Protoporphyrin Hepatopathy

EFFECTS OF CHOLIC ACID INGESTION IN MURINE GRISEOFULVIN-INDUCED PROTOPORPHYRIA

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ABSTRACT Short-term effects of cholic acid ingestion on hepatic accumulation, fecal excretion, and blood levels of protoporphyrin were studied in vivo in griseofulvin-induced protoporphyrin mice. Experimental mice that received feed with 2% griseofulvin and 0.5% cholic acid were compared with control mice that received feed with 2% griseofulvin for 4 wk. Five mice from each group were assessed each week for liver and blood porphyrin levels. Fecal protoporphyrin was compared weekly in the total pooled output of each population. Mean protoporphyrin levels were significantly lower for liver ($P < 0.0001$), erythrocytes ($P < 0.05$), and plasma ($P < 0.05$), and higher for feces ($P < 0.001$) for the mice that were fed cholic acid. Microscopic protoporphyrin deposits, inflammation, necrosis, and dysplasia were more severe in livers of control mice. A second experimental design compared four regimens in the feed given to all mice after 1-wk induction with 2% griseofulvin: (a) 0.5% cholic acid, (b) no adulterant, (c) 2% griseofulvin and 0.5% cholic acid, and (d) 2% griseofulvin. No difference in protoporphyrin removal from livers of mice in groups 1 and 2 was observed after 1 and 2 wk of these regimens. The apparent reduction in hepatic protoporphyrin content in mice of group 3 as compared with group 4 at weeks 2 and 3 was not significant at $P < 0.05$.

These data suggest that in selected circumstances, hepatic protoporphyrin secretion may be enhanced in protoporphyrin disease states by bile salt supplementation.

INTRODUCTION

Cutaneous photosensitivity is no longer considered the most important symptom of human protoporphyrin. The progressive functional deterioration and death of hepatocytes associated with massive crystalline protoporphyrin deposition and cirrhosis are now well-recognized complications of this disease (1-7). Partial deficiency of the enzyme heme synthase (8) results in accumulation of protoporphyrin, a hydrophobic heme precursor. In human protoporphyrina, the majority of the excess protoporphyrin is produced in the eryron; however, variable contributions to the total may derive from disordered hepatic heme synthesis in different patients (9, 10). Protoporphyrin from both sources is excreted by the hepatobiliary-fecal route (11) and undergoes enterohepatic recirculation (12). Patients with protoporphyrin are prone to early cholelithiasis (13).

Apparent reduction of intrahepatic protoporphyrin stores after prolonged ingestion of cholestyramine occurred in two cases (14, 15); however, this has not been confirmed in further clinical trials. Hematin infusion (10, 16) and hypertransfusion (17) may diminish protoporphyrin overproduction, but are not suitable for chronic use in the general population of patients with protoporphyrina.

Addition of bile salts to perfusates that contained protoporphyrin increased both the rate of flow and the protoporphyrin concentration of bile collected from isolated, in situ perfused rat liver preparations (18). Bile salts are used in humans as cathartics, to promote
bile flow after biliary surgery and to reverse gallstone formation (19). Effects of bile salt ingestion have not been studied in human protoporphyrin. It can be hypothesized that such treatment might enhance protoporphyrin secretion by the livers of these patients, and thereby reduce its insidious accumulation and resultant toxicity.

To examine short-term in vivo effects of bile acid supplementation on hepatic protoporphyrin accumulation and secretion, the following studies were performed by using the griseofulvin-induced murine protoporphyrin model.

**METHODS**

Three experiments were performed. In the first experiment, 50 Swiss-Webster 8-wk-old female mice were divided randomly into experimental and control groups. All were made protoporphyrinogenic by feeding ad lib. with powdered feed (Purina Rat Chow, Ralston Purina Co., St. Louis, MO) containing 2% griseofulvin (Schering Corp., Kenilworth, NJ) by weight. Feed for the experimental group was also adulterated with 0.5% cholic acid (Sigma Chemical Co., St. Louis, MO) by weight. At weekly intervals, several mice, usually five, from each group were killed. Each mouse was anesthetized with ether and then exsanguinated by intracardiac puncture into a heparinized syringe to yield 0.5–1.5 ml blood. Plasma and erythrocytes were immediately separated by centrifugation to preclude hemolytic contamination of the plasma. The entire liver was removed, examined grossly, and weighed. A weighed portion (0.15–0.30 g) of each liver was assayed for protoporphyrin content. The entire fecal output of all mice in each group was collected in weekly pools, weighed, homogenized, and assayed for protoporphyrin content. Normal values were determined from similar assays that were carried out by using specimens from four untreated mice and fecal specimens collected three times from untreated murine populations.

