Biochemical Pharmacology, Vol. 26, pp. 1125–1130. Pergamon Press, 1977. Printed in Great Britain.

ALTERATION OF HEPATIC MICROSOMAL ENZYMES BY GRISEOFULVIN TREATMENT OF MICE*

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(*Received* 1 *July* 1976; *accepted* 21 *October* 1976)

Abstract—Feeding mice a diet containing 2.5% griseofulvin (GF) for 12 days caused an increase in liver weight to 9 per cent of the body weight with a proportional decrease in microsomal protein/g of liver wet weight. With respect to microsomal protein, the cytochrome P-450 content was 50 per cent and cytochrome b_5 was 200 per cent of that in control mouse liver microsomes. The amount of increase in cytochrome b_5 was approximately the same as the amount of decrease in cytochrome P-450, and there was no change in the total microsomal heme content. The microsomal content of NADH–cytochrome b_5 reductase, as measured by ferricyanide reduction, was unchanged, but in agreement with the elevated cytochrome b_5 content, NADH–cytochrome *c* reductase activity was doubled. While the cytochrome P-450 level was low in microsomes after GF feeding, the NADPH–cytochrome *c* reductase was significantly elevated. Since this enzyme is generally considered to be rate limiting for many mixed function oxidase reactions, its increase may explain the normal to slightly elevated rates of metabolism *in vitro* of several type I and type II substrates. Although cytochrome b_5 has been suggested as being rate limiting for input of a second electron to cytochrome P-450 linked mixed function oxidations, elevation of cytochrome b_5 in the microsomes did not change the extent of NADH-synergism of NADPH-supported aminopyrine demethylation. NADH-supported Δ-9,10-fatty acid desaturase activity, which requires cytochrome b_5, was elevated several-fold after GF feeding. In contrast, NADPH-supported lipid peroxidation showed a slower onset after GF treatment; the NADH-supported reaction, however, was slightly elevated.

The antibiotic griseofulvin (GF) is used effectively as an oral anti-fungal agent in man [1]. Despite its widespread use, relatively little is known about its effect *in vivo* on biochemical pathways in the recipient of the drug. In mice, GF feeding promotes disturbances in porphyrin metabolism, as first shown by DeMatteis and Rimington [2]. During prolonged treatment with GF, an enlargement and darkening of the liver were observed with histologic evidence of liver cell damage, porphyrinstasis, and cholestasis, and, on an electron microscopic level, evidence for membrane destruction associated with the appearance of protoporphyrin crystals [3–5]. More recently, DeMatteis and Gibbs [6] have reported a diminution in the hepatic ferro chelatase of mice and rats treated with GF (1% in the diet) when measured by Co^{2+}-mesoporphyrin formation in isolated mitochondria. This latter report suggests that a decreased ability to convert the more rapidly forming protoporphyrins into heme is an explanation for the accumulation of porphyrin in the liver.

The GF molecule has two methoxy groups on an aromatic ring structure, at positions 4 and 6. As was expected from earlier studies *in vivo* and with liver slices [7], dealkylation at both positions was found to occur aerobically with liver microsomes in the presence of NADPH [8]. Since the enzyme system in the liver microsomes which catalyzes such reactions is a Fe^{3+}-protoporphyrin hemoprotein, a number of studies have been carried out in the past to examine the effects of GF and other porphyrogenic drugs on this mixed function oxidase [8–12].

Besides cytochrome P-450, liver microsomes contain another hemoprotein, cytochrome b_5. This pigment, a component in fatty acid desaturation [13, 14], is present at about half to equal the concentration of cytochrome P-450 in the liver microsomes of the untreated animal. Although induction of the mixed function oxidase generally elevates the microsomal content of cytochrome P-450 several-fold, the content of cytochrome b_5 rarely undergoes much alteration during such treatments.

The purpose of this communication is to describe studies aimed at determining the mechanism of GF-induced hepatotoxicity. The results indicate that GF treatment does not change total microsomal heme content, but instead causes an alteration in the relative amounts of the two microsomal cytochromes. Attendant with these changes are increases in liver weight and decreases in the microsomal content. In addition, cytochrome P-450 and cytochrome b_5-dependent enzyme reactions were affected to a different degree.

MATERIALS AND METHODS

Animals. Male Swiss Albino mice (Charles River Breeders) of 20–30 g body weight were fed a standard

*Supported in part by U.S. Public Health Service Grant GM17021.

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‡ U.S.P.H.S. Research Career Development Awardee, GMK419,601.

