Biochemical Pharmacology, Vol. 28, pp. 621–626

0006-2952/79/0301-0621 $02.00/0

SULFOXIDATION OF CHLORPROMAZINE AND THIORIDAZINE BY BOVINE LIVER—PREFERENTIAL METABOLIC PATHWAYS

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(*Received* 15 *April* 1978; *accepted* 5 *July* 1978)

Abstract—The sulfoxidation of chlorpromazine (Thorazine), thioridazine (Mellaril), and some of their metabolites by a crude microsomal preparation from bovine liver was examined in an *in vitro* system which enables a rapid assessment of phenothiazine metabolism by various mammalian tissues. The enzymatic sulfoxidation of these drugs was found to require the presence of certain nicotinamide dinucleotide factors in their reduced form. Maximum activity was observed in the presence of NADPH. Preferential sulfoxidation pathways were found to differ between these two antipsychotic agents governing whether they are converted to pharmacologically active or inactive metabolites. Chlorpromazine undergoes sulfoxidation to form its inactive ring-sulfoxide; in contrast, the sulfoxidation of thioridazine occurs preferentially at its side-chain sulfur residue to form mostly mesoridazine, a pharmacologically active metabolite. The ring-sulfur residue of thioridazine, unlike that of chlorpromazine, was very resistant toward this enzymatic process. In addition, various drugs including chloroquine and some of its analogues were tested for their ability to inhibit the formation of phenothiazine ring-sulfoxide.

There has been increasing evidence suggesting that the metabolism of antipsychotic drugs is of major importance in their action and therapeutic efficacy [1–7]. Enzymes associated with the liver cell endoplasmic reticulum catalyze a variety of drug-metabolizing reactions including hydroxylation, dealkylation, epoxide and *N*-oxide formation [8, 9]. More important in the metabolism of phenothiazines is sulfoxidation which represents a major metabolic pathway in humans. This is clinically important since sulfoxidation of these drugs can produce both pharmacologically active and inactive metabolites [10]. Nonetheless, no comprehensive work has been carried out to study the nature of these enzymes, including their mechanism of action and substrate specificity. Recently, however, Prema and Gopinathan [11] have purified an enzyme characterized as a mono-oxygenase from guinea pig liver which catalyzes the sulfoxidation of ethionamide as well as chlorpromazine. This enzyme is a flavoprotein and specifically requires nicotinamide adenine dinucleotide phosphate in the reduced form (NADPH) as a cofactor. The oxidative mechanism was found to be mediated by superoxide anions (O_2^-), since complete inhibition was observed in the presence of superoxide dismutase.

In view of the fact that many chlorpromazine non-responders have been found to have higher plasma levels of chlorpromazine-sulfoxide than responders [1–3, 12–15] and that EKG abnormalities in some cases during thioridazine treatment are associated with high plasma levels of ring-sulfoxide [16], we have compared the sulfoxidation of chlorpromazine to that of thioridazine since this latter drug has an active side-chain ring-sulfoxide [10]. A preliminary study was carried out to investigate some possible inhibitors of phenothiazine ring-sulfoxidation which might be applicable as adjuncts to phenothiazine therapy in those drug non-responders who are found to have high plasma concentrations of the inactive ring-sulfoxide. These studies were performed in an *in vitro* system, enabling a rapid assessment of phenothiazine metabolism.

MATERIALS AND METHODS

Materials. Pure chlorpromazine (Thorazine) and chlorpromazine-5-sulfoxide (ring-sulfoxide) were gifts from Smith, Kline & French Labs, Philadelphia, PA. Authentic samples of thioridazine (Mellaril), thioridazine-5-sulfoxide (ring-sulfoxide), thioridazine-2-sulfoxide [side-chain sulfoxide (Mesoridazine)] thioridazine-2-sulfone [side-chain sulfone (Sulforidazine)] and thioridazine-2,5-disulfoxide were kindly supplied by Sandoz Co., East Hanover, NJ. Chloroquine diphosphate, quinacrine dihydrochloride, piperazine hexahydrate, hexylresorcinol, riboflavin, quinine sulfate, NADPH, NADP, α-NADH, α-NAD, β-NADH, β-NAD, FMN, FAD, NH_xDPH (nicotinamide hypoxanthine dinucleotide phosphate) and bovine serum albumin were purchased from Sigma Chemical Co., St. Louis, MO. Galactoflavin was a gift from Merck, Sharp & Dohme, Rahway, NJ. Pyrimethamine was from Burroughs Wellcome, Research Triangle Park, NC, and chlorpheniramine was from Schering Co., Kenilworth, NJ. Cyclophosphamide was a gift from Mead Johnson, Evansville, IN.

