Biochemical Pharmacology, Vol. 25, pp. 1355 1360. Pergamon Press, 1976. Printed in Great Britain.

IMPAIRMENT OF HEPATIC MICROSOMAL DRUG METABOLISM IN THE RAT DURING DAILY DISULFIRAM ADMINISTRATION*

MICHAEL A. ZEMAITIS†‡ and FRANK E. GREENE§

Department of Pharmacology, The Milton S. Hershey Medical Center, The Pennsylvania State University College of Medicine, Hershey, Pa. 17033, U.S.A.

(*Received* 31 *May* 1975; *accepted* 15 *August* 1975)

Abstract—Male rats were given 0.1 or 0.4 g/kg of disulfiram (DS) daily by gavage for up to 12 days, in order to study the effects of chronic DS administration on hepatic microsomal drug metabolism. Administration of 0.4 g/kg of DS resulted in significant impairment of aniline (ANL) hydroxylase after 1 day, but 2 days of DS treatment were required for significant inhibition of ethylmorphine (EtM) metabolism and depression of cytochrome P-450 levels. At this time, maximum impairment of ANL and EtM metabolism and maximum reduction of cytochrome P-450 levels were seen. Continued administration of DS for 10 additional days produced no further change in these parameters. ANL hydroxylase was also significantly reduced in treated animals throughout a 12-day period during which 0.1 g/kg of DS was given. EtM *N*-demethylase activity and cytochrome P-450 levels were also reduced in animals so treated, but not until DS had been given for at least 5 days. However, by the end of the 12-day experimental period, EtM metabolism and cytochrome P-450 levels had returned almost to control levels and only ANL hydroxylase was significantly different from control activity. Daily DS administration (0.4 g/kg) produced small but significant increases in microsomal cytochrome b_5 levels and in NADPH-cytochrome *c* reductase activity, whereas NADPH oxidase and NADPH-cytochrome P-450 reductase activities were significantly lower in treated rats. In addition to these effects *in vivo*, DS competitively inhibited EtM *N*-demethylase *in vitro* and bound to cytochrome P-450, producing a type I difference spectrum, thus providing additional mechanisms to account for impairment *in vivo* of drug metabolism by DS.

Disulfiram (Antabuse, DS) has been used for over 20 years in avoidance therapy for certain patients with chronic alcoholism. This usage is based on the observation that DS inhibits aldehyde dehydrogenase resulting in acetaldehyde accumulation upon ingestion of ethanol [1]. This elevation in blood acetaldehyde levels appears to be primarily responsible for the unpleasant symptoms of the so-called "Antabuse reaction" [1, 2]. Since the early work with this compound, several other mechanisms for its effectiveness in alcoholism therapy have been proposed [3, 4] and DS has been shown to inhibit other enzymes such as: dopamine β-hydroxylase [5], hexokinase [6], glyceraldehyde 3-phosphate dehydrogenase [7] xanthine oxidase [8], and D-amino acid oxidase [9].

Of particular clinical significance are the observations that DS administration to man prolongs the half-life of antipyrine [10] and may result in toxic accumulation of diphenylhydantoin and warfarin by inhibiting the metabolism of these drugs by hepatic microsomal enzymes [11, 12]. More recently, DS has been shown to inhibit rat hepatic microsomal and plasma esterases that are responsible for hydrolysis of many ester and amide drugs [13]. Oxidative drug metabolism has also been studied in rat liver microsomes after DS administration, but previous work has focused primarily on the acute effects of a single dose of the drug [14–16]. Since DS is given in daily doses during therapy for chronic alcoholism [17], the present study was undertaken to more clearly define the effects of daily DS administration on the metabolism *in vitro* of drugs and on the various components of the microsomal enzyme systems in rat liver.

MATERIALS AND METHODS

Animals. Male Sprague–Dawley rats weighing 180–200 g at the beginning of the dosing period were used in all studies. Animals had free access to Purina lab chow and water. The average weights for each group were not statistically different at the beginning of the dosing period or at any subsequent sacrifice time. Both control and treated rats ate normally; the average weight gain for the control animals was 7.8 g/day, while rats receiving 0.1 or 0.4 g/kg of DS daily gained 8.6 g/day and 7.1 g/day respectively.

