Acknowledgements—We are grateful to Dr. H. Felsenfeld of the University of Connecticut for the initial tumor-bearing mice and to Dr. A. Dannenberg of Johns Hopkins University for the pepstatin.

Department of Pharmacology,
College of Physicians and Surgeons,
Columbia University,
630 *West* 168*th Street,*
New York, N.Y. 10032, *U.S.A.*

MARTHA M. JOHNSTON*
LOWELL M. GREENBAUM

REFERENCES

1. L. M. GREENBAUM, R. FREER, J. CHANG, G. SEMENTE and K. YAMAFUJI, *Br. J. Pharmac. Chemother.* **36,** 623 (1969).
2. L. M. GREENBAUM, *Am. J. Path.* **68,** 3 (1972).
3. J. CHANG, R. FREER, R. STELLA and L. M. GREENBAUM, *Biochem. Pharmac.* **21,** 3095 (1972).
4. R. FREER, J. CHANG and L. M. GREENBAUM, *Biochem. Pharmac.* **21,** 3107 (1972).
5. C. TANAKA, N. J. GIARMAN, K. BENSCH and H. FELSENFELD, *Biochem. Pharmac.* **19,** 963 (1970).
6. H. UMEZAWA, T. AOYAGI, H. MORISHIMA, M. MATSUZAKI, M. HAMADA and T. TAKEUCHI, *J. Antibiot.* **23,** 259 (1970).
7. A. A. MILES and E. M. MILES, *J. Physiol., Lond.* **118,** 228 (1952).

* Smith, Kline and French Fellow.

Biochemical Pharmacology, Vol. 22, pp. 1389–1392. Pergamon Press, 1973. Printed in Great Britain.

Oxidative demethylation of ^{14}C-griseofulvin by liver microsomes of rats and mice

(*Received* 4 *June* 1972; *accepted* 17 *November* 1972)

GRISEOFULVIN is an orally effective antifungal agent and is used widely in the treatment of fungal infections in animals and man.[1] The metabolism of griseofulvin *in vivo* has been studied in man, rat, mouse, rabbit and dog.[2–7] Limited metabolic studies of griseofulvin in tissue slices[8,9] and liver microsomes[10] have also been reported. However, the metabolism of griseofulvin by liver microsomes has not yet been elucidated. It was therefore of interest to determine the kinetic parameters for *O*-demethylations of griseofulvin in the rat and mouse liver microsomes.

Charles River male mice (20 g) and rats (180 g) were sacrificed by decapitation. Livers were homogenized and the homogenate was centrifuged at 9000 *g* for 20 min and the precipitate was discarded. The microsomes were sedimented by centrifugation at 100,000 *g* for 60 min. The enzyme activities of 4-demethylation and 6-demethylation of griseofulvin were determined by incubation of ^{14}C-griseofulvin with the liver microsomes, $MgCl_2$ ($5{\cdot}5 \times 10^{-3}$M) and NADPH-generating system (1 mg NADP, 8·8 mg glucose 6-phosphate and 2 Kornberg units glucose 6-phosphate dehydrogenase) in 0·05 M potassium phosphate buffer, pH 7·4. The reactions were stopped by acidifying the mixtures to pH 1, and griseofulvin and its metabolites were extracted with diethyl ether. The ^{14}C-labeled griseofulvin, 4-desmethylgriseofulvin and 6-desmethylgriseofulvin were separated by thin-layer chromatography using chloroform–methanol (10:1, v/v) as the solvent system. The radioactivity associated with each component was then measured by liquid scintillation counting.

Griseofulvin was converted to 4-desmethylgriseofulvin and 6-desmethylgriseofulvin by rat and mouse liver homogenates (Table 1). Both demethylating enzyme activities were localized in the microsomal fraction. When the liver microsomes were boiled, there was no evidence of conversion of griseofulvin to its demethylated derivatives. NADPH-generating system was essential for these enzymatic reactions. A linear relationship was obtained between the metabolic rate and the enzyme concentration for both demethylation reactions in the rat and mouse (Fig. 1). The amount of product formed for both reactions and in both species increased linearly during the first 8 min, and then declined (Fig. 2).

TABLE 1. CONVERSION OF GRISEOFULVIN TO 4-DESMETHYLGRISEOFULVIN AND 6-DESMETHYLGRISEOFULVIN BY VARIOUS PREPARATIONS*

	Enzyme activity‡			
	Rats		Mice	
Preparation†	4-Demethylation	6-Demethylation	4-Demethylation	6-Demethylation
Total homogenates	37·2	27·8	44·8	52·2
Microsomes	30·6	27·2	35·6	49·8
Soluble fraction	0	0	0	0
Boiled microsomes§	0	0	0	0
Microsomes without NADPH-generating system	6·4	3·2	3·6	1·2

* ^{14}C-Griseofulvin ($3{\cdot}3 \times 10^{-4}$ M) was incubated with various preparations listed, NADPH-generating system and $MgCl_2$ in phosphate buffer (0·05 M, pH 7·4) at 37° for 5 min.