Tissue samples. Fresh bovine livers were washed with an ice-cold solution of 1.15% (w/v) NaCl to remove excess blood. The livers, after blotting dry, were then weighed and resuspended in 3 vol. of the fresh ice-cold saline solution, and all subsequent operations were carried out at 0–4°. The tissues were scissor-minced, and then homogenized in a cold Kontes–Duall, glass–Teflon homogenizer operated by an electric motor (1500 rev/min).

To prepare a crude microsomal fraction, the homogenates were subjected to 40,000 *g* centrifugation for

15 min. The supernatant fraction was decanted into a clean tube and centrifuged once more at 48,000 *g* for 20 min. The protein content was determined by the method of Lowry *et al.* [17] using bovine serum albumin as a standard.

Enzyme assay and quantitation of phenothiazine metabolites. In order to provide a rapid and simple *in vitro* assessment of chlorpromazine and thioridazine sulfoxidation, the following method was developed. Enzyme activity was assessed by the level of decreasing substrate, as well as by the amount of each metabolite formed. A typical reaction mixture contained, in a final volume of 500 μl, 1.19 μmoles NADPH or other nicotinamide adenine dinucleotide and flavin analogues as specified in Fig. 5, 0.1 M sodium phosphate buffer containing 1.87 mM KCl, 0.80 mM $CaCl_2 \cdot 2H_2O$, 0.50 mM $MgCl_2 \cdot 6H_2O$ titrated to pH 6.5 with Na_2CO_3, and 75 μl of tissue preparation (3.5 mg protein and 150 μg phenothiazine substrate). Incubations were carried out in 10 × 1.5 cm glass tubes in an oscillating water bath (80 oscillations/min) at 37° for 60 min under an atmosphere of 95% O_2/5%CO_2. Tubes without tissue and/or with tissue in the absence of NADPH were used as controls. Termination of the reaction was carried out by placing the assay tubes in a boiling water bath for 2.0 min, and then, after cooling, centrifuging at 5000 *g* for 10 min. The reaction was linear with all protein (1–5 mg) time (30–120 min), and substrate conditions (50–200 μg) tested.

Separation and quantitation of metabolic products were carried out by applying 60 μl of the supernatant fraction directly onto the diatomaceous earth spotting pad on a Kontes quantum, silica gel thin-layer chromatography (t.l.c.) plate (LQD No. 5090, 19 channel). The t.l.c. plate was developed in a tank equilibrated with the following solvent system: acetone–chloroform–dichloroethane–NH_4OH (20:10:10:1). This solvent system has the advantage that added NADPH and soluble liver components remain at the origin or migrate with the solvent front, thereby providing a large uncontaminated surface for the measurement of phenothiazines and their sulfoxides (Table 1).

For chlorpromazine and metabolites, color development was carried out by spraying the air-dried t.l.c. plate with a chromogenic agent containing 50 ml of 18 N H_2SO_4, 150 ml ethanol and 0.5 g ferric chloride [2]. The plate was then heated in an oven at 90–100° for 10–15 min (chlorpromazine and metabolites appear as red spots). Since thioridazine and its metabolites are not as responsive to this chromogenic spray as chlorpromazine, fluorescence techniques were employed as described previously [5]. The plate containing thioridazine and metabolites was sprayed with a solution of H_2O_2 in aqueous acetic acid (2.0 ml of 30% H_2O_2 + 2.0 ml glacial acetic acid + 96 ml H_2O) and allowed to remain at room temperature for 5–10 min (spot development can be monitored under u.v. light). Both plates were then scanned automatically with the use of a Schoeffel double-beam spectrodensitometer (model SD 3000-2) in conjunction with a Schoeffel density integrator computer (model SDC-300). Chlorpromazine and metabolites were scanned at a wavelength of 530 nm in the transmission mode, while thioridazine and metabolites were scanned at λ_{ex} 325 nm in the fluorescence mode. For fluorescence measurement the spectrodensitometer was affixed with a $\lambda_{fl} > 420$ nm filter. Standard curves were prepared by using a mixture of authentic samples of these compounds. A unit of enzyme activity is defined as the amount of enzyme required to catalyze the formation of 1 nmole of the phenothiazine sulfoxide/min at 37°. Specific activity is expressed as enzyme units/mg of protein.