At the end of wk 4, three control and two experimental (cholic acid-fed) mice remained. These were fed as previously for one additional week, and the livers were then processed for light and electron microscopic analysis. The pathologist who evaluated these liver specimens was not provided the group assignment identities until after the assessments were completed.

Technical problems were encountered with the erythrocyte and plasma porphyrin assays (a variable pipetting calibration error and hemolysis of several plasma specimens) that invalidated these data during the initial 2–3 wk of the study. 50 additional mice were obtained and the entire experiment was repeated (experiment 2).

In experiment 3, 50 8-wk-old Swiss-Webster albino female mice were all fed ad lib. with 2% griseofulvin (Ayerst Laboratories, New York) for 1 wk. After this induction period, the 50 mice were randomly divided into four groups and each group received a different feed mixture for the subsequent 2 wk. Feed for group 1 contained 0.5% cholic acid, for group 2 contained no adulterant, for group 3 contained both 2% griseofulvin and 0.5% cholic acid, and for group 4 contained 2% griseofulvin.

The liver protoporphyrin content was determined for each of five mice at the end of week 1, and for five mice from each of the groups at the end of weeks 2 and 3. Blood from mice in groups 3 and 4 was used for comparison of serum griseofulvin levels. This was done to exclude the possibility that cholic acid effects which were seen in these experiments could be explained by interference with griseofulvin absorption in cholic acid-fed mice to the extent that hepatic bioavailability of the porphyrin inducer was less in cholic acid-fed mice.

**Assays of erythrocyte and plasma protoporphyrin.** Erythrocyte protoporphyrin was assayed by the method of Piomelli (20). Plasma protoporphyrin was assayed by the same method, which was modified to the use of 200 μl plasma. All assays were performed in duplicate.

**Assay for liver protoporphyrin.** Each preweighed liver portion was homogenized in phosphate-buffered saline pH 7.4 (0.15 M NaCl, 0.1 M Na₂HPO₄, 0.1 M NaH₂PO₄) in an homogenizer (Polytron Corp., Elkhart, IN), and then lyophilized. 5 ml of a mixture of 0.5 N perchloric acid/methanol (1:1 by vol) was added to the residue, and agitated for 30 min in a 37°C water bath to extract the porphyrin. The particulate matter was then pelleted by centrifugation and 20 μl of the clear supernatant was diluted with 10 ml of the same solvent in duplicate. The fluorescence emissions of these solutions were measured in an Aminco fluorometer (American Instrument Co., Inc., Silver Springs, MD) routinely standardized in our laboratories with a working standard of 0.05 μg coproporphyrin I/ml 1.5 N hydrochloric acid. This standard is preferred to protoporphyrin standards because of its greater stability. Excitation light in the 405-nm range was provided by an Aminco 4-7112 primary filter (American Instrument Co., Inc.). Fluorescence emission was detected in the 600-nm range. The calculation for liver protoporphyrin content included conversion constants of 0.64 for the relative fluorences of protoporphyrin to coproporphyrin, and 1.7 to equate the enhanced fluorescence of protoporphyrin in perchloric acid/methanol to that of protoporphyrin in hydrochloric acid in this instrument.

Recovery of protoporphyrin by this method was determined by exhaustive re-extraction of the pellet that remained after the first extraction in 15 specimens. Recovery in the initial extract was 78.7±3.8% (±±SD) of the total extractable porphyrin. Data shown have not been corrected to 100%.

Reproducibility of the assay was tested by assaying five livers in triplicate portions. Standard deviations calculated for the triplicate values averaged 7.5% (range 3.4–13.2) of the mean total protoporphyrin content measured.

**Assay for fecal coproporphyrin.** Each 7–9 g of feces from the two different populations in experiments 1 and 2 was weighed and then homogenized with a known volume of distilled H₂O. Duplicate samples (~0.2 g) were weighed and assayed essentially as described by Rimington (21). The method was modified in that the coproporphyrin fraction was not separated before extraction of the protoporphyrin, since the total fecal porphyrin output is predominated by protoporphyrin in this disorder (22).