Dosing. Disulfiram (Sigma Chemical Co., St. Louis, Mo.) was finely powdered with a mortar and pestle and was suspended in 0.5% carboxymethyl-cellulose (CMC) solution. Rats were given DS (0.1 or 0.4 g/kg) daily by gavage with a stainless steel feeding tube fitted to a 5-cm^3 disposable syringe. The concentration of DS was adjusted in the solution so that the final volume given was 2–3 ml. Control animals received an equal volume of vehicle (0.5% CMC).

* This research was supported by NIEHS Grant ES00638.

† Portions of this work were submitted in partial fulfillment of the requirements for the Ph.D. degree at The Pennsylvania State University.

‡ Present address: Department of Pharmacology, School of Pharmacy, University of Pittsburgh, Pittsburgh, Pa. 15261, U.S.A.

§ Author to whom reprint requests should be addressed.

Tissue preparation and assays. After various periods of daily DS administration, groups of control and treated animals were decapitated; their livers were removed and immediately homogenized in 2 vol. of ice-cold Tris–KCl buffer (0.02 M Tris–HCl, pH 7.4–1.15% KCl). Microsomal suspensions were prepared as previously described [14]. The protein content of the microsomes was determined by the Biuret method [18].

Cytochrome P-450 and cytochrome b_5 contents of the microsomal suspensions were determined with an Aminco Chance spectrophotometer as described by Omura and Sato [19, 20]. NADPH-cytochrome *c* reductase activity was estimated according to the method of Phillips and Langdon [21] as modified by Gigon *et al.* [22]. NADPH oxidase activity was estimated according to the method of Gillette *et al.* [23] and NADPH-cytochrome P-450 reductase activity was determined as described by Gigon *et al.* [22]. A Gilford model 2400 spectrophotometer with a cell block maintained at 37° was used for these determinations.

Drug metabolism was determined in a 3.0-ml incubation mixture consisting of 5 mM $MgCl_2$, 0.43 mM NADP, 10 mM glucose 6-phosphate, 1.5 enzyme units of glucose 6-phosphate dehydrogenase, 50 mM Tris HCl buffer, 2–4 mg of microsomal protein, and appropriate substrate at optimal concentration. The mixtures were incubated in a Dubnoff metabolic incubator at 37° for 10 min (ethyl-morphine *N*-demethylase assay) or 15 min (aniline hydroxylase assay) in an air atmosphere.

The *N*-demethylation of ethylmorphine (5.0 mM) was measured in the described incubation mixture by the method of Nash [24], which measures the amount of formaldehyde formed from the cleaved methyl group. The metabolism of aniline (1.0 mM) to *p*-aminophenol (paP) was measured by the method of Imai *et al.* [25] as modified by Chhabra *et al.* [26].

Studies in vitro. In the kinetic study, the K_m and V_{max} for ethylmorphine *N*-demethylation in the presence and absence of DS were obtained as described by Davies *et al.* [27]. The binding of DS to cytochrome P-450 was determined in an Aminco Chance spectrophotometer operated in the split beam mode [28]. For this study, washed microsomes were prepared by centrifuging the original microsomal suspension at 78,000 *g* for 30 min and resuspending the pellet in fresh Tris KCl buffer (2 mg protein/ml). Washed microsomes (3 ml) were placed in each of two matched cuvettes, and a baseline was recorded from 340 to 500 nm. Microliter additions of 30 mM DS in absolute ethanol were made to the sample cuvette with equal volumes of ethanol to the reference cuvette. The absolute spectra of DS in Tris–KCl buffer were graphically subtracted from the difference spectra recorded in microsomes to correct for the absorbance of the compound in the range of 340–420 nm.

Statistics. Student's *t*-test was used to make comparisons between the control and treated animals.

