDC) on ferrochelatase activity were compared in the 17-day-old chick embryo. While DDC reduced enzyme activity markedly, griseofulvin did not exert an inhibitory effect. These results are consistent with the fact that DDC, but not griseofulvin, causes hepatic porphyrin accumulation in this species. The effects of griseofulvin and DDC on ferrochelatase activity were compared in chick embryo liver cell culture. DDC reduced ferrochelatase activity markedly while griseofulvin caused no significant inhibition. Both DDC and griseofulvin cause marked porphyrin accumulation in this system. The porphyrins were separated and quantitated using high-performance liquid chromatography. Protoporphyrin was the major porphyrin which accumulated in response to DDC, a result consistent with the inhibition of ferrochelatase. In contrast, protoporphyrin was a minor porphyrin accumulating in response to griseofulvin, a result consistent with the failure of griseofulvin to inhibit ferrochelatase; in this case, coproporphyrin was the major porphyrin to accumulate. These findings in the chick embryo contrast with those reported in rodents, where both DDC and griseofulvin exert an inhibitory effect on ferrochelatase. Clearly, species differences exist in the effects of griseofulvin on the enzymes of the heme biosynthetic pathway.

INTRODUCTION

There are several mechanisms by which porphyrinogenic compounds disrupt the regulation of the heme biosynthetic pathway. Of all the drugs known to cause porphyrin accumulation, DDC[1] and the antifungal agent griseofulvin have been reported to inhibit the terminal enzyme of the pathway, ferrochelatase (EC 4.99.1.1), in rats and mice (1, 2). It has been proposed that this results in decreased heme formation, diminished heme-mediated feedback repression of the rate-limiting enzyme, ALA synthetase (EC 2.3.1.37) followed by increased ALA synthetase activity leading to an accumulation of porphyrins (3). We have recently shown that DDC is a potent inhibitor of ferrochelatase in the intact 17-day-old chick embryo (4), a result which is in accordance with that reported by Anderson (5) and Rifkind (6). We have previously shown that griseofulvin, unlike DDC, does not cause porphyrin accumulation in the 17-day-old chick embryo (7), and it was of interest to determine the effect of griseofulvin on ferrochelatase activity in this species. Therefore, the first objective of this study was to determine whether griseofulvin inhibits ferrochelatase in the intact chick embryo as it has been reported to do in rats and mice (2).

In contrast to the intact 17-day-old chick embryo, both griseofulvin and DDC are highly active porphyrin-inducing agents in the 17-day-old chick embryo liver cell culture system (8). Our second objective was to determine whether DDC and griseofulvin inhibit ferrochelatase in chick embryo liver cell cultures.

METHODS

Source of compounds. Waymouth MD 705/1 medium was obtained in powder form from the Grand Island Biological Company, Grand Island, N. Y. Insulin (bovine pancreas, 23.6 IU/mg), L-thyroxine sodium pentahydrate (T_4), penicillin G sodium, streptomycin sulfate, and griseofulvin were purchased from Sigma Chemical Company, St. Louis, Mo. DDC was prepared previously in our laboratory by the procedure of Loev and Snader (9).

This work was supported by the Medical Research Council of Canada.

[1] The abbreviations used are: DDC, 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine; ALA, δ-aminolevulinic acid; DMSO, dimethyl sulfoxide.

0026-895X/81/030477-04$02.00/0

Copyright © 1981 by The American Society for Pharmacology and Experimental Therapeutics.
All rights of reproduction in any form reserved.

MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Universidade do Estado do Rio de Janeiro on December 6, 2012

MOL PHARM

MOLECULAR PHARMACOLOGY

Free porphyrins and porphyrin methyl esters were purchased from Porphyrin Products, Logan, Utah.

Effects of DDC and griseofulvin on ferrochelatase activity, ALA synthetase activity, and porphyrin accumulation in the intact 17-day-old chick embryo. DDC (10 μg) or griseofulvin (8 mg) dissolved in DMSO (0.1 ml) was injected into 17-day-old chick embryos through a small hole in the eggshell above the air sac. Controls received DMSO (0.1 ml). The embryos were killed 10 hr later. In a second series of experiments, griseofulvin (8 mg) dissolved in DMSO (0.1 ml) was administered to the embryos at 48 hr and then again at 24 hr before the animals were killed. Controls received DMSO (0.1 ml) alone at each time period.