Table 1. Thin-layer chromatography of chlorpromazine, thioridazine and their sulfoxide metabolites

Compound	R_f*
Chlorpromazine	0.60
Chlorpromazine-5-sulfoxide	0.41
Thioridazine	0.60
Thioridazine-5 sulfoxide	0.38
Thioridazine-2-sulfoxide (mesoridazine)	0.28
Thioridazine-2-sulfone (sulforidazine)	0.50
Thioridazine 2, 5-disulfoxide†	0.15

* Solvent system: acetone–chloroform–dichloroethane–NH_4OH (20:10:10:1).

† See Ref. 18.

RESULTS

Chlorpromazine was metabolized mainly to its pharmacologically inactive ring-sulfoxide by the crude microsomal preparation when supplied with NADPH (Fig. 1). No sulfoxidation was observed in the absence of NADPH; however, the production of chlorpromazine-5-sulfoxide was related to the dose of NADPH added to the incubation. Significant increases in the ring-sulfoxide occurred with as little as 0.5 μmoles NADPH (26 per cent conversion), while maximum

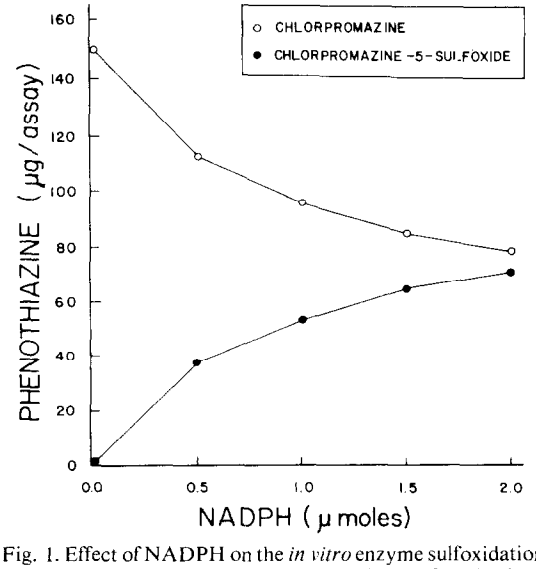

Fig. 1. Effect of NADPH on the *in vitro* enzyme sulfoxidation of chlorpromazine. Incubations were carried out for 90 min at 37° with 75 μl tissue (3.5 mg protein) and 150 μg chlorpromazine/assay tube. Various doses of NADPH as indicated were added to the incubation. Each value is the mean of six experiments; S.E. < 4.3.