† Liver homogenates were fractionated into microsomes and soluble fraction by step-wise centrifugation, 9000 *g* for 20 min and 100,000 *g* for 60 min.

‡ Expressed as millimicromoles of products formed per min per g of liver.

§ Liver microsomes were boiled at 100° for 20 min.

From double reciprocal plots, K_m values of $3{\cdot}1 \times 10^{-4}$ and $2{\cdot}7 \times 10^{-4}$ M and V_{max} values of 0·37 and 0·27 nmole/min/g of protein were obtained in rats for 4- and 6-demethylation respectively (Fig. 3). In mice, K_m values of $3{\cdot}3 \times 10^{-4}$ and $3{\cdot}2 \times 10^{-4}$ M and V_{max} values of 0·66 and 0·75 nmole/min/mg protein were obtained for 4- and 6-demethylation respectively (Fig. 3). In double reciprocal plots (Fig. 4), addition of SKF 525-A resulted in elevation of the slope and also in decrease of the intercept of $1/S$. These changes are proportional to the amount of SKF 525-A added. However, in all cases, the intercept of $1/v$ did not change, indicating that SKF 525-A is a competitive inhibitor for the 4-demethylation and 6-demethylation of griseofulvin in mice liver microsomes.

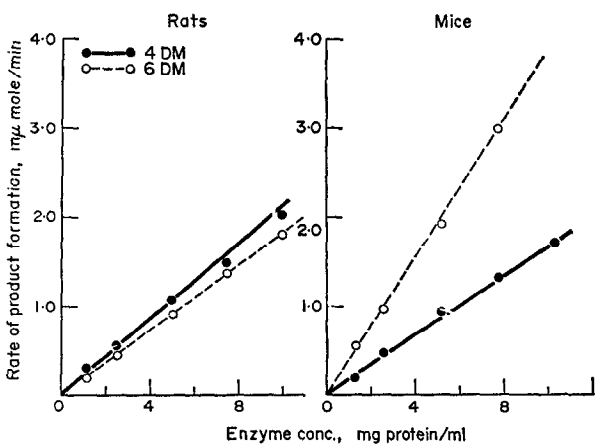

FIG. 1. Conversion of griseofulvin to 4-desmethylgriseofulvin and 6-desmethylgriseofulvin with different concentrations of enzyme preparations. ^{14}C-Griseofulvin ($3{\cdot}0 \times 10^{-4}$ M) was incubated with different concentrations of liver microsomes, NADPH-generating system and $MgCl_2$ in phosphate buffer (0·05 M, pH 7·4) at 37° for 5 min.

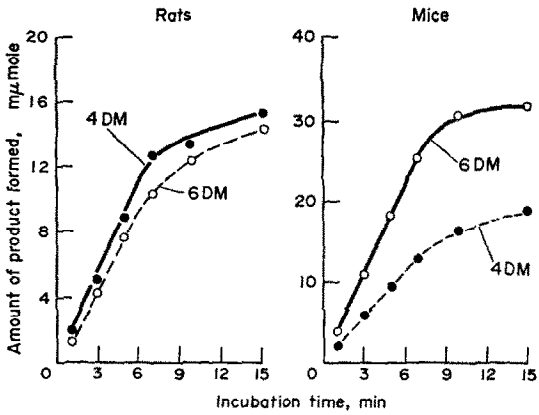

FIG. 2. Conversion of griseofulvin to 4-desmethylgriseofulvin and 6-desmethylgriseofulvin at different incubation periods. ^{14}C-Griseofulvin ($3{\cdot}0 \times 10^{-4}$ M) was incubated with microsomal protein (6·6 mg protein/ml in rats, 5·0 mg protein/ml in mice), NADPH-generating system and $MgCl_2$ in phosphate buffer (0·05 M, pH 7·4) at 37°.