For the determination of ferrochelatase activity, the livers of eight similarly treated animals were pooled. A solubilized ferrochelatase preparation was obtained and ferrochelatase activity was measured by a modification of the pyridine hemochromogen method as described previously (4, 10). The spectrum of the reduced minus oxidized pyridine mesohemochromogen was recorded from 600 to 500 nm on a Cary 219 ultraviolet spectrophotometer. Activity was calculated as nanomoles of mesoheme formed per milligram of protein per 10 min and results are expressed as a percentage of control activity. ALA synthetase and porphyrin accumulation were estimated in single livers as described previously (11).

Effect of DDC and griseofulvin on ferrochelatase activity in 17-day-old chick embryo liver cell cultures. The details of the cell culture technique have been described previously (12, 13). The cells were maintained in serum-free Waymouth MD 705/1 medium supplemented with 60 mg of penicillin G, 100 mg of streptomycin sulfate, 1 mg of insulin, and 1 mg of thyroxine per liter.

For the assay of ferrochelatase activity, the cells were maintained in 10-cm diameter disposable plastic dishes containing 15 ml of the medium. After an initial incubation of 24 hr, the medium was discarded and replaced with fresh medium and the drugs were added. DDC (5 μg/ml of medium) was dissolved in 95% ethanol (30 μl) for addition to the 10-cm diameter dishes. Controls received 95% ethanol (30 μl). Griseofulvin (20 μg/ml of medium) was dissolved in DMSO (30 μl) and controls received DMSO (30 μl). The cells were reincubated for 24 hr after addition of the drugs. The medium was then discarded and 5.0 ml of a solution containing 0.25 M sucrose, 0.05 M Tris-HCl, and 1 mM EDTA at pH 8.2 were added to each dish. The cells were removed from the dish by scraping with a rubber policeman and the cells from two similarly treated dishes were pooled and centrifuged (500 × *g*) for 5 min. The cells were washed once with 2.0 ml of the sucrose/Tris buffer and the cell pellet was suspended in 2.0 ml of ice-cold 0.02 M Tris-HCl buffer, pH 8.2. The suspension was homogenized using a Polytron homogenizer, and the ferrochelatase activity of a 0.8-ml aliquot of the homogenate was measured by the pyridine hemochromogen method, using mesoporphyrin and iron as substrates as described previously (4). Activity was calculated as nanomoles of mesoheme formed per milligram of protein per 10 min and results are expressed as a percentage of control activity.

Porphyrin profiles produced by DDC and griseofulvin in chick embryo liver cell cultures. For the determination of porphyrin profiles by high-performance liquid chromatography, the cells were maintained in 6-cm diameter disposable plastic dishes containing 5 ml of the medium. After an initial incubation period of 24 hr, the medium was discarded and replaced with fresh medium and the drugs were added. DDC (5 μg/ml of medium) and griseofulvin (20 μg/ml of medium) were dissolved in acetone (10 μl) for addition to 6-cm diameter dishes. Controls received 10 μl of acetone. The cells were reincubated for 24 hr after addition of the drugs. The contents of each dish were then lyophilized and esterified with 5% sulfuric acid in methanol for 24 hr at −15°. Chloroform was added to each sample followed by water. The aqueous layer was washed with chloroform and the pooled chloroform extracts were washed with 5% sodium bicarbonate, water, and then dried over anhydrous sodium sulfate. The samples were evaporated to dryness under nitrogen at 37°C. The residue was taken up in chloroform and the porphyrin methyl esters were separated and quantitated by the second-derivative high-performance liquid chromatography method of Zelt *et al.* (14). Results were expressed as a percentage of total porphyrins produced .

Estimation of protein. Protein was assayed by the method of Lowry *et al.* (15) as modified by Miller (16).