RESULTS

The effect of daily administration of 0.4 g/kg of DS on the level of microsomal cytochrome P-450 and on the metabolism *in vitro* of ethylmorphine (EtM) and aniline (ANL) is shown in Fig. 1. Treated animals were given the first dose of DS by gavage at 7:00 a.m. on day 0 and they received subsequent doses at the same time each day. Groups of control and treated animals (N = 4) were sacrificed 24 hr after the last dose of DS. Enzyme activities and cytochrome P-450 levels are presented as percentage of decrease; values (mean ± S. E. M.) for the 20 control animals sacrificed during the experiment were: cytochrome P-450, 0.601 ± 0.024 nmole/mg of protein; Etm *N*-demethylase, 10.10 ± 0.40 nmoles HCHO formed/mg of protein/min; and ANL hydroxylase, 0.45 ± 0.02 nmole pAP formed/mg of protein/min.

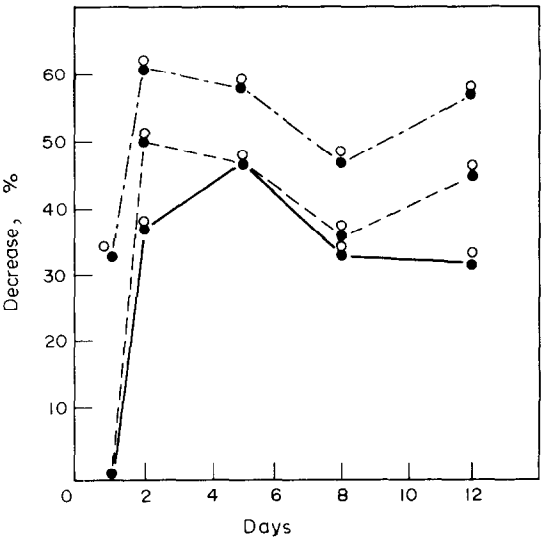

Fig. 1. Effects of daily oral administration of 0.4 g/kg of DS on cytochrome P-450 (——), EtM *N*-demethylase (— · — · —), and ANL hydroxylase (— — —) activity in rat liver microsomes. The asterisk (*) indicates different from control, $P < 0.05$.

As shown in Fig. 1, after 1 day of DS administration, no significant differences in cytochrome P-450 levels or EtM *N*-demethylase activity were observed

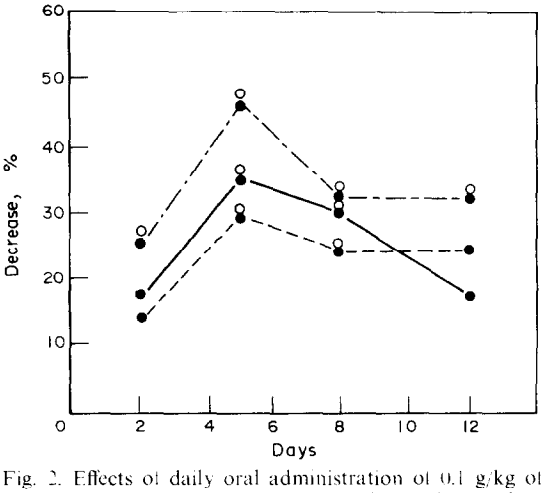

Fig. 2. Effects of daily oral administration of 0.1 g/kg of DS on cytochrome P-450 (——), EtM *N*-demethylase (— — —), and ANL hydroxylase (— · — · —) activity in rat liver microsomes. The asterisk (*) indicates different from control, $P < 0.05$.

Table 1. Effect of daily disulfiram administration on heme components and oxido-reductase activity of rat liver microsomes

Parameter measured*	Experimental group	Days of DS administration (0.4 g/kg) 2 Days	8 Days
Cytochrome P-450 (nmoles/mg)	Control	0.546 ± 0.015	0.645 ± 0.017
	DS	0.343 ± 0.23†	0.429 ± 0.040†
Cytochrome b_5 (nmoles/mg)	Control	0.288 ± 0.014	0.266 ± 0.014
	DS	0.337 ± 0.015	0.402 ± 0.019†
NADPH-cytochrome *c* reductase (nmoles cytochrome *c* reductase/mg/min)	Control	87.1 ± 2.1	122.1 ± 3.6
	DS	98.8 ± 0.7†	135.7 ± 6.5
NADPH oxidase (nmoles NADPH oxidase/mg/min	Control	12.2 ± 0.4	12.7 ± 0.5
	DS	9.5 ± 0.4†	8.9 ± 0.5†
NADPH cytochrome P-450 reductase (nmoles cytochrome P-450 reductase/mg/min)	Control	6.2 ± 0.5	7.3 ± 0.7
	DS	3.9 ± 0.3	4.0 ± 0.3†