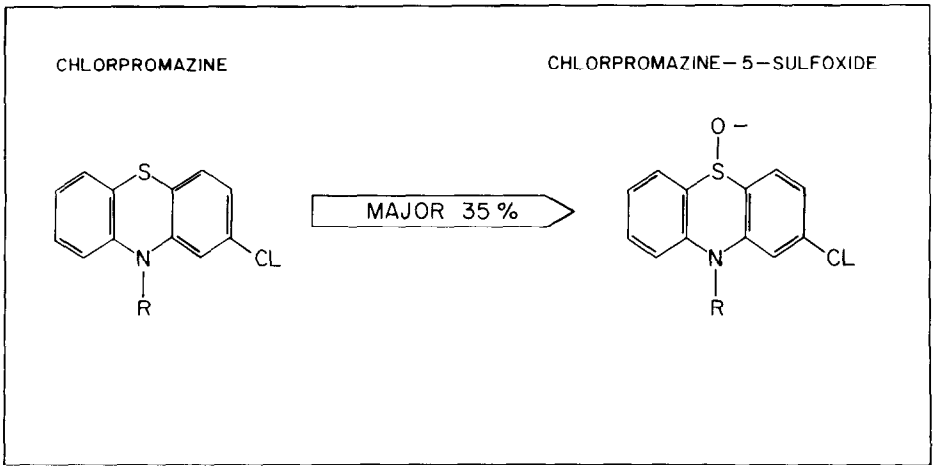

Fig. 2. Preferential major sulfoxidation pathways of chlorpromazine (see text). R = $-(CH_2)_3-N(CH_3)_2$.

enzyme activation was observed with 2.0 μmoles of this factor (47 per cent conversion). The decrease in chlorpromazine concentration was linearly related to the increase in its sulfoxide (specific enzyme activity was 0.49).

To determine if the ring-sulfoxide could undergo further sulfoxidation (i.e. to sulfone), authentic chlorpromazine-5-sulfoxide was used as the starting substrate in place of chlorpromazine, and incubations were carried out as described in Materials and Methods with various doses of NADPH. The results of this experiment showed no further sulfoxidation of chlorpromazine-5-sulfoxide. Figure 2 summarizes the major preferential route of sulfoxidation of this phenothiazine by liver in the presence of 1.19 μmoles NADPH.

When thioridazine was used as a substrate, contrasting results were observed (Fig. 3). Although sulfoxidation was similarly dependent on the quantity of NADPH present, this *in vitro* system preferentially metabolized to the pharmacologically active thioridazine-2-sulfoxide (mesoridazine, 43 per cent maximum conversion) instead of the inactive thioridazine-5-sulfoxide (10 per cent maximum conversion). In addition, up to 4 per cent conversion to the pharmacologically active thioridazine-2-sulfone (sulforidazine) was also detected (specific enzyme activity was 0.44). levels of parent thioridazine decreased proportionally to the sum of increasing levels of these three metabolites. The total per cent conversion to mesoridazine closely resembled the amount of chlorpromazine-5-sulfoxide formed from chlorpromazine under equivalent conditions (compare Figs. 1 and 3).

In order to determine the major and minor preferential metabolic routes of these thioridazine metabolites, each authentic compound, mesoridazine, sulforidazine thioridazine-5-sulfoxide, was individually used as a substrate in this system. The preferential metabolic sulfoxidation pathways of these compounds are summarized in Fig. 4. In the presence of 1.19 μmoles NADPH, both mesoridazine and thioridazine-5-sulfoxide favored thioridazine-2, 5-disulfoxide formation with 7 and 8 per cent conversion, respectively, while mesoridazine also produced small quantities of sulforidazine (4 per cent conversion). Interestingly, sulforidazine was preferentially metabolized to mesoridazine with over 19 per cent conversion via the removal of one oxygen residue from the side-chain sulfur group.

The effects of other nicotinamide nucleotide analogues and flavin compounds were studied (Fig. 5). The addition of NADPH and NH_xDPH produced highest activities with 40 and 37 per cent of chlorpromazine to its sulfoxide respectively. The unphosphorylated analogues produced about half the activity with β-NADH producing 21 per cent conversion and α-NADH producing about 13 per cent conversion. The unreduced forms of all these compounds were inactive. Of the flavin compounds examined, riboflavin and flavin mon-

