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INHIBITION OF HEPATIC DRUG-METABOLIZING ENZYMES BY PYRIDOXINE-5-DISULFIDE ·2HCl·2H$_2$O (PYRITHIOXINE)

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Abstract—Pyrithioxine inhibits hepatic drug-metabolizing enzymes in male mice and rats both *in vivo* and *in vitro*. When given *in vivo* it does not affect the activity *in vitro* of G-6-P dehydrogenase, nor the level of microsomal cytochrome P-450. Pyrithioxine inhibits the hepatic drug-metabolizing enzymes competitively *in vitro*; in male and female rats, the *N*-demethylation of aminopyrine was inhibited by pyrithioxin, but the metabolism of hexobarbital was inhibited only in the male. Pyrithioxine accelerates *in vivo* the uptake of ^{14}C-amino acids into acid-insoluble proteins. Among the metabolites of pyrithioxine, 5-methylthiomethyl-4-hydroxymethyl-2-hydroxy-2-methylpyridine strongly inhibits hepatic drug-metabolizing enzymes both *in vivo* and *in vitro*. Comparable effects can be exhibited by 5′-thiopyridoxal.

PYRITHIOXINE is a derivative of vitamin B_6, and comprises 2 molecules of pyridoxine linked to each other by a disulfide linkage (Fig. 1). It has been shown that pyrithioxine has neither vitamin B_6 nor anti-vitamin B_6 activity.[1–3] A variety of pharmacological

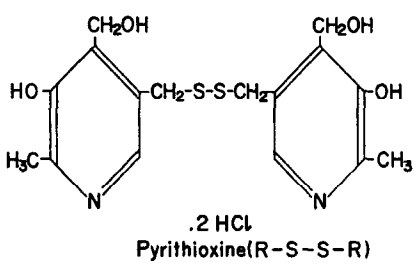

FIG. 1. The structure of pyrithioxine.

actions have been reported for pyrithioxine: it blocks perhenazine-induced catalepsy in rats,[4] modifies the EEG patterns of cats,[5] increases blood flow through *A. carotis* in dogs,[2] and has been claimed to be of value in the treatment of psycho-neurotic conditions.[6–9] Other effects reported for pyrithioxine include barbiturate potentiation and acceleration of intracerebral uptake of glucose.[3]

The present report describes the effect of pyrithioxine upon drug-metabolizing enzymes.

MATERIALS AND METHODS

Chemicals

The following compounds were studied: Pyrithioxine (R—S—S—R), 2-methyl-3-hydroxy-4-hydroxymethyl-5-methyl-thiopyridine·HCl (R—S—CH_3), 2-methyl-3-hydroxy-4-hydroxymethyl-5-methysulfonylpyridine·HCl (R—$\overset{\overset{O}{\|}}{S}$—$CH_3$), 2-methyl-3-hydroxy-4-hydroxymethyl-5-methylsulfinylpyridine·HCl (R—$\overset{\overset{O}{\|}}{S}$—$CH_3$), and 5′-thiopyridoxal·HCl (R—SH), obtained from Chugai Pharmaceutical Co., Japan; and pyridoxine·HCl (R—OH), a product of Wako Pure Chemicals Co., Japan. Glucose 6-phosphate (G-6-P) and nicotinamide adenine dinucleotide phosphate (NADP) were purchased from Sigma Chemical Co. ^{14}C-protein hydrolysate (specific radioactivity, 19·9 mc/m-mole, uniformly labelled) were obtained from Daiichi Chemical Co., Japan.

Treatment of animals

Male ddY strain mice, weighing 20–25 g, and Wister strain rats, weighing 120–150 g, were used. Pyrithioxine was given either intraperitoneally (i.p.) or orally at a dose of either 100 mg/kg or 200 mg/kg dissolved in 0·9 per cent NaCl solution. As the control, 0·9 per cent NaCl solution was given; the pH was adjusted with 0·01 N HCl to that of the test solution (pH 3·8). The dose consisted of a single dose of either 100 mg/kg or 200 mg/kg in experiments to determine variations with time in activities of drug-metabolizing enzymes and to determine effects on protein synthesis. For other experiments, the dose consisted of two doses of 200 mg/kg separated by an interval of 2 hr. Enzyme activities were measured 2 hr after receiving the second dose of pyrithioxine. Quantities of R—S—CH_3, etc., equimolar to that of pyrithioxine, were given to some animals.