* Mean ± S. E. M.
† Different from control, $P < 0.01$.

in microsomes isolated from control or treated animals. However, ANL hydroxylase activity was significantly inhibited by 33 per cent in the treated group. After 2 days of DS treatment, cytochrome P-450 levels as well as EtM and ANL metabolism were significantly reduced in treated rats. Moreover, significant impairment of all three parameters was observed throughout the remainder of the 12-day study. Although EtM *N*-demethylase activity expressed per mg of microsomal protein was significantly inhibited in treated animals from days 2 through 12, when demethylase activity was calculated in terms of cytochrome P-450 content, it was significantly reduced in treated rats only at the 2-day determination ($P < 0.05$). In contrast, ANL hydroxylase was significantly below control activity at all time points when calculated in terms of microsomal protein or cytochrome P-450 content.

Impairment of demethylase activity and cytochrome P-450 levels was also observed during daily administration of 0.1 g/kg of DS (Fig. 2); however, the reductions were not statistically significant until after 5 days of treatment. Demethylase activity and cytochrome P-450 levels were also lower in treated rats after 8 and 12 days, but the 12-day decreases were not statistically significant. On the other hand, ANL hydroxylase was significantly inhibited in treated animals at all times from 2 to 12 days. When metabolic activity was expressed in terms of cytochrome P-450 content, EtM *N*-demethylase activity was not significantly reduced by 0.1 g/kg of DS daily, while ANL hydroxylase activity was significantly below control values after 5 or 12 days of treatment.

Table 1 summarizes the effect of daily DS (0.4 g/kg) administration for 2 or 8 days on microsomal heme components and oxido-reductase activity. In contrast to the decrease in cytochrome P-450 levels produced by DS administration, no significant changes occurred in cytochrome b_5 levels after 2 days of DS administration; after 8 days the cytochrome b_5 levels in treated rats were actually increased by about 50 per cent. Daily treatment with DS slightly increased NADPH-cytochrome *c* reductase activity while it significantly decreased NADPH oxidase and NADPH-cytochrome P-450 reductase activities by approximately 25 and 40 per cent respectively.

During the studies summarized in Figs. 1 and 2, impairment of EtM *N*-demethylase closely paralleled corresponding decreases in cytochrome P-450 levels. Although lower cytochrome P-450 levels probably account for the decreased EtM *N*-demethylase activity in microsomes isolated from DS-treated rats, other direct interactions between DS and the microsomal mixed function oxidase system may also account for impaired metabolism *in vivo* of drugs such as aminopyrine when administered after DS pretreatment [15]. Since compounds administered to animals prior to isolation of hepatic microsomes may be "washed out" during preparation of the microsomal suspension [29], EtM metabolism was also measured in pooled control microsomes in the presence and absence of added DS. Disulfiram was added to appropriate incubations (as described in Materials and Methods) in 10 μl acetone; control incubations contained an equal amount of acetone. In order to evaluate changes in the kinetics of EtM *N*-demethylation, six concentrations of EtM from 0.1 to 1.0 mM were employed. The results of this study are summarized in the Lineweaver–Burk plots [30, 31] presented in Fig. 3. The addition of 1×10^{-5} or 5×10^{-5} M DS to control microsomes increased the K_m for EtM *N*-demethylation by about 170 and 580 per cent, respectively, while the V_{max} for the reaction was only slightly decreased (by 10 and 30 per cent respectively). Therefore, inhibition *in vitro* of EtM metabolism by DS appears to be competitive in nature with an inhibitor constant (K_i) of approximately 1.2×10^{-5} M calculated according to the formula $K_i = K_m[I]/K_m' - K_m$, where K_m represents the Michaelis constant in the absence of inhibitor and K_m' represents the apparent Michaelis constant in the presence of DS at a given concentration $[I]$ [30, 31].