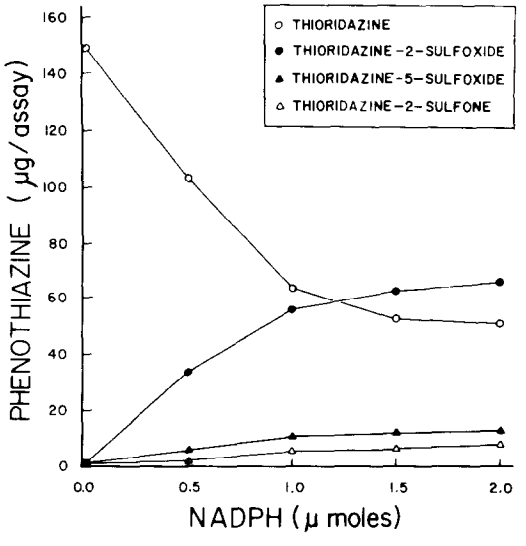

Fig. 3. Effect of NADPH on the *in vitro* sulfoxidation of thioridazine. Incubations were carried out for 90 min at 37° with 75 μl tissue (3.5 mg protein) and 150 μg chlorpromazine/assay tube. Various doses of NADPH as indicated were added to the incubation. Each value is the mean of six experiments; S. E. < 3.1.

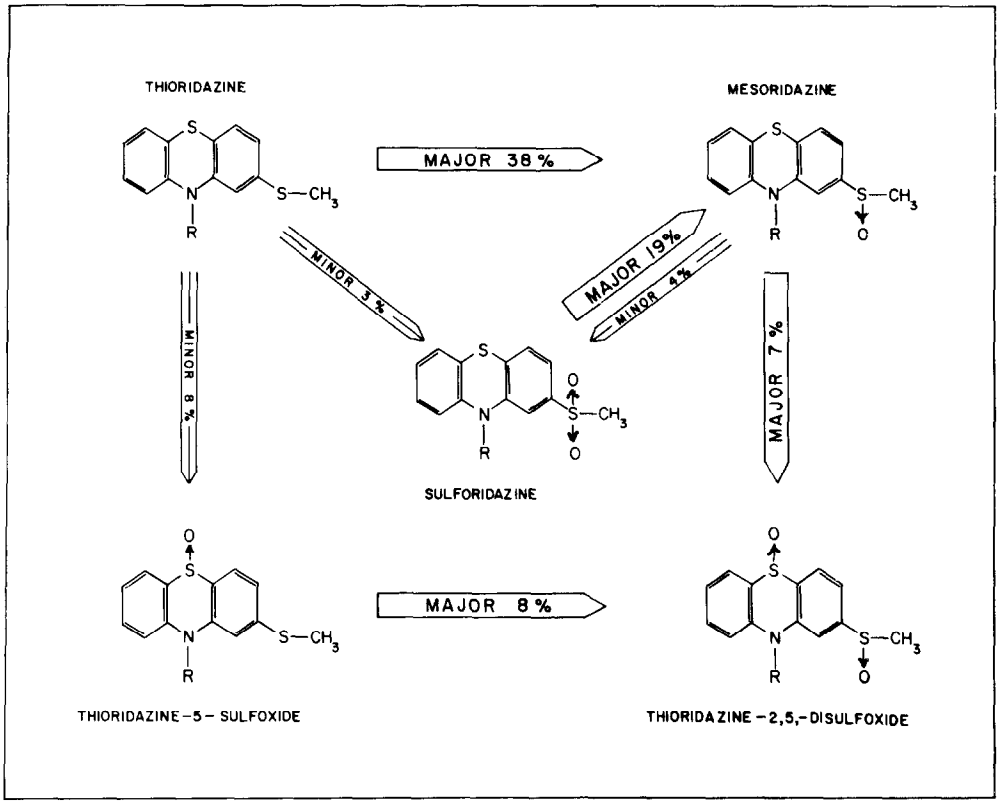

Fig. 4. Preferential major and minor sulfoxidation pathways of thioridazine, thioridazine-5-sulfoxide, mesoridazine and sulforidazine (see text).