Enzyme preparation

Test animals were decapitated and the livers were immediately homogenized in 4 vol. of cold 1·15 per cent KCl solution, using a Teflon-glass homogenizer. The liver homogenates thus obtained were centrifuged at 9000 *g* for 30 min with a Tominaga model S-60 centrifuge. The supernatant was designated the post-mitochondrial supernatant (9000 *g* supernatant). The liver microsomes and the soluble fraction were prepared by ultracentrifuging the above-mentioned 9000 *g* supernatant at 105,000 *g* for 60 min with a Hitachi preparative ultracentrifuge. The supernatant was designated the soluble fraction (105,000 *g* supernatant) and the microsomal pellet was resuspended in the original volume of 1·15 per cent KCl solution. The entire procedure as described above was conducted below 4°.

Enzyme assays

Drug-metabolizing enzyme activity. The incubation mixture consisted of 2·0 ml of the 9000 *g* liver supernatant, 1·0 ml of 0·1 M phosphate buffer, 50 μmoles of $MgCl_2$,

50 μmoles of nicotinamide, 0·27 μmole of NADP, 1·92 μmoles of G-6-P, and the substrate in final volume of 5·0 ml. When rats were used as test animals, 0·68 μmole of NADP and 5·0 μmoles of G-6-P replaced their corresponding amounts in the above system. The quantity of substrate was 5 μmoles each of aminopyrine, meperidine, aniline, procaine, *p*-nitroanisole, and *p*-nitrobenzoic acid, 4 μmoles of hexobarbital, and 1 μmole of *O*-ethyl-*O*-*p*-nitrophenyl phenylphosphonothioate (EPN). The pH of the buffer solution was 7·85 for aminopyrine, EPN, and *p*-nitroanisole, and 7·40 for the remainder. The incubation was conducted at 37° for 30 min, except in the case of procaine where the incubation time was 15 min. Hydrolysis of procaine was linear for 20 min, and the remainder were linear for 40–50 min. We did not observe that nicotinamide had any inhibitory effects on the drug-metabolizing enzymes studied. The enzyme activities were determined by the rate of appearance of products, except for hexobarbital where the activity was followed by the rate of utilization of substrate. In the case of aminopyrine and meperidine, the resulting formaldehyde was quantitatively assayed by the method of Cochin and Axelrod.[10]

In assays with *p*-nitroanisole and EPN, the *p*-nitrophenol produced was determined by the methods of Netter and Siedel[11] and Neal and DuBois.[12] With procaine and *p*-nitrobenzoic acid, the resulting *p*-aminobenzoic acid was determined by the method of Fouts and Brodie.[13] Hexobarbital was quantitatively assayed by the method of Cooper and Brodie.[14]

Assay of G-6-P dehydrogenase. The assay used was a modification of the method of Glock and McLean.[15] Dehydrogenase activity was determined spectrophotometrically by following the rate of reduction of NADP at 340 mμ in 1-cm cells at room temperature. The reaction mixture consisted of 1·0 ml of the 105,000 *g* supernatant, 450 mμmoles of G-6-P, 150 mμmoles of NADP, 50 μmoles of $MgCl_2$, and 1·0 ml of 0·1 M Tris-HCl buffer (pH 7·8) in a total volume of 3·0 ml.

Assay of alcohol dehydrogenase. The assay was carried out by the method of Theorell and Bonnichsen.[16]

Determination of cytochrome b_5 and cytochrome P-450. These assays were conducted by the methods of Omura and Sato.[17] Protein content was assayed by a modification of the method of Gornall and Bardawill.[18]

Uptake of ^{14}C-amino acids. After intraperitoneal administration of 200 mg/kg of pyrithioxine, ^{14}C-chlorella hydrolysates were given intraperitoneally to the animals at a dose of 1 μc/20 g of body weight. Thirty min thereafter, ^{14}C-amino acid incorporated into acid-insoluble protein was assayed by the methods of Koike *et al.*[19] and Otaka *et al.*[20] The cellular fractionation was carried out by the method of Kuroiwa *et al.*[21]

RESULTS

Changes in aminopyrine-N-demethylase activity after pyrithioxine administration. Microsomal *N*-demethylation of aminopyrine was measured at various times after i.p. administration of pyrithioxine (100 or 200 mg/kg). As shown in Fig. 2, the *N*-demethylation of aminopyrine was markedly inhibited by the administration of pyrithioxine. When 200 mg/kg of pyrithioxine was given orally, similar effects were observed. With both routes of administration, the inhibition was found to be maximum at