Since the first step in oxidative metabolism of foreign compounds by hepatic microsomal enzymes appears to be the binding of substrate to ferric cytochrome P-450 [33], the experiment summarized in Fig. 4 was carried out. Disulfiram, like EtM, binds to cytochrome P-450 producing a type I spectrum, and may therefore inhibit EtM metabolism by competing with the substrate for binding sites on cytochrome P-450. The affinity constant (K_s) and maximum spectral shift (ΔA_{max}) between 390 nm (peak) and

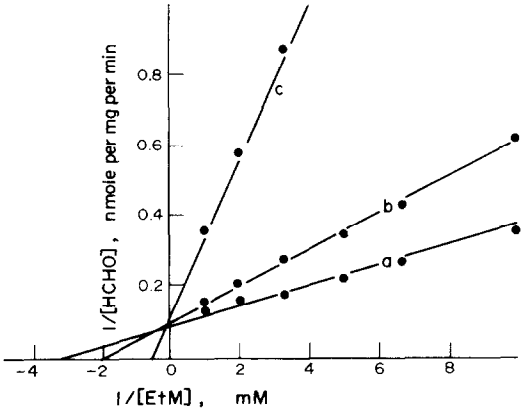

Fig. 3. Effect of DS on the kinetics of Etm metabolism *in vitro*. Key: (a) control; (b) 1×10^{-5} M DS; and (c) 5×10^{-5} M DS. The K_m (mM) and V_{max} (nmoles HCHO/mg of protein/min) were calculated by means of a computer program [32]. These values ($K_m \pm$ S.E.M./$V_{max} \pm$ S.E.M.) under the described incubation conditions were: (a) 0.29 ± 0.01/10.99 ± 0.14; (b) 0.49 ± 0.01/9.88 ± 0.13; and (c) 1.69 ± 0.08/7.33 ± 0.25.

423 nm (trough) for DS were calculated for microsomes prepared from five male rats. These values (mean ± S. E. M.) were: K_s ($M \times 10^5$) = 1.17 ± 0.19 and ΔA_{max} ($A_{390-423\ nm}$/mg protein) = 0.028 ± 0.004.

Since DS is reduced to the corresponding thiol, diethyldithiocarbamate (DDTC), upon administration *in vivo* to the rat [4], studies *in vitro* were also carried out with this metabolite. Unlike DS, DDTC produced no detectable binding spectrum when the compound was added to control microsomes or to microsomes prepared from phenobarbital-treated rats. However, DDTC was found to competitively inhibit microsomal EtM *N*-demethylation, but it was considerably less potent than equimolar concentrations of DS; the K_i for DDTC was calculated to be 1.8×10^{-4} M, an order of magnitude greater than the K_i for DS (1.2×10^{-5} M).

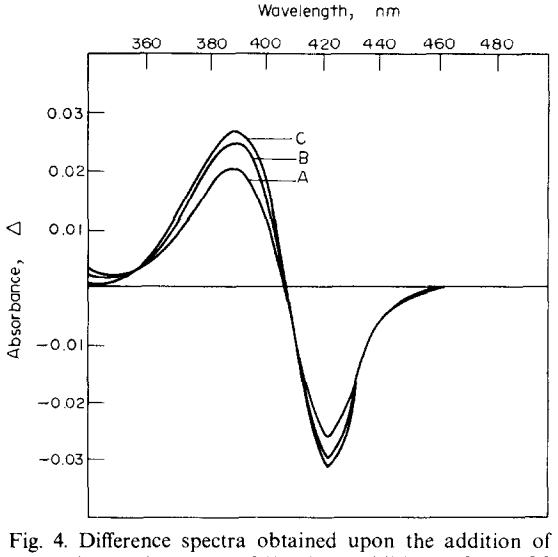

Fig. 4. Difference spectra obtained upon the addition of DS to liver microsomes. Microliter additions of 30 mM DS in ethanol were made to microsomes from a control male rat containing 0.733 nmole cytochrome P-450/mg of protein, and difference spectra were recorded. The final DS concentrations were: (A) 2×10^{-5} M; (B) 4×10^{-5} M; and (C) 8×10^{-5} M.