On the basis of NADPH requirement, inhibition by SKF 525-A and localization of enzyme activity in microsomes, it can be concluded that mixed-function oxidase catalyzed the conversion of griseofulvin to 4-desmethylgriseofulvin and 6-desmethylgriseofulvin in liver microsomes. Since 4-desmethylgriseofulvin and 6-desmethylgriseofulvin have been previously shown[6,7] to be major metabolites of

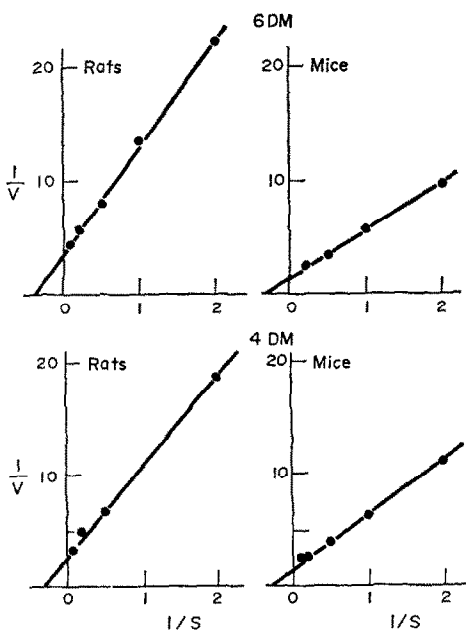

FIG. 3. Formation of 4-desmethylgriseofulvin and 6-desmethylgriseofulvin at different substrate concentrations. ^{14}C-griseofulvin at different concentrations (0·5, 1, 2, 5 and 10×10^{-4} M) was incubated with microsomal enzyme (3·0 mg protein/ml in rats, 7·1 mg protein/ml in mice) in the presence of NADPH-generating system and $MgCl_2$ in phosphate buffer (0·05 M, pH 7·4) at 37° for 5 min. Velocities are given as millimicromoles of 6-desmethylgriseofulvin or 4-desmethylgriseofulvin formed per min per milligram of microsomal protein.

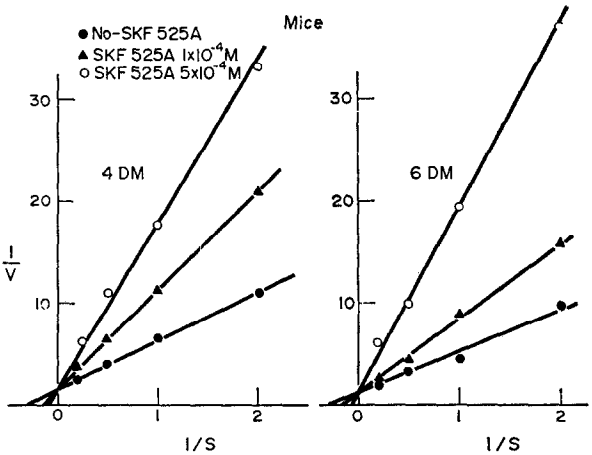

FIG. 4. Effect of SKF 525-A on the kinetics of 4-demethylation and 6-demethylation of griseofulvin. ^{14}C-griseofulvin (0·5, 1, 2, 5 and 10 × 10^{-4} M) was incubated with microsomal enzyme (6·6 mg protein/ml in rats, 11·4 mg protein/ml in mice), NADPH-generating system, $MgCl_2$ and different concentrations of SKF 525-A (1 and 5 × 10^{-4} M) in phosphate buffer (0·05 M, pH 7·4) at 37° for 5 min. Velocities are given as millimicromoles of 6-desmethylgriseofulvin or 4-desmethylgriseofulvin formed per min per milligram of microsomal protein.

griseofulvin in the urine of rats and mice, it is apparent from this investigation that there is good correlation between *in vitro* and *in vivo* metabolism of griseofulvin in mice and rats.

Department of Biochemistry,
Schering Corp.,
Bloomfield, N.J. 07003, *U.S.A.*

RICHARD L. CHANG
SAMSON SYMCHOWICZ
CHIN-CHUNG LIN

REFERENCES

1. M. L. ELGART, *Med. Ann. Distr. Columbia* **36,** 331 (1967).
2. M. J. BARNES and B. BOOTHFOYD, *Biochem. J.* **78,** 41 (1961).
3. S. A. KAPLAN, S. RIEGELMAN and K. H. LEE, *J. pharm. Sci.* **49,** 14 (1960).
4. P. A. HARRIS and S. RIEGELMAN, *J. pharm. Sci.* **58,** 93 (1969).
5. W. L. CHIOU and S. RIEGELMAN, *J. pharm. Sci.* **60,** 1376 (1971).
6. S. SYMCHOWICZ and K. K. WONG, *Biochem. Pharmac.* **15,** 1596 (1966).
7. C. LIN, R. CHANG, J. MAGAT and S. SYMCHOWICZ, *J. Pharm. Pharmac.*, in press.
8. C. BEDFORD, P. BUSFIELD, K. J. CHILE, I. MACGREGOR, P. SUTHERLAND and E. G. TOMICH, *A.M.A. Archs Derm.* **81,** 735 (1961).
9. S. SYMCHOWICZ and K. K. WONG, *Biochem. Pharmac.* **15,** 1601 (1966).
10. S. A. KAPLAN, S. RIEGELMAN and K. H. LEE, *J. pharm. Sci.* **55,** 14 (1966).