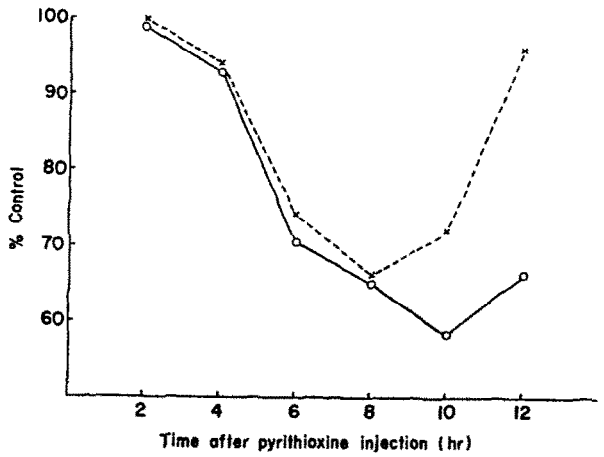

FIG. 2. Time course of the metabolism of aminopyrine in liver after the intraperitoneal administration or pyrithioxine. Male mice were given 100 mg/kg (×———×) and 200 mg/kg (○———○) of pyrithioxine intraperitoneally. The incubation conditions were described in Methods. Nine animals were used in each experiment. Three livers were combined and enzyme activity was measured. Each value represents the average obtained from three different groups of livers.

8–10 hr. In the case of two doses of 200 mg/kg, the inhibitory action was maximum at 2 hr after receiving the second dose of pyrithioxine and lasted for 16 hr.

Inhibitory actions of pyrithioxine on other hepatic microsomal drug-metabolizing enzymes. The effects of pyrithioxine were studied on the following important drug metabolizing reaction, demethylation, hydroxylation, reduction and hydrolysis. As shown in Table 1, all the reactions examined (demethylation, hydroxylation, reduction and hydrolysis) were markedly inhibited by pyrithioxine in male mice. Similarly, it was found that the metabolism of aminopyrine and hexobarbital was inhibited by

TABLE 1. EFFECT OF PYRITHIOXINE TREATMENT ON SOME MICROSOMAL DRUG-METABOLIZING ENZYMES*

Substrate	Enzyme activity		Inhibition (%)
	Control	Pyrithioxine	
Aminopyrine	4·392 ± 0·084	2·876 ± 0·120	34·5
Meperidine	4·816 ± 0·108	2·276 ± 0·148	52·8
p-Nitroanisole	2·228 ± 0·056	1·488 ± 0·032	36·5
EPN	0·048 ± 0·002	0·028 ± 0·001	41·7
Procaine	3·292 ± 0·052	2·368 ± 0·044	28·1
p-Nitrobenzoic acid	0·328 ± 0·044	0·152 ± 0·008	53·7
Hexobarbital	0·750 ± 0·018	0·307 ± 0·029	58·1

* Male mice were injected *i.p.* with pyrithioxine (200 mg/kg) twice. The incubation conditions were described in Methods. The enzyme activities were represented by the mean ± S.E. of metabolites produced in μmoles per g liver per hr, and in the case of hexobarbital, in the mean ± S.E. of the substrate utilized in μmoles per g liver per hr. Groups of six animals were used, the activity being estimated in duplicate on three paired 9000 *g* supernatants.

TABLE 2. EFFECT OF PYRITHIOXINE ADMINISTRATION ON THE METABOLISM OF AMINOPYRINE AND HEXOBARBITAL BY LIVER MICROSOMES FROM MALE AND FEMALE RATS*

Substrate	Enzyme activity		Difference (%)
	Control	Pyrithioxine	
	Males		
Aminopyrine	4·574 ± 0·056	2·179 ± 0·006	−52·4
Hexobarbital	9·667 ± 0·323	1·713 ± 0·141	−82·7
	Females		
Aminopyrine	1·665 ± 0·125	1·117 ± 0·101	−32·9
Hexobarbital	1·171 ± 0·169	1·069 ± 0·139	−8·7

* The rats were treated with pyrithioxine (200 mg/kg, i.p.) twice. The incubation conditions were described in Methods. Results are expressed as the mean ± S.E. of the metabolites produced or substrate utilized in μmoles per g liver per hr. Six animals were used; 9000 *g* supernatants were prepared from pairs of animals and estimated in triplicate.