DISCUSSION

These studies clearly demonstrate that repeated administration of DS to rats results in prolonged impairment of microsomal drug metabolism. Prolonged impairment of drug oxidation during chronic DS treatment is in contrast to results obtained with several other "inhibitors" of drug metabolism. For example, daily administration of SKF-525A (2-diethylaminoethyl 2,2-diphenyl-valerate HCl) for 5 days produced initial inhibition followed by induction of drug oxidation [34]. Moreover, in a study of several other inhibitors, Kato *et al.* [35] found that DEPA(2,4-dichloro-6-phenyl-phenoxyethylamine), Lilly 18947 (2,4-dichloro-6-phenyl-phenoxyethyl-*N*-*N*-diethylamine HBr) and CTF 1201 (diethylaminoethylphenyldiallyl acetate) inhibited microsomal drug oxidation shortly after administration but induced drug metabolism 48 hr after pretreatment. No such biphasic pattern was observed during chronic DS administration (Figs. 1 and 2) or from 4 to 168 hr after a single oral dose of 1.0 or 2.0 g/kg (data not shown).

Data presented in Figs. 1 and 2 also indicate that the rate of onset, maximum effect and persistence of impairment of drug metabolism by DS are different during administration of the high (0.4 g/kg) or low (0.1 g/kg) dose of the compound. After 0.4 g/kg, maximum effects were achieved after two daily doses and persisted without significant change throughout the experimental period. In contrast, the maximum effects produced by 0.1 g/kg of DS were not seen until 5 days of treatment and both EtM *N*-demethylation and cytochrome P-450 levels had returned to near control levels by day 12, despite continued DS treatment. These data suggest that administration of low therapeutic doses of DS to man may result in relatively small effects on drug metabolism during chronic therapy.

When EtM *N*-demethylase activity during daily DS treatment was calculated in terms of cytochrome P-450 levels, activity in treated animals was significantly below control activity in only one case (0.4 g/kg of DS for 2 days). In contrast, ANL hydroxylase was generally lower in treated rats even when activity was calculated in terms of microsomal cytochrome P-450 content. Therefore, impairment of EtM metabolism appears to be due primarily to depression of cytochrome P-450, at least after 48 hr, while additional factors are involved in impairment of ANL metabolism. The exact nature of the interactions responsible for the more "selective impairment" of ANL hydroxylase are not known. However, it should be emphasized that the metabolism of a type II compound such as aniline is often affected differently from that of type I compounds after exposure of an animal to chemicals such as polycyclic hydrocarbons [36] and urethane [37]. Moreover, since microsomes prepared from DS-pretreated rats metabolize ANL at a reduced rate in the absence of overt changes in cytochrome P-450 levels, the interaction could involve covalent binding of DS or a metabolite to a microsomal component essential for ANL hydroxylation or may represent a "permanent inactivation" of this component.

In addition to the depression of cytochrome P-450 levels produced by DS administration, studies *in vitro* indicate that DS may also impair drug metabolism by competing with the drug substrate for hepatic microsomal enzymes. DS was shown to bind to cytochrome P-450 producing a type I spectrum (Fig. 4). Therefore, it appears that the two type I compounds, DS and EtM, compete for the same binding site on cytochrome P-450, since DS competitively inhibits EtM *N*-demethylation (Fig. 3). Moreover, results *in vitro* indicate that the reduced metabolite of DS, diethyldithiocarbamate, is a comparatively weak inhibitor of EtM metabolism and does not bind to cytochrome P-450 as does the oxidized disulfide.

Although daily DS administration depresses microsomal cytochrome P-450 levels, it increases or does not alter cytochrome b_5 content (Table 1). These results are consistent with the observations of Stripp *et al.* [14] who found that, 24 hr after a single dose of DS (200 mg/kg, i.p.), there was a significant decrease in cytochrome P-450 and no change in cytochrome b_5 levels in microsomes isolated from treated rats. Although the actual mechanism by which DS lowers cytochrome P-450 levels is not known, the above observations indicate that the mechanism does not involve a generalized heme destruction or impairment of heme synthesis in the liver.