RESULTS

Intact 17-day-old chick embryo. The effect of DDC and griseofulvin on ferrochelatase activity in 17-day-old chick embryos 10 hr after drug administration is shown in Fig. 1. While DDC (10 μg/egg) reduced ferrochelatase activity to less than 10% of control values, griseofulvin (8 mg/egg) was without effect on the enzyme. Administration of 8 mg of griseofulvin per egg in DMSO (0.1 ml)

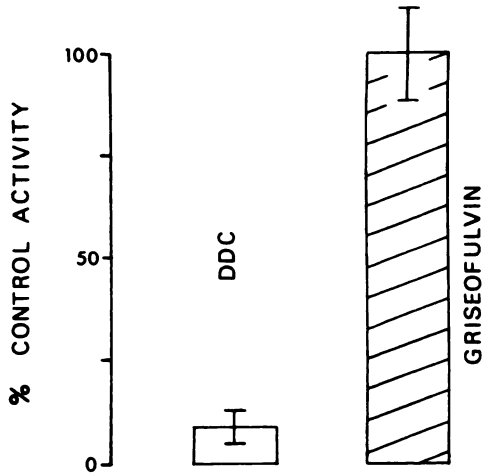

FIG. 1. *Percentage of control ferrochelatase activity in chick embryo livers 10 hr after administration of DDC (10 μg/egg) (open bar) and griseofulvin (8 mg/egg) (hatched bar)*

In the DDC experiments, control activity was 1.79 nmoles of mesoheme per milligram of protein per 10 min. In the griseofulvin experiments, control activity was 1.46 nmoles of mesoheme per milligram of protein per 10 min. Each bar represents the mean (± standard error of the mean) of three determinations.

Downloaded from molpharm.aspetjournals.org at Universidade do Estado do Rio de Janeiro on December 6, 2012

resulted in an increase in ALA synthetase activity (72.9 ± 22.7 nmoles of ALA per 100 mg of protein per hour) as compared with controls (8.0 ± 0.8 nmoles of ALA per 100 mg of protein per hour). Only a slight increase in porphyrins was observed. Porphyrin levels were 0.26 ± 0.04 μg/100 mg of protein in griseofulvin-treated embryos as compared with 0.14 ± 0.02 μg/100 mg of protein in control embryos.

Following administration of griseofulvin (8 mg) at 48 hr and again at 24 hr prior to killing the animals, no significant reduction in ferrochelatase activity was noted, and no elevation of porphyrin levels was detected; ALA synthetase activity was 79.1 ± 8.0 nmoles of ALA per 100 mg of protein per hour as compared with control activity of 22.3 ± 1.4 nmoles of ALA per 100 mg of protein per hour.

Chick embryo liver cell cultures. Ferrochelatase activity in 17-day-old chick embryo liver cell cultures was measured in whole homogenates of the cells and was found to be approximately linear with respect to time for 10 min. The effect of DDC and griseofulvin on ferrochelatase activity in chick embryo liver cell cultures 24 hr after addition of the drugs is shown in Fig. 2. DDC at a concentration of 5 μg/ml of medium reduced ferrochelatase activity to about 20% of control values. Griseofulvin at a concentration of 20 μg/ml of medium caused no significant inhibition of ferrochelatase.

The porphyrin profiles produced by DDC and griseofulvin are shown in Fig. 3. After DDC administration (5 μg/ml of medium), protoporphyrin accounted for about 79% of the total porphyrin while most of the remainder was coproporphyrin. The pattern of porphyrin accumulation obtained after the addition of griseofulvin (20 μg/ml of medium) was markedly different (Fig. 3). Corproporphyrin was the major porphyrin to accumulate (about 49%) while only about 7% of the total porphyrin was protoporphyrin. Smaller amounts of pentacarboxylic, hexacarboxylic, heptacarboxylic, and uroporphyrins were present.

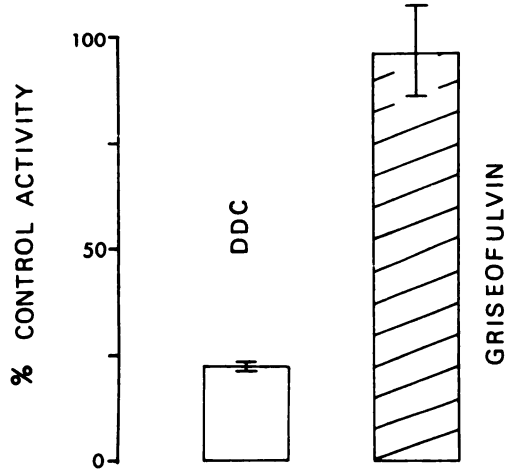

FIG. 2. *Percentage of control ferrochelatase activity in chick embryo liver cell cultures 24 hr after administration of DDC (5 μg/ml of medium) (open bar) and griseofulvin (20 μg/ml of medium) (hatched bar)*

In the DDC experiments, control activity was 2.54 nmoles of mesoheme per milligram of protein per 10 min. In the griseofulvin experiments, control activity was 3.26 nmoles of mesoheme per milligram of protein per 10 min. Each bar represents the mean (± standard error of the mean) of three determinations.