**Quantitative determination of griseofulvin in plasma.** Plasma griseofulvin content was measured fluorometrically essentially by the method described by Shah et al. (23), which was modified to the use of 10-μl plasma samples, and then read from a linear standard curve. The curve was constructed by preparing dilutions of a stock standard of griseofulvin in methanol (200 µg/ml) with distilled water over a range of 1–100 µg/ml, and then assaying 10-µl samples of each dilution by the method to be used for the 10-μl plasma specimens.

**Statistical methods.** Statistical analyses that compared protoporphyrin levels in erythrocytes, plasma, and liver in experiments 1 and 2 were performed by using the logarithm.
mic transformations of the data. The variability within each week's cluster of transformed data was tested weekly for cholic and acid-treated mice and for control mice in experiment 1 as well as experiment 2. The differences in the observed variances within each cluster were not significant (24). Therefore, this transformation was used for further analyses. Fecal porphyrin data required no transformation.

To assess whether data for hepatic and fecal porphyrin levels from experiments 1 and 2 could be pooled for further analyses, regression lines were fitted to the means of the data for transformed liver protoporphyrin and the nontransformed fecal protoporphyrin (for cholic acid-treated mice and for control mice over weeks 0–4 separately) for each experiment. Since the lines for data for the same treatment in the separate experiments did not differ significantly (25), pooling of these data could be justified. Exclusion of the technically inaccurate data for plasma and erythrocyte porphyrin levels for the first 2–3 wk of experiment 1 precluded a similar construction of comparable linear regressions which would rigorously justify pooling the valid data from experiment 1 with the data from experiment 2. Therefore, only data from experiment 2 was submitted to regression analysis.

In comparing erythrocyte or plasma porphyrin levels between mice in different treatment groups, regression lines were then fitted to the means of these data over weeks 0–4. The significance of the differences in these linear regressions was tested (25).

In experiment 3, means and SEM were determined for the nontransformed hepatic protoporphyrin data for the four treatment groups. In weeks 2 and 3, significance was assessed with the Wilcoxon rank sum test (26).

**RESULTS**

**Experiments 1 and 2.** Quantitative data are summarized in Table I for each experiment separately. Mean values for both experiments for hepatic protoporphyrin content rose progressively from the normal value of 0.9 μg/g liver to 1,176.5 μg/g by week 4 in the mice fed 2% griseofulvin. Hepatic protoporphyrin content in the mice also fed 0.5% cholic acid never exceeded 28 μg/g. The differences in the regression lines fitted to the means of the transformed data (Fig. 1) were highly significant (P < 0.0001).

By week 4, erythrocyte protoporphyrin levels had risen from the normal range (63±23 μg/dl) to a mean (for both experiments) of 1,406/8 μg/dl in mice fed only 2% griseofulvin, as compared with 275.8 μg/dl for the cholic acid-tested mice. Differences in the regression lines fitted to the means of the transformed data from experiment 2 (Fig. 2) were significant (P < 0.05).

Similarly, plasma protoporphyrin levels rose less in the cholic acid-treated mice, as compared with levels of mice in the 2% griseofulvin control group (Fig. 3) (P < 0.05).

Both cholic acid-treated mice and those fed with only 2% griseofulvin excreted large amounts of pro-

**Table I**

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<th>Week</th>
<th>Erythrocytes</th>
<th>Plasma</th>
<th>Liver</th>
<th>Feces</th>
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<tr>
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<td>μg PP/g</td>
<td>μg PP/g dry wt.</td>
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PP, protoporphyrin.

* Parentheses indicate number of mice; if not indicated, five mice were used for each mean value.

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Differences in regression lines fitted to the means of the natural log transformations of the pooled hepatic protoporphyrin content data of experiments 1 and 2 were highly significant ($P < 0.0001$). *, mice fed 2% griseofulvin; ●, mice fed 0.5% cholic acid and 2% griseofulvin.

Protoporphyrin in feces. However, the cholic acid-treated populations consistently produced larger amounts of fecal protoporphyrin (micrograms per gram dry weight) in weeks 2–4. At the fourth week, the comparative mean values for both experiments were 701.2 μg/g dry wt for cholic acid-treated mice vs. 508.3 μg/g dry wt for mice fed griseofulvin.