Table 1. Response of mice to 2.5% dietary GF*

	Body wt (g)	Liver wt (g/10 g body wt)	Total liver (g/mouse)	Microsomal protein (mg/g liver)	Total microsomal protein (mg/mouse)	Heme (nmoles/liver)
GF	32.25 ± 1.87	0.90 ± 0.03†	3.36 ± 0.18†	38.87 ± 2.13†	129.57 ± 6.20	182.47 ± 20.79
C	34.75 ± 0.52	0.48 ± 0.03	1.93 ± 0.05	61.03 ± 5.67	118.50 ± 14.00	190.21 ± 25.24

* Each value is expressed as the mean ± S. E. M. from four experiments each with the pooled livers of seven to eight mice. Abbreviations: GF, griseofulvin-treated mice; and C, control mice.

† Significantly different from control, $P < 0.005$ (Student's t-test).

Table 2. Effect of 2.5% dietary GF on mouse microsomal enzymes and cytochromes*

	Cytochrome P-450 (nmoles/mg microsomal protein)	Cytochrome b_5 (nmoles/mg microsomal protein)	Total heme (nmoles/mg microsomal protein)	Ferricyanide reductase (nmoles/sec/mg microsomal protein)		Cytochrome c reductase (nmoles/sec/mg microsomal protein)	
				NADH	NADPH	NADH	NADPH
GF	0.57 ± 0.07†	0.84 ± 0.03†	1.49 ± 0.03	79.15 ± 3.70†	7.87 ± 0.15†	18.27 ± 0.58†	3.41 ± 0.24†
C	1.10 ± 0.07	0.44 ± 0.04	1.61 ± 0.09	61.28 ± 2.23	4.13 ± 0.09	7.33 ± 0.74	0.91 ± 0.04

* Each value is expressed as the mean ± S. E. M. from four experiments each with the pooled livers of seven to eight mice. Abbreviations: GF, griseofulvin-mice; and C, control mice.

† Significantly different from control, $P < 0.005$ (Student's t-test).

diet (Purina) containing 2.5% GF for 12 days. Control mice received the powdered standard diet without the antibiotic. Food intake was about equal in the GF and the control group.

Microsome preparation. The mice were sacrificed by decapitation and the livers were removed, chilled on ice, weighed and gently perfused with cold isotonic saline in order to remove residual blood. Seven to eight mouse livers were pooled for each determination, and were homogenized in sucrose (0.25 M) containing 10 mM Tris/HCl buffer, pH 7.5. The microsomes were prepared by a rapid centrifugation technique [15]. Microsomal protein was measured by a biuret method [16]. Total microsomal protein was determined as described by Greim *et al.* [17].

Assays. Mixed function oxidase activity was measured in a medium containing either 1 mg of microsomal protein/ml (aminopyrine demethylase) or 1.67 mg/ml (aniline hydroxylase) suspended in 0.05 M Tris/HCl buffer, pH 7.5, 5 mM $MgCl_2$, 1 mM NADPH and aniline (5 mM) or aminopyrine (8 mM) as substrates. Assays were performed as described previously [18]. Incubation times were 6 min for aminopyrine and 14 min for aniline at 37° in a Dubnoff shaking incubator. Lipid peroxidation was measured as the malondialdehyde produced by the method of Ottolenghi [19] with 1 mM NADH or NADPH and 1 mg microsomes/ml as described by Jansson and Schenkman [20]. Stearyl-CoA desaturase activity was determined by the method of Oshino *et al.* [21], measuring oleyl-CoA formation from stearyl-CoA. The assay medium contained 1 mM NADH in 0.1 M Tris/HCl buffer, pH 7.25, and 70 μM [^{14}C]stearyl-CoA (362 μCi/ml). The reaction was started by addition of microsomes (2 mg protein/ml) and samples were removed at half-minute intervals up to 2.5 min after addition of microsomes. Spectra were recorded with an Aminco DW 2 spectrophotometer as previously described [18]. Kinetic measurements of spectral changes were done with the instrument in the dual wavelength mode. Cytochrome *c* reductase and ferricyanide reductase assays were performed and evaluated as described by Jansson and Schenkman [20]. Total heme was determined as pyridine hemochromogen according to Paul *et al.* [22].