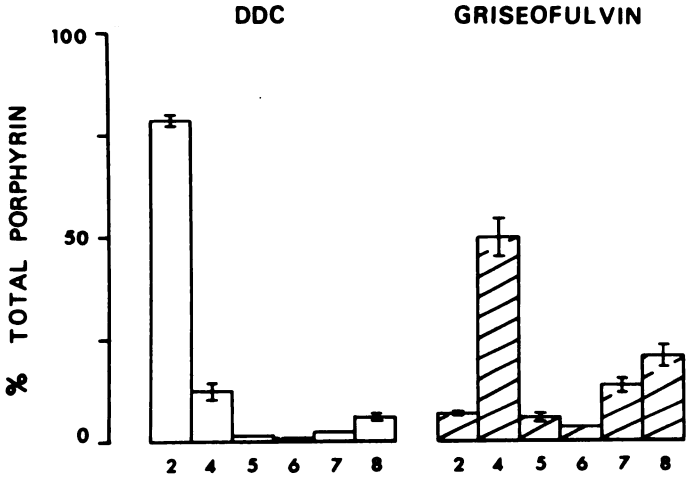

FIG. 3. *Porphyrin profiles produced by DDC (5 μg/ml of medium) (open bars), and griseofulvin (20 μg/ml of medium) (hatched bars) in chick embryo liver cell culture*

Numbers under the bars indicate the number of carboxyl groups (*8*, uroporphyrin; *7*, heptacarboxylic porphyrin; *6*, hexacarboxylic porphyrin; *5*, pentacarboxylic porphyrin; *4*, coproporphyrin; *2*, protoporphyrin). Each bar represents the mean (± standard error of the mean) of at least 14 determinations.

DISCUSSION

De Matteis and co-workers (2, 3) have demonstrated that griseofulvin and DDC produce a marked dimunition of mouse hepatic ferrochelatase. An analogous but less marked effect was produced by these chemicals on rat liver ferrochelatase. It was proposed that inhibition of the enzyme results in decreased heme formation and therefore increased ALA synthetase activity and porphyrin accumulation (1, 3). Tephly *et al.* (17) and De Matteis and co-workers (18, 19) have shown that, when DDC and griseofulvin are injected into rodents, these drugs lead to the accumulation, apparently from heme breakdown, of green porphyrin-like pigments which exert an inhibitory effect on ferrochelatase. They have shown that the inhibitory green pigments closely resemble *N*-monomethylporphyrins and suggest that they bind to the active site of ferrochelatase, thus interfering with the normal incorporation of iron into protoporphyrin.

In view of the results described above, it is curious that in the 17-day-old chick embryo, DDC causes marked porphyrin accumulation while griseofulvin is inactive (7). Despite its inability to cause porphyrin accumulation, griseofulvin was found in the present study to produce a moderate elevation of ALA synthetase activity. The inability of griseofulvin to cause hepatic porphyrin accumulation relative to DDC is understandable in light of the results of the ferrochelatase inhibition experiments (Fig. 1). In these experiments, griseofulvin (8 mg/egg) did not affect ferrochelatase activity while DDC (10 μg/egg) reduced the enzyme to less than 10% of control values. The dose of griseofulvin selected in this study (viz., 8 mg/egg) was the maximal possible dose due to the limited solubility of the drug. The dose of DDC selected, viz., 10 μg/egg, had been found in a previous

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Universidade do Estado do Rio de Janeiro on December 6, 2012

study (4) to produce maximal inhibition of ferrochelatase. It has been shown that rodents require a considerably longer period of exposure to griseofulvin than to DDC in order to elicit ferrochelatase inhibition (2, 18). For this reason griseofulvin (8 mg) was administered to the chick embryo 48 hr and 24 hr prior to sacrifice. In spite of this increase in dose and in duration of exposure to griseofulvin, no significant decrease in ferrochelatase activity nor elevation of porphyrin levels was noted. It is noteworthy that DDC (10 μg/egg) produced a maximal decrease in ferrochelatase activity as early as 0.5 hr after adminstration to the chick embryo (4). It was therefore concluded that, unlike the results reported in rats and mice, griseofulvin does not inhibit ferrochelatase activity in the 17-day-old chick embryo.