Regression lines fitted to the means of the natural log transformations of the erythrocyte (RBC) protoporphyrin data for experiment 2 were significantly different ($P < 0.05$). ■, mice fed 2% griseofulvin; ●, mice fed 0.5% cholic acid and 2% griseofulvin.

Regression lines fitted to the means of the natural log transformations of the plasma protoporphyrin data for experiment 2 were significantly different ($P < 0.05$). *, mice fed 2% griseofulvin; ●, mice fed 0.5% cholic acid and 2% griseofulvin.

Regression lines fitted to the fecal porphyrin excretion data for experiments 1 and 2 were significantly different ($P < 0.001$). ●, mice fed 2% griseofulvin; ■, mice fed 0.5% cholic acid and 2% griseofulvin.


μg/g dry wt for the control mice. Regression lines fitted to the means of the two values obtained weekly in the two experimental trials (Fig. 4) were significantly different \((P < 0.001)\) between the two groups of mice.

By week 3 of both trials, livers from mice that were fed only 2% griseofulvin were darkly pigmented and firm compared with the normal appearance and turgor of livers from the cholic acid-treated mice. Liver specimens from both groups examined by light and electron microscopy at 5 wk are described in detail in a separate communication (27). In brief, examination of control livers showed moderate to marked red-brown pigment deposits in bile ducts, canaliculi, Kupffer cells, and hepatocytes. These deposits were birefringent when examined by polarized light, which is consistent with protoporphyrin (28). Other histologic changes observed in livers of control mice included portal infiltrates of neutrophils and mononuclear cells, scattered zones of necrosis, and dysplastic liver changes. In contrast, in livers of cholic acid-treated mice, no pigment deposits or birefringence on polarization were detected. Portal tracts appeared normal in livers of cholic acid-treated mice. Livers of both control and cholic acid-treated mice showed centrilobular liver cell dysplasia, increased mitotic activity, and small foci of acidophilic necrosis with neutrophilic inflammation (Fig. 5).

Striking differences between livers of control and cholic acid-treated mice were observed with transmission electron microscopy. The control animals' livers showed masses of radially arranged, electron dense crystals are consistent with protoporphyrin (7) in liver cell cytoplasm (Fig. 6), in the space of Dissé and sinusoids, and phagocytosed in Kupffer cell lysosomes. Marked abnormalities of bile canaliculi that are char-

![Figure 5](image_url)

**Figure 5** (A) Liver specimen from a mouse fed with 2% griseofulvin for 5 wk. Dark pigment deposits are seen in bile ducts and ductules (curved arrows) and in sinusoids and bile canaliculi (straight arrows) (hematoxylin and eosin, × 94). **Inset**: The same specimen under polarized light shows pigment birefringence with a “Maltese cross” pattern (arrow) (× 375). (B) Liver specimen from a mouse fed both 0.5% cholic acid and 2% griseofulvin in feed for 5 wk. Note the complete absence of visible pigment and the normal nuclei of dysplastic hepatocytes in centrilobular zones (arrow) (hematoxylin and eosin, × 94). CV, central vein; P, portal tract.
Figure 6 (A) Transmission electron micrograph of liver from a mouse fed with 2% griseofulvin in the feed for 5 wk. Numerous arrays of electron dense protoporphyrin crystals (arrows) are present in hepatocyte cytoplasm (×12,000). (B) Transmission electron micrograph of liver from a mouse fed both 0.5% cholic acid and 2% griseofulvin in feed for 5 wk. Note the paucity of intracellular protoporphyrin crystals (arrows). Lysomes with irregular myelin-like whorls are present (×10,500). G, Golgi apparatus; M, mitochondrion; RER, rough endoplasmic reticulum; L, lipid vacuole.

Characteristic of cholestasis were present, including dilation, loss and blunting of microvilli, membrane blebs and protrusion, increased pericanalicular microfilaments, and intraluminal accumulations of granular and membranous biliary material. Mitochondria showed variable degeneration, myelin-like membranous whorls, and disorganization of cristae. In contrast, the cholic acid-treated mice showed only rare clusters of protoporphyrin crystals in hepatocyte cytoplasm (Fig. 6). Although less severe than in the control group, some mitochondrial and bile canalicular abnormalities persisted in the cholic acid-treated animals. In addi-
tion, frequent membrane-bound lipid vacuoles were noted in their liver cells.