RESULTS

Consumption of GF at 2.5% in the diet for 12 days did not affect the weight gain of the mice (Table 1). The liver weight, in agreement with earlier reports [2], nearly doubled, reaching 9 per cent of the body weight, as compared with 4.8 per cent in untreated mice. The content of microsomal protein/g of liver, however, decreased about 40 per cent, resulting in no real change in liver microsomal protein/animal (Table 1). On the other hand, the specific amounts of some individual microsomal enzymes did change markedly (Table 2). For example, the total cytochrome P-450 content dropped almost 50 per cent as a result of GF feeding, despite reports that there is a transient rise [11] or no change [12] in this hemoprotein. Most striking of all was the almost 2-fold increase in cytochrome b_5 content in the microsomes. This is the first report, to our knowledge, that b_5 is specifically inducible by administered drugs. Phenobarbital treatment, while causing a large elevation of cytochrome P-450, causes no change [20, 23] to a small (about 25 per cent) elevation of cytochrome b_5 content [17] in the microsomes. The amount of increase in one hemoprotein after GF is matched by the amount of decrease in the other hemoprotein with the result that in agreement with an earlier report [2] there was no change in hepatic protoheme content (Table 2).

Measurement of hepatic microsomal NADH–cytochrome b_5 reductase content by its ability to reduce ferricyanide indicated that there was no change in the specific microsomal content of this enzyme. However, because of the increased microsomal content of b_5 in the treated animals, NADH–cytochrome *c* reductase was more than doubled (Table 2). One unexpected finding was the GF-mediated induction of NADPH–cytochrome *c* reductase content; when measured by its ability to reduce ferricyanide, the enzyme was found to be doubled. However, when measured in the manner usually employed for this enzyme, cytochrome *c* reduction, the activity was more than tripled. Clearly, then, this enzyme does not follow the level of cytochrome P-450 (Table 2).

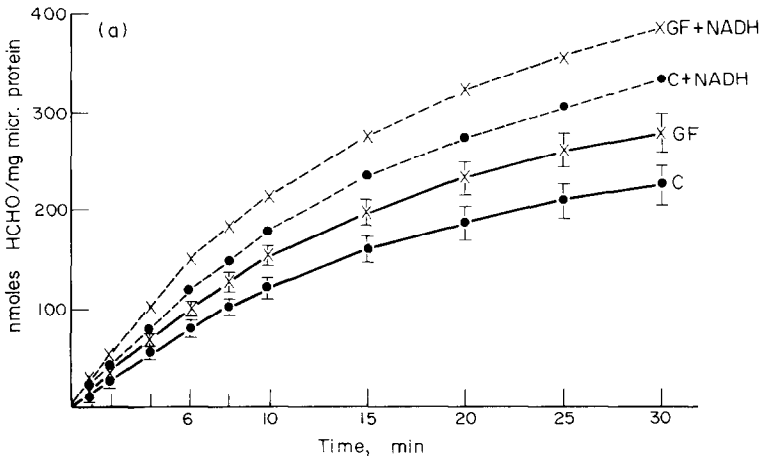
Examination of the mixed function oxidase activity using aminopyrine (Fig. 1a) and aniline (Fig. 1b) as type I and type II substrates [18], respectively, revealed slight elevation of these activities/mg of microsomal protein. However, if expressed on the basis of microsomal cytochrome P-450 content, there is a doubling of activity of the microsomal mixed function oxidase with both aminopyrine and aniline as substrates after GF treatment. No change in the extent of NADH-synergism (Fig. 1a) of NADPH-supported aminopyrine demethylation was seen (aniline hydroxylation does not usually show an NADH-synergism).

When NADPH-supported lipid peroxidation was measured, no difference was seen in the extent of the reaction by 50 min; however, the onset of peroxidation was slower in the microsomes from treated animals (Fig. 2a), revealing a 2-min lag time. The NADH-supported lipid peroxidation (Fig. 2b) showed a lag of about 2 min with GF mouse liver microsomes and a lag of about 4 min with untreated mouse microsomes; the initial rate of peroxidation was slower with NADH than with NADPH; however, in 50 min, the NADH-supported peroxidation reached the same extent as the NADPH-supported peroxidation.

Examination of NADH-supported fatty acid desaturase indicated (Fig. 3) a 12-fold increase in stearyl-CoA desaturase activity. This induction is similar to that observed earlier with a fat-free diet [20], but unlike that study, in which cytochrome b_5 was decreased by 50 per cent, in this study it is doubled.