TABLE 3. RECOMBINATION OF HEPATIC SUBFRACTIONS OBTAINED FROM CONTROL AND PYRITHIOXINE TREATED ANIMALS*

Substrate	Fraction		Activity	Inhibition (%)
	Microsomes	Supernatant		
	Male mice			
Aminopyrine	Control	Control	0·196 ± 0·002	
	Control	Pyrithioxine	0·198 ± 0·002	
	Pyrithioxine	Control	0·095 ± 0·002	51·5
	Pyrithioxine	Pyrithioxine	0·092 ± 0·001	53·1
	Male mice			
EPN	Control	Control	2·32 ± 0·02	
	Control	Pyrithioxine	1·68 ± 0·07	37·6
	Pyrithioxine	Control	0·48 ± 0·03	79·3
	Pyrithioxine	Pyrithioxine	0·47 ± 0·03	79·8
	Male rats			
Aminopyrine	Control	Control	0·158 ± 0·002	
	Control	Pyrithioxine	0·135 ± 0·003	14·6
	Pyrithioxine	Control	0·053 ± 0·001	63·3
	Pyrithioxine	Pyrithioxine	0·043 ± 0·002	72·8
	Female rats			
Aminopyrine	Control	Control	0·066 ± 0·003	
	Control	Pyrithioxine	0·053 ± 0·002	19·4
	Pyrithioxine	Control	0·042 ± 0·002	36·3
	Pyrithioxine	Pyrithioxine	0·029 ± 0·001	56·1

* Animals were treated with pyrithioxine (200 mg/kg, i.p.) twice. One ml of microsomal suspension and 1 ml of 105,000 *g* supernatant fraction obtained from control and pyrithioxine-treated animals were recombined as indicated. The incubation conditions were described in Methods. Results were expressed as the mean ± S.E. of the metabolites produced in μmoles per mg of microsomal protein per hr. In experiments with mice, 10 animals were used in the control and pyrithioxine-treated groups; in experiments with rats, four animals were used in each group. The activity was determined in triplicate on each recombined system.

pyrithioxine in male rats (Table 2). However, in female rats, though the *N*-demethylation of aminopyrine was inhibited by pyrithioxine, the hydroxylation of hexobarbital was not inhibited (Table 2). The following experiments were carried out in an attempt to clarify the mechanism of the inhibitory action of pyrithioxine on drug-metabolizing enzymes.

Effect of pyrithioxine upon G-6-P dehydrogenase. Netter reported that a decrease of the concentration of cofactors was one of the mechanisms inhibiting reactions of drug-metabolizing enzymes.[22] It was felt that changes of co-factor supplied to the system might bring about the inhibition described above, and, therefore, recombination experiments were carried out with the microsomal and 105,000 *g* supernatant fractions. As shown in Table 3, the activity of the enzyme dropped markedly in both mice and rats when the microsomes were prepared from the animals pretreated with pyrithioxine. It appears, therefore, that the effect of pyrithioxine is exerted directly on the microsomes. However, it was also observed (Table 3) that when EPN was used as the substrate, effects of pyrithioxine were also observed in the supernatant. Therefore, the effects of pyrithioxine on the activities of G-6-P dehydrogenase and of alcohol dehydrogenase were examined as examples of oxidizing enzymes present in the supernatant fraction. It was found, as shown in Table 4, that pyrithioxine was almost without effect on the activities of these two enzymes.

TABLE 4. EFFECT OF PYRITHIOXINE ON G-6-P DEHYDROGENASE AND ALCOHOL DEHYDROGENASE ACTIVITIES IN LIVER*

Animals	Activities	
	G-6-P dehydrogenase	
	Mouse	Rat
Control	4·91 ± 0·25	5·23 ± 0·21
Pyrithioxine-treated	4·79 ± 0·04	5·30 ± 0·23
	Alcohol dehydrogenase	
	Mouse	Rat
Control	0·43 ± 0·02	0·63 ± 0·02
Pyrithioxine-treated	0·37 ± 0·04	0·59 ± 0·05

* Male mice and rats were injected i.p. with pyrithioxine (200 mg/kg) twice. Enzyme activities were measured in 105,000 *g* supernatant fraction of the liver homogenate prepared from control and pyrithioxine-treated animals. Results were expressed as the mean ± S.E. of NADPH produced in mμmoles per mg protein per min in the case of G-6-P dehydrogenase, and of NADH produced in the case of alcohol dehydrogenase. Five livers, each assayed in triplicate were used for rats; three groups consisting of five livers, each assayed in triplicate, were used for mice.