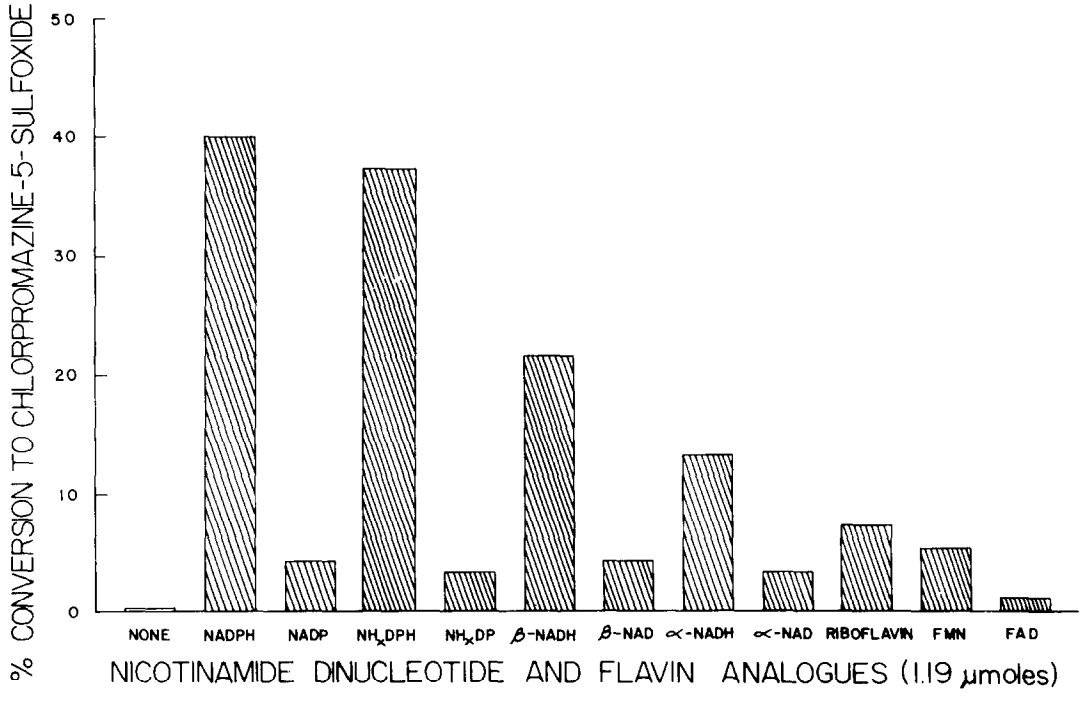

Fig. 5. Effect of nicotinamide adenine dinucleotide and flavin analogues on the *in vitro* sulfoxidation of chlorpromazine. Incubations were carried out for 90 min at 37° with 75μl tissue (3.5 mg protein) and 150μg chlorpromazine/assay tube with the addition of 1.19μ moles of each analogue as specified.

onucleotide gave 8 and 5 per cent conversion, respectively, while flavin adenine dinucleotide was not effective.

As previously reported [19], chloroquine was found to potentiate the neuroleptic effects of thioridazine in both rats and drug-resistant schizophrenic patients, possibly by the inhibition of thioridazine-5-sulfoxide. In light of this observation, we decided to test the effects of chloroquine and some related compounds in our *in vitro* system for their ability to inhibit the formation of the pharmacologically inactive phenothiazine ring-sulfoxide. These results are summarized in Table 2.

While the presence of chloroquine (10^{-3}M) resulted in 10 per cent inhibition of ring sulfoxide formation, both quinacrine and hydroxychloroquine produced about 30 per cent inhibition at the same concentration. The most effective of this class was quinine sulfate with over 60 per cent inhibition at 10^{-3} M. Galactoflavin, a more specific riboflavin anagonist, produced 55 per cent at a similar concentration. Finally, cyclophosphamide was tried in light of recent reports suggesting that this drug improves the clinical response of phenothiazine non-responders [20] via an immunosuppression mechanism. This drug had no effect on ring-sulfoxidation even at 10^{-3} M.

DISCUSSION

The results of this study suggest that metabolic sulfoxidation pathways in liver may differ for chlorpromazine and thioridazine, governing whether these phenothiazines are converted to pharmacologically active or inactive metabolites. The sulfoxidation of chlorpromazine and thioridazine by a crude microsomal fraction from bovine liver specifically requires NADPH for maximum activity. Slight changes in the molecular structure of this nicotinamide dinucleotide, i.e. replacing the amino group in adenine with an OH group to form hypoxanthine (NH_xDPH), resulted in slightly lower activity. Furthermore, the unphosphorylated nicotinamide dinucleotides, α-NADH and β-NADH, were only about half as active as NADPH. The unreduced form of each individual nicotinamide dinucleotide factor tested did not activate the microsomal enzyme(s). Flavin compounds could not substitute for the nicotinamide factors. The order of decreasing effectiveness to activate the enzymatic sulfoxidation of chlorpromazine was: NADPH > NH_xDPH > β-NADH > α-NADH > riboflavin > FMN > FAD. The exact physiological utilization of these factors in the regulation of *in vivo* human phenothiazine sulfoxidation remains to be determined.