DISCUSSION

Previous investigations by several authors have disclosed that treatment of mice with GF causes porphyria and severe disturbance of liver cell structure and function [2–5]. The endoplasmic reticulum in the hepatocytes was increased after GF treatment [12]. Microsomal benzphetamine-*N*-demethylase and benz-



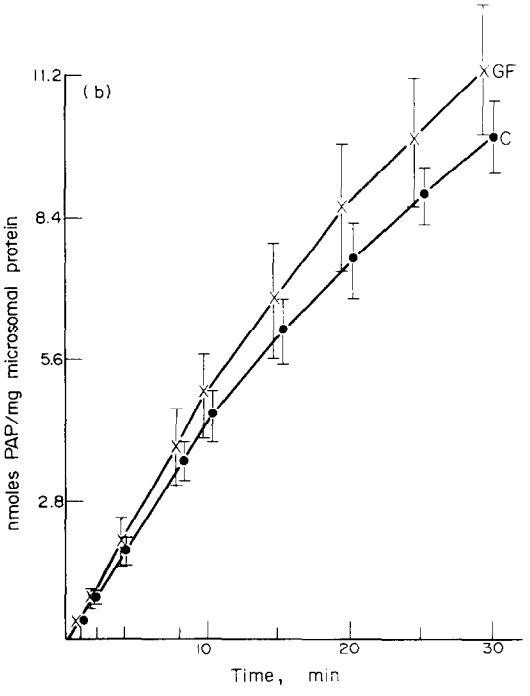
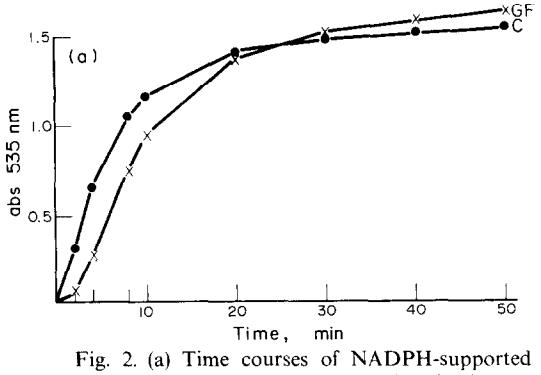


Fig. 1. (a) Time course of aminopyrine-*N*-demethylase assays with GF (× —— ×) and control (●——●) microsomes (NADPH-supported reaction). The reactions were performed as described in the methodology section. Each point represents a mean value ± S. D. from four experiments each done with the microsomes of eight pooled mouse livers. A dashed line (– – –) shows the synergistic effect of NADH (0.5 mM) on the NADPH-supported reaction. Each point represents a mean value from two experiments with the microsomes of eight pooled mouse livers. (b) Time courses of aniline hydroxylase by GF(× —— ×) and control (●——●) microsomes *in vitro*. Each point represents a mean value ± S. D. from four experiments each done with the microsomes of eight pooled mouse livers. PAP = paraaminophenol.

pyrene hydroxylase specific activities were increased despite considerable loss of cytochrome P-450 from the microsomal membranes [12]. This was, in essence, confirmed in the present study with respect to metabolism of the substrates aminopyrine and aniline (Fig. 1, panels a and b), which was slightly enhanced. Despite the lower content of cytochrome P-450 in GF-treated mouse microsomes, aminopyrine demethylase and aniline hydroxylase activities *in vitro* were slightly higher than the controls, when calculated on a mg



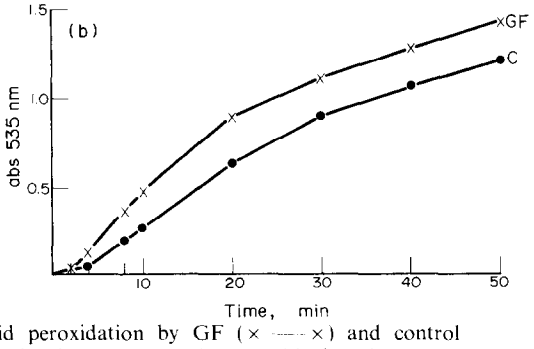


Fig. 2. (a) Time courses of NADPH-supported lipid peroxidation by GF (× —— ×) and control (●——●) microsomes *in vitro*. Each point is a mean value from two experiments with the microsomes of eight pooled mouse livers. (b) NADH-supported reaction. Conditions are the same as in Fig. 1a.