Since cytochrome *c* reduction was catalyzed equally well by microsomes from control or DS-treated rats (Table 1), chronic DS administration apparently had no direct effect on NADPH-cytochrome *c* reductase of rat liver microsomes. However, microsomal reductase activity measured with cytochrome P-450 as the terminal electron acceptor (NADPH-cytochrome P-450 reductase) was significantly decreased by 37 or 45 per cent after 2 or 8 days of treatment (Table 1). Since NADPH-cytochrome *c* reductase and NADPH-cytochrome P-450 reductase are now assumed to be the same enzyme [38], apparent differences depend on the substrate used to assay its activity. Cytochrome *c* is added to microsomes at approximately 10 times its K_m concentration [39] and, therefore, the reaction proceeds at maximum velocity. However, in the NADPH-cytochrome P-450 reductase assay, the substrate concentration is determined by the level of cytochrome P-450 in the microsomal membrane and cannot be varied as can cytochrome *c*. Although the K_m for cytochrome P-450 in the reaction is unknown, if the cytochrome P-450 content of control or treated microsomes is near the K_m, lower levels in microsomes from DS-treated animals would be expected to result in proportionally lower NADPH-cytochrome P-450 reductase activity. Such appears to be the case since after 2 or 8 days of DS treatment, decreases in NADPH-cytochrome P-450 reductase activity roughly paralleled decreases in microsomal cytochrome P-450 levels. Therefore, chronic DS administration does not appear to decrease the level of microsomal flavoprotein reductase, but does decrease the rate at which cytochrome P-450 is reduced, presumably by depressing microsomal cytochrome P-450 content.

Since several investigators demonstrated that alterations in the fatty acid (FA) content of the microsomal membrane can affect drug oxidation [40, 41], microsomal FA content was determined by gas–liquid chromatography during chronic DS administration (data not shown). The total FA content of microsomes isolated from DS-treated rats was slightly lower than control animals (by approximately 10–20 per cent). However, the slight decrease in total FA content was not due to loss of a particular class of FA, since each FA measured (16:0, 18:0, 18:1, 18:2, 20:4, 22:6) was decreased in treated animals to approximately the same extent. Therefore, significant alterations in the content or relative distribution of microsomal fatty acids apparently do not contribute to the observed impairment of microsomal drug metabolism by DS.

Recent studies in man suggest that daily administration of DS results in a relatively constant degree of impairment in drug metabolism. For example, the prolongation of the half-life of antipyrine in persons receiving DS (7 mg/kg) for 4 days was practically identical to that observed after 10 days of treatment [10]. Moreover, a similar inhibition of warfarin hydroxylation has also been reported in normal volunteers given 250 or 500 mg of DS daily from 3 to 21 days [42]. These observations in man and our observations in rats given 0.4 g/kg of DS daily indicate that, during chronic therapy with relatively high doses of DS, a prolonged impairment of drug oxidation occurs. Therefore, the dose of other drugs given concurrently with commonly used doses of DS (250–500 mg/day) must be closely evaluated. On the other hand, results obtained after administration of 0.1 g/kg of DS daily to the rat indicate that at lower doses the impairment of drug oxidation by DS is not cumulative with continuing therapy; in fact, microsomal enzyme activity may return to near control levels after several days of continued DS administration. Since administration of relatively low doses of DS to the rat results in only transitory impairment of hepatic microsomal enzymes, it would be of interest to determine the effect of low daily doses of DS on drug biotransformation in man. If such studies revealed that low doses of DS did not produce cumulative impairment of drug metabolism in man, these data would be valuable in treating the alcoholic patient with DS while minimizing adverse drug reactions resulting from impaired bio-transformation of drugs given concurrently with DS.

REFERENCES

1. J. Hald and E. Jacobsen, *Acta pharmac. tox.* **4,** 304 (1948).
2. J. Hald, E. Jacobsen and V. Larsen, *Acta pharmac. tox.* **4,** 285 (1948).
3. H. Casier and E. Merlevede, *Archs int. Pharmacodyn. Thér.* **139,** 165 (1962).
4. J. H. Strömme, *Biochem. Pharmac.* **14,** 393 (1965).
5. M. Goldstein, *Pharmac. Rev.* **18,** 77 (1966).
6. J. H. Strömme, *Biochem. Pharmac.* **12,** 157 (1963).
7. A. Nygaard and J. Sumner, *Archs Biochem. Biophys.* **39,** 119 (1952).
8. D. Richert, R. Vanderline and W. Westerfield, *J. biol. Chem.* **186,** 261 (1950).
9. A. H. Neims, D. S. Coffey and L. Hellerman, *J. biol. Chem.* **241,** 5941 (1966).
10. E. S. Vesell, G. T. Passananti and C. H. Lee, *Clin. Pharmac. Ther.* **12,** 785 (1971).
11. O. V. Olesen, *Acta pharmac. tox.* **24,** 317 (1966).
12. E. Rothstein, *J. Am. med. Ass.* **206,** 1574 (1968).