**Experiment 3.** After 1 wk of feeding with 2% griseofulvin, the mean hepatic protoporphyrin level of five mice was $258.8 \pm 65.1 \, \mu g/g \, liver \, (\bar{x} \pm SEM)$. These levels continued to rise in weeks 2 and 3 for mice in group 4 (2% griseofulvin). Mean hepatic protoporphyrin levels rose more slowly in mice in group 3 (2% griseofulvin and 0.5% cholic acid). The apparent difference did not reach significance at the $P < 0.05$ level due to the variability of the individual data points around the means and the limited number of animals (five from each of four groups) that could be assayed in each week. There was essentially no difference for mean hepatic protoporphyrin levels in weeks 2 and 3 for mice in group 1 (no griseofulvin, 0.5% cholic acid) vs. mice in group 2 (unadulterated feed). At week 2, these levels were only slightly higher than at week 1 and by week 3, they had fallen below the levels of week 1, in both groups (Fig. 7).

Serum griseofulvin levels from the mice of groups 3 and 4 assayed at weeks 2 and 3 are shown in Table...
II. A wide range of values was observed in both groups, which indicates different efficiencies of absorption among individual animals; this data is similar to that previously documented in rabbits fed griseofulvin (29). However, the similarity of the mean values suggests overall equivalent absorption of griseofulvin by cholic acid-fed mice and those not fed cholic acid.

DISCUSSION

Symptomatic hepatopathy develops in a minority of patients with protoporphyrin (30); however, once manifested, it is characterized by rapid deterioration and death (1–7). Protoporphyrin deposits are visible in the liver specimens of the majority of patients with protoporphyria, and even in asymptomatic individuals with normal liver function profiles (13). Deposits in livers of patients dying of liver failure have been massive, up to 57,500 μg/g liver (15).

Although the determinants of the onset of hepatic dysfunction and failure remain uncertain, the degree of protoporphyrin deposited appears to correlate directly with the severity of the disease. Protoporphyrin deposited in the human disorder is thought to derive primarily from disordered heme synthesis in the erythron. The excess erythropoietic protoporphyrin disappears rapidly from juvenile erythrocytes and their precursors and appears in the plasma (31). That the liver is able to clear protoporphyrin rapidly from plasma, while secretion into bile is limited, was shown in rat liver perfusion studies by Avner and Berenson (32) and confirmed by Poh-Fitzpatrick and Javitt (33).

The observations of Avner and Berenson that protoporphyrin secretion into bile is enhanced by choleric bile salts in hepatic perfusion studies (18) supports the hypothesis that bile salt-induced choleresis might also increase protoporphyrin secretion in protoporphyrin disease states. In this study, we have examined selected short-term effects of cholic acid supplementation in an animal model for protoporphyrin.

Griseofulvin-induced murine protoporphyrina (22) has served as a model for previous studies of several aspects of human protoporphyrina (34–36). This model, although primarily an hepatic rather than an erythropoietic form of porphyria (37), is similar in many respects to the human disease. Abnormally large amounts of protoporphyrin accumulate in the livers, erythrocytes, and plasmas of both species, although the tissue sources and directions of flux may differ (36). Hepatobiliary-fecal excretion occurs in both, and their hepatic histopathologic abnormalities are indistinguishable by light or electron microscopy (34, 35).

The most significant observation was the limitation of hepatic protoporphyrin accumulation in the mice fed both cholic acid and griseofulvin from the onset of experiments 1 and 2. After examining these data, the question was raised whether cholic acid ingestion might have interfered with the ability of the mice receiving it to absorb as much griseofulvin as the control mice. It was not possible to then perform assays for serum griseofulvin because all the blood had been used for porphyrin assays. Therefore, blood from mice in experiment 3 was assayed to address this question. Wide variability occurred among individual values; however, the mean values suggested that overall, griseofulvin serum levels in the cholic acid-fed mice were not lower than in mice fed only 2% griseofulvin. This observed variability, which is similar to that noted in absorption efficiencies among rabbits fed griseofulvin (28), rationalizes the wide range about the means noted for each data cluster for porphyrin levels derived from five mice in these experiments.