In contrast to the results observed in the 17-day-old chick embryo, both DDC and griseofulvin are potent porphyrin-inducing agents in the chick embryo liver cell culture system (8). It was thus anticipated that inhibition of ferrochelatase activity would be observed with both DDC and griseofulvin in this system. We were therefore surprised to find that, while DDC (5 μg/ml of medium) reduced ferrochelatase activity to 20% of control values, griseofulvin (20 μg/ml of medium) caused no significant inhibition of the enzyme (Fig. 2). The doses of DDC and griseofulvin selected for use in these inhibition experiments were known to produce maximal elevation of porphyrin levels in the chick embryo liver cell culture.[2]

Blockade of heme synthesis by inhibition of ferrochelatase is thought to explain the large amounts of protoporphyrin which are observed in DDC and griseofulvin porphyria in mice (1, 20). In a similar manner, hexachlorobenzene-mediated inhibition of uroporphyrinogen decarboxylase is consistent with the accumulation of uroporphyrin (21, 22). Since griseofulvin failed to inhibit ferrochelatase, it was of interest to compare the porphyrins accumulating in response to griseofulvin with those accumulating in response to DDC in the chick embryo liver cells. The results of this study (Fig. 3) show that, as expected, protoporphyrin is the major porphyrin (79%) to accumulate in response to DDC. In contrast, protoporphyrin was found to be only a minor porphyrin (7%) accumulating in response to griseofulvin; this fact is consistent with its inability to inhibit ferrochelatase. The major porphyrin which accumulated in response to griseofulvin was coproporphyrin (49%). There are at least two possible explanations for the porphyrin pattern observed with griseofulvin: (a) it inhibits coproporphyrinogen oxidase and (b) it acts directly to stimulate ALA-synthetase activity and coproporphyrinogen accumulates and is oxidized to the porphyrin when the ability of coproporphyrinogen oxidase to handle the increased flux of intermediates through the pathway is exceeded. Further studies are required to test these two possibilities.

An observation that remains unexplained is the inability of griseofulvin to cause porphyrin accumulation in the intact embryo as compared with its marked activity in chick embryo liver cell culture. This difference between the *in vivo* and *in vitro* systems has been observed with several drugs and has been attributed, at least in part, to the unique pharmacokinetics of the cell culture system; unlike the intact embryo, the isolated cells cannot terminate the action of the drug by redistribution to other organs or by excretion (23).

[2] G. S. Marks, unpublished observations.