13. M. A. Zemaitis and F. E. Greene, *Biochem. Pharmac.*, **25**, 453 (1976).
14. B. Stripp, F. E. Greene and J. R. Gillette, *J. Pharmac. exp. Ther.* **120**, 347 (1969).
15. T. Honjo and K. J. Netter, *Biochem. Pharmac.* **18**, 2681 (1969).
16. M. A. Zemaitis, W. R. Blackburn and F. E. Greene, *Pharmacologist* **15** (No. 2), 191 (1973).
17. L. Lundwall and F. Baekeland, *J. nerv. ment. Dis.* **153**, 381 (1971).
18. A. G. Gornall, C. S. Bardawill and M. M. David, *J. biol. Chem.* **177**, 751 (1949).
19. T. Omura and R. Sato, *J. biol. Chem.* **239**, 2370 (1964).
20. T. Omura and R. Sato, *J. biol. Chem.* **239**, 2379 (1964).
21. A. H. Phillips and R. G. Langdon, *J. biol. Chem.* **237**, 2652 (1962).
22. P. L. Gigon, T. E. Gram and J. R. Gillette, *Biochem. biophys. Res. Commun.* **31**, 558 (1968).
23. J. R. Gillette, B. B. Brodie and B. N. LaDu, *J. Pharmac. exp. Ther.* **119**, 532 (1957).
24. I. Nash, *Biochem. J.* **55**, 416 (1953).
25. Y. Imai, A. Ito and R. Sato, *J. Biochem., Tokyo* **60**, 417 (1966).
26. R. S. Chhabra, T. E. Gram and J. R. Fouts, *Toxic. appl. Pharmac.* **22**, 50 (1972).
27. D. S. Davies, P. L. Gigon and J. R. Gillette, *Biochem. Pharmac.* **17**, 1865 (1968).
28. H. Remmer, J. B. Schenkman, R. W. Estabrook, H. A. Sesame, J. R. Gillette, S. Narasimhulu, D. Y. Cooper and O. Rosenthal, *Molec. Pharmac.* **2**, 187 (1966).
29. G. J. Mannering, in *Handbook of Experimental Pharmacology* (Eds. B. B. Brodie and J. R. Gillette), Vol. 28, Part 2, pp. 452–76. Springer, New York (1971).
30. M. Dixon and E. C. Webb, in *Enzymes*, 2nd Edn, pp. 63–9. Longmans, Greene & Co., London (1964).
31. M. Dixon and E. C. Webb, in *Enzymes*, 2nd Edn, pp. 327–31. Longmans, Greene & Co., London (1964).
32. W. W. Cleland, *Nature, Lond.* **198**, 463 (1963).
33. J. R. Gillette, D. C. Davis and H. A. Sesame, *A. Rev. Pharmac.* **12**, 57 (1972).
34. M. W. Anders and G. J. Mannering, *Molec. Pharmac.* **2**, 341 (1966).
35. R. Kato, E. Chiesara and P. Vassanelli, *Biochem. Pharmac.* **13**, 69 (1964).
36. H. Remmer, *Revue Can. Biol.* **31**, 193 (1972).
37. R. I. Glazer, D. L. Cinti, R. I. Marahata, J. B. Schenkman and A. C. Sartorelli, *Biochem. Pharmac.* **21**, 2867 (1972).
38. B. S. S. Masters, E. B. Nelson, B. A. Schacter, J. Baron and E. L. Isaacson, *Drug Metab. Dispos.* **1**, 121 (1973).
39. C. H. Williams and H. Kamin, *J. biol. Chem.* **237**, 587 (1962).
40. B. Century, *J. Pharmac. exp. Ther.* **185**, 185 (1973).
41. W. P. Norred and A. E. Wade, *Biochem. Pharmac.* **21**, 2887 (1972).
42. R. A. O'Reilly, *Ann. intern. Med.* **78**, 73 (1973).