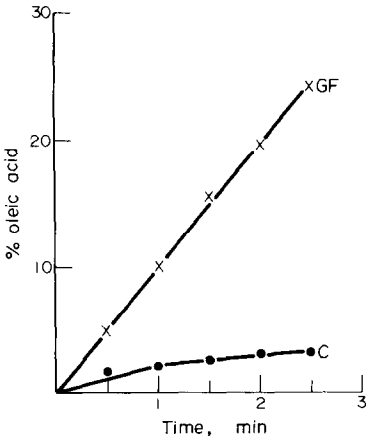


Fig. 3. Time courses of microsomal NADH-supported fatty acid desaturation of stearyl-CoA by GF (×——×) and control (●——●) microsomes. Each point is a mean value from two experiments with the microsomes of eight pooled mouse livers.

protein basis and doubled when related to cytochrome P-450 content. The other microsomal hemoprotein, cytochrome b_5, was doubled within 12 days of GF feeding. Although it is premature to conclude that GF treatment increases cytochrome b_5 at the expense of cytochrome P-450, it appears that GF manifests unique effects upon the liver microsomal cytochrome levels. No other xenobiotic is reported to increase cytochrome b_5 to this extent while simultaneously decreasing cytochrome P-450.

The lack of decrease in mixed function oxidase activities with decreased cytochrome P-450 levels after GF treatment caused us to consider the possibility that elevated cytochrome b_5 levels in GF microsomes might compensate for the diminished cytochrome P-450 content by facilitating the input of a second electron into the P-450-dependent mixed function oxidase pathway [24]. This latter possibility is unlikely because it was shown in microsomes [20, 25] as well as in a reconstituted cytochrome P-450-containing mixed function oxidase system [26] that aminopyrine, aniline and several other drug-metabolizing activities are actually diminished after supplementation of the preparation with extra b_5. However, it should be noted that the inhibition to be expected from the increase in b_5 after GF and the decreased P-450/b_5 ratio may have been relieved by the 3-fold elevation of the NADPH–cytochrome *c* reductase; studies by Lu *et al.* [26] indicated that the inhibitory effect of excess b_5 could be reversed by the addition of excess NADPH–cytochrome *c* reductase. Alternatively, GF may induce synthesis of a P-450 cytochrome which utilizes cytochrome b_5, as seen in chlorobenzene metabolism [27]. One other possibility which must be considered is that this study provides further evidence that NADPH–cytochrome *c* reductase, and not cytochrome P-450 is the rate-limiting enzyme in the mixed function oxidase reaction, and the decrease in cytochrome P-450 level is compensated for by the increase in the reductase.

Cytochrome b_5 is a component of the desaturase system [13, 14] and increasing the microsomal cytochrome b_5 resulted in an increase in desaturase activity in rats [20]. This observation *in vitro* is consistent with our present data where an increase in microsomal cytochrome b_5 by GF treatment *in vivo* is accompanied by elevated fatty acid desaturase activity. However, the elevation in desaturase is much greater (12-fold) than the cytochrome b_5 elevation (2-fold) suggesting that a component other than cytochrome b_5 determines the activity of the desaturase. Similar conclusions were reached by Jansson and Schenkman [20] on the basis of their studies with rats fed a fat-free diet. They agree with the suggestion of Oshino and Sato [28] that the "cyanide sensitive factor," rather than cytochrome b_5, is rate limiting in the desaturase reaction.

GF-provoked porphyria in mice has been suggested by DeMatteis and Gibbs [6] as due to a decrease in hepatic ferro chelatase activity, the enzyme responsible for insertion of iron into the protoporphyrin ring. Our present results indicate, however, that the situation in GF-induced porphyria in mice may be more complex. While the total microsomal heme is virtually unchanged, a shift occurred in the hemoprotein composition of the microsomes. This could mean that the specificity of the GF effect lies in an alteration of apocytochrome P-450 synthesis rather than of microsomal heme synthesis and that, as suggested by Correia and Meyer [29], apocytochrome P-450 is rate limiting in the formation of P-450. On the other hand, apocytochrome b_5 is known to be present in the microsomes [30, 31], and may account for the increase in this cytochrome.

Morphologic investigations of GF mouse livers revealed severe liver cell damage and membrane destruction accompanied by "brown pigment" deposition [4, 5], all of which could eventually point to a lipid peroxidation process being involved in the pathology [32]. In this context, it is interesting that GF feeding caused a several-fold increase in stearyl-CoA desaturase.

Acknowledgements—This work was supported in part by National Institutes of Health Grant GM 17021, and American Cancer Society Grant BC 164; it was also supported by Fonds zur Förderung der Eissenschaftlichen Forschung (Austria) Grant 1543 and Adolf-Sonnleitner Fonds of the Austrian Academy of Sciences (to H. D.).

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