REFERENCES

1. Onisawa, J., and R. F. Labbe. Effects of diethyl-1,4-dihydro-2,4,6-trimethylpyridine-3,5-dicarboxylate on the metabolism of porphyrins and iron. *J. Biol. Chem.* **238**:724–727 (1963).
2. De Matteis, F., and A. H. Gibbs. Stimulation of the pathway of porphyrin biosynthesis in the liver of rats and mice by griseofulvin, 3,5-diethoxycarbonyl-1,4-dihydrocollidine and related drugs: evidence for two basically different mechanisms. *Biochem. J.* **146**:285–287 (1975).
3. De Matteis, F., G. Abritti, and A. H. Gibbs. Decreased liver activity of porphyrin-metal chelatase in hepatic porphyria caused by 3,5-diethoxycarbonyl-1,4-dihydrocollidine: studies in rats and mice. *Biochem. J.* **134**:717–727 (1973).
4. Cole, S. P. C., E. J. Vavasour, and G. S. Marks. Drug-induced porphyrin biosynthesis. XIX. Potentiation of the porphyrin-inducing effects of SKF 525-A in the chick embryo liver by 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine, an inhibitor of ferrochelatase. *Biochem. Pharmacol.* **28**:3533–3538 (1979).
5. Anderson, K. E. Effects of antihypertensive drugs on hepatic heme biosynthesis and evaluation of ferrochelatase inhibitors to simplify testing of drugs for heme pathway induction. *Biochim. Biophys. Acta* **543**:313–327 (1978).
6. Rifkind, A. B. Maintenance of microsomal hemoprotein concentration following inhibition of ferrochelatase activity by 3,5-diethoxycarbonyl-1,4-dihydrocollidine in chick embryo liver. *J. Biol. Chem.* **254**:4636–4644 (1979).
7. Racz, W. J., and G. S. Marks. Drug-induced porphyrin biosynthesis. II. Simple procedure for screening drugs for porphyrin-inducing activity. *Biochem. Pharmacol.* **18**:2009–2018 (1969).
8. Granick, S. The induction *in vitro* of the synthesis of δ-aminolevulinic acid synthetase in chemical porphyria: a response to certain drugs, sex hormones and foreign chemicals. *J. Biol. Chem.* **241**:1359–1375 (1966).
9. Loev, B., and K. M. Snader. The Hantzch reaction. I. Oxidative dealkylation of certain dihydropyridines. *J. Org. Chem.* **30**:1914–1916 (1965).
10. Porra, R. J., K. S. Vitols, R. F. Labbe, and N. A. Newton. Studies on ferrochelatase. The effects of thiols and other factors on the determination of activity. *Biochem. J.* **104**:321–327 (1967).
11. Marks, G. S., V. Krupa, and M. W. Roomi. Drug-induced porphyrin biosynthesis. VIII. Investigation of the importance of an allyl group for activity in substituted acetamides *Can. J. Physiol. Pharmacol.* **51**:863–868 (1973).
12. Fisher, P. W. F., R. O. Morgan, V. Krupa, and G. S. Marks. Drug-induced porphyrin biosynthesis in chick embryo liver cells maintained in serum-free Waymouth medium. *Biochem. Pharmacol.* **25**:687–693 (1976).
13. Morgan, R. O., P. W. F. Fisher, J. K. Stephens, and G. S. Marks. Thyroid enhancement of drug-induced porphyrin biosynthesis in chick embryo liver cells maintained in serum-free Waymouth medium. *Biochem. Pharmacol.* **25**:2609–2612 (1976).
14. Zelt, D. T., J. A. Owen, and G. S. Marks. Second-derivative high-performance liquid chromatographic-fluorometric detection of porphyrins in chick embryo liver cell culture medium. *J. Chromatogr.* **189**:209–216 (1980).
15. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Fandall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
16. Miller, G. L. Protein determination for large numbers of samples. *Anal. Chem.* **31**:964 (1959).
17. Tephly, T. R., A. H. Gibbs, and F. De Matteis. Studies on the mechanism of experimental porphyria produced by 3,5-diethoxycarbonyl-1,4-dihydrocollidine. *Biochem. J.* **180**:241–244 (1979).
18. De Matteis, F., and A. H. Gibbs. Drug-induced conversion of liver haem into modified porphyrins. *Biochem. J.* **187**:285–288 (1980).
19. De Matteis, F., A. H. Gibbs, and T. R. Tephly. Inhibition of protohaem ferrolyase in experimental porphyria. *Biochem. J.* **188**:145–152 (1980).
20. De Matteis, F., and C. Rimington. Disturbance of porphyrin metabolism caused by griseofulvin in mice. *Br. J. Dermatol.* **75**:91–104 (1963).
21. Elder, G. H., J. O. Evans, and S. A. Matlin. The effect of the porphyrogenic compound, hexachlorobenzene, on the activity of hepatic uroporphyrinogen decarboxylase in the rat. *Clin. Sci. Mol. Med.* **51**:71–80 (1976).
22. Elder, G. H. Porphyria caused by hexachlorobenzene and other polyhalogenated aromatic hydrocarbons. *Handbook Exp. Pharmacol.* **44**:157–200 (1978).
23. Marks, G. S. The effect of chemicals on hepatic heme biosynthesis. *Handbook Exp. Pharmacol.* **44**:201–237 (1978).

Send reprint requests to: Dr. Gerald S. Marks, Department of Pharmacology, Queen's University, Kingston, Ontario, Canada K7L 3N6.

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Universidade do Estado do Rio de Janeiro on December 6, 2012