Equivalent exposure of livers of experimental and control mice in experiments 1 and 2 is also suggested

![Figure 7](image_url)
by estimates of total protoporphyrin accumulated in blood and liver as compared with total protoporphyrin excreted. For example, in experiment 2, 25 mice contributed to the total fecal output for week 1, and 20 mice contributed to the total in week 2, for each group of animals. By dividing weekly total outputs by numbers of mice contributing, the mean total output yielded was 3,584 μg/mouse for mice fed griseofulvin only and 5,098 μg/mouse for mice fed cholic acid in addition, at the end of 2 wk. The difference (1,514 μg) represents an estimate of how much more porphyrin was excreted by the average mouse fed both cholic acid and griseofulvin over the 2-wk period, as compared with the average mouse fed only 2% griseofulvin.

The average amount of porphyrin accumulated by each mouse at the end of the 2 wk was approximated by the sum of the blood and liver contents, although this does not take into account any protoporphyrin deposited in other body tissues. Assuming a total blood volume of 3 ml/mouse and a hematocrit of 50%, the mean blood protoporphyrin burden in mice fed griseofulvin only was calculated to be 6.2 μg, while that in mice fed cholic acid also was 2.8 μg. Mean liver weight for mice killed in week 2 was 1.50±0.53 g for mice fed griseofulvin only, and 1.25±0.24 g for mice fed cholic acid in addition. Multiplying these weights by the mean hepatic protoporphyrin levels (micrograms per gram), corrected to 100% recovery, yielded a mean total hepatic protoporphyrin accumulations of 609 μg for griseofulvin-only mice, and 20 μg for mice fed cholic acid in addition.

Thus, mice fed 2% griseofulvin and 0.5% cholic acid appeared to have excreted amounts of porphorpyrin during the 2-wk period that could completely account for the difference in its accumulation in blood and liver between mice fed only griseofulvin and mice fed cholic acid in addition, at the end of the 2-wk period. This indicates that the livers of the experimental (cholic acid) mice indeed were exposed to similar levels of griseofulvin, but were able to excrete the bulk of the porphyrin produced in the feces.

Differences between hepatic protoporphyrin levels were much less evident when the mice were fed griseofulvin for 1 wk before starting cholic acid; however, even in this comparison, mice fed cholic acid appeared to accumulate less hepatic protoporphyrin. However, in the comparison of cholic acid feeding vs. no treatment in mice that were induced for 1 wk but then given no further griseofulvin, cholic acid did not appear to enhance protoporphyrin removal from the livers.

Quantitative differences in hepatic protoporphyrins assayed by extraction were reflected in the microscopic assessments. The pathologist correctly identified which liver specimens were from control and which were from experimental animals by the marked differences in degrees of protoporphyrin present and toxic changes observed.

These data suggest that choleretic enhancement of protoporphyrin secretion into bile may occur in vivo and may reduce hepatic protoporphyrin deposition in some circumstances. Since this enhancement only seemed to occur in those experiments in which griseofulvin-induced porphyrogenesis was continuously stimulated, it may be speculated that "newer" protoporphyrin in the hepatocyte is more readily mobilized for such accelerated secretion, while "older" deposits are less accessible. Since large amounts of protoporphyrin in livers of patients with protoporphyria are thought to be cleared from the plasma and transported into bile relatively efficiently (as reflected by the large amounts of fecal protoporphyrin excreted daily) (1, 3, 4, 6, 8, 9, 13, 15, 16, 30), an important fraction of total intrahepatic protoporphyrin of these patients may be of such a relatively labile type. Other explanations may be speculated as well.

These studies clearly do not address several important issues. For instance, effects of chronic bile salt supplementation on protoporphyrin hepatopathy cannot be inferred from these preliminary studies. Whether these effects are more adverse than the toxicity of chronic protoporphyrin deposition remains to be determined. Nevertheless, since there are currently no reliable means of preventing deaths due to hepatic failure in these patients, further investigation of these considerations is warranted.

ACKNOWLEDGMENTS

The authors are grateful to Dr. Robert Palmer for helpful discussions about the selection of the bile acid chosen for this study. Schering Corp. and Ayerst Laboratories generously supplied the powdered griseofulvin used in this study. This work was supported by research grant AM 18549 from the National Institute of Arthritis, Diabetes, Digestive and Kidney Diseases.

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