Table 2. *In vitro* inhibition of chlorpromazine-5-sulfoxide formation by various drugs

Drug	Final concn (M)		
	10^{-3}	10^{-4}	10^{-5}
Chloroquine	10*	6	0
Quinacrine	34	11	0
Hydroxychloroquine	30	9	0
Quinine	63	25	8
Pyrimethamine	0	—†	—
Chlorpheniramine	0	—	—
Piperazine	14	0	—
Hexylresorcinol	0	—	—
Riboflavin	0	—	—
Galactoflavin	55	—	—
Cyclophosphamide	0	—	—

* Per cent inhibition of chlorpromazine-5-sulfoxide formation.

† Not tested.

In this *in vitro* enzyme system, chlorpromazine was metabolized mostly to its pharmacologically inactive metabolite, chlorpromazine-5-sulfoxide. No further sulfoxidation of this metabolite was detected. In contrast, thioridazine undergoes sulfoxidation preferentially at its side-chain residue to form mesoridazine, an active metabolite. Only small amounts of thioridazine-5-sulfoxide (inactive) and sulforidazine (active) were detected. These results suggest that *in vitro* phenothiazine ring-sulfoxidation differs for chlorpromazine and thioridazine. The side-chain sulfur atom of the thiorida zine appears to be the preferred site of sulfoxidation rather than the ring-sulfur. This preference may possibly be due to conformational or electron charge density differences in the phenothiazine nucleus of thioridazine and chlorpromazine. Further sulfoxidation of the thioridazine metabolites themselves revealed both thioridazine-5-sulfoxide and mesoridazine to favor the formation of thioridazine-2, 5-disulfoxide although only about 8 per cent conversion was observed in both cases. In addition, mesoridazine also produced small quantities of sulforidazine (4 per cent). Surprisingly, sulforidazine converts back to mesoridazine (19 per cent) via the removal of one oxygen from the side-chain sulfone. These results suggest that mesoridazine is the more preferred metabolite.

Finally, the effects of various drugs on the *in vitro* sulfoxidation of chlorpromazine were examined since in previous studies [19] we found chloroquine to potentiate the effects of thioridazine in rats and in some drug-resistant schizophrenic patients, possibly by ring-sulfoxidation inhibition. Although chloroquine at 10^{-3} M caused only 10 per cent inhibition, the most potent of this drug class was quinine sulfate, which produced 63 per cent inhibition at 10^{-3} M while quinacrine and hydroxychloroquine were half as active at the same concentration, producing 34–30 per cent lowering of ring-sulfoxidation. Galactoflavin, a more specific riboflavin antagonist, produced 53 per cent inhibition at 10^{-3} M. Other drug classes examined had little or no effect. In conclusion, research in the development of an adjunct to phenothiazine therapy in schizophrenic patients who produce high plasma levels of phenothiazine ring-sulfoxide might begin with the investigation of various derivatives of these antimalarial agents.

The significance of our findings lies in the future possibility of controlling the metabolism of phenothiazines to direct them along the desired metabolic pathway so that these compounds might be administered more efficaciously, thereby increasing the per cent of active versus inactive metabolites, thus improving patient response and possibly reducing undesirable side effects. Although preferential metabolic pathways in humans may not proceed in the same manner that they do in bovine liver, our preliminary studies show similar preferential metabolic sulfoxidation pathways of chlor-

promazine and thioridazine in *in vitro* human liver investigations and in *in vivo* dog studies.*

Acknowledgement—We wish to thank Dr. B. Kinon for his keen interest and excellent comments concerning this research.

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* L. J. Traficante *et al.*, manuscript in preparation.