Effects of pyrithioxine upon microsomal hemoproteins. It is established that 2 hemoproteins (cytochrome b_5 and cytochrome P-450) are present in hepatic microsomes, utilizing NADH and NADPH, respectively, as the electron donor, and that the latter (cytochrome P-450) plays an important role in drug-metabolizing reactions. Some reports show that changes in cytochrome P-450 level of microsomes are related to

changes in the activities of drug-metabolizing enzymes.[23–25] Because of this relationship, the cytochrome b_5 and P-450 levels of microsomes from the livers of rats to which pyrithioxine had been given were determined. As shown in Table 5, the

TABLE 5. EFFECT OF PYRITHIOXINE ON THE CONTENT OF CYTOCHROME P-450 AND CYTOCHROME b_5 IN MALE RAT LIVER MICROSOMES*

Rat liver	Cytochrome b_5	Cytochrome P-450
Control	0·326 ± 0·015	0·607 ± 0·006
Pyrithioxine-treated	0·338 ± 0·026	0·600 ± 0·013

* Microsomes obtained from control and pyrithioxine treated (200 mg/kg × 2, i.p.) rat liver were resuspended in pH 7·0 phosphate buffer at a concentration of 2 mg of microsomal protein per ml. The oxidized-reduced differential spectra (for cytochrome b_5) and CO-differential spectra (for cytochrome P-450) were determined. Results were expressed as the mean ± S.E. of the concentrations in mμmoles per mg of microsomal protein. The values were obtained from groups of six animals, the activity being determined in triplicate on three paired liver preparations.

levels of the two hemoproteins showed no changes despite the administration of pyrithioxine.

Effects of pyrithioxine upon the incorporation of ^{14}C-amino acids. It has been shown by many investigators[23,24,26,27] that changes in the rate of protein synthesis play an important role in the alteration of enzyme activities. As indicated in Fig. 3, after the administration of pyrithioxine, the incorporation of ^{14}C-amino acids into protein

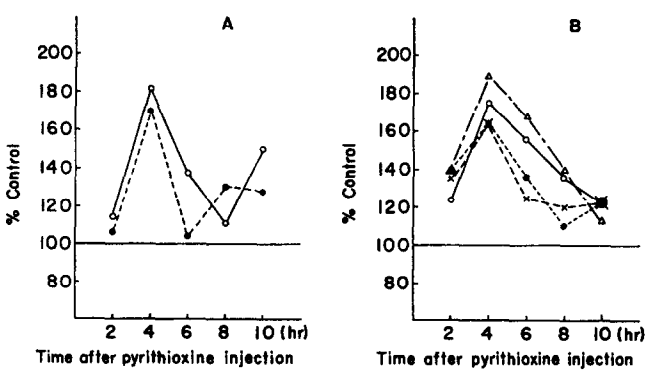

FIG. 3. Effect of pyrithioxine on the incorporation of ^{14}C-amino acids into acid-insoluble protein. (A) Time course of the incorporation of ^{14}C-amino acids into pyrithioxine-treated mouse tissues *in vivo*. Pyrithioxine (200 mg/kg) was injected i.p. ^{14}C-amino acids (1 μc/20 g of body weight) were given i.p. to mice 30 min before sacrifice, and the radioactivity incorporated into acid-insoluble protein was measured by means of a gas-flow counter. ○ = liver; ● = kidney. (B) Time course of incorporation of ^{14}C-amino acids into hepatic subcellular fractions of pyrithioxine-treated mice *in vivo*. × = nuclei and cell debris fraction; ○ = mitochondrial fraction; ● = microsomal fraction; △ = soluble fraction. Groups of nine animals were used, the radioactivity being estimated in duplicate on three paired livers.

increased in liver, kidneys and all the hepatic subcellular fractions, i.e. in nuclei and cell debris, mitochondrial, microsomal and soluble fractions.

Effects of pyrithioxine upon hepatic drug-metabolizing enzymes in vitro. Demethylation of aminopyrine by the 9000 *g* supernatant from male mouse liver was determined after pre-incubation at 37° for 10 min with levels of pyrithioxine ranging from 1 μM to 1 mM. Saline solution, adjusted to pH 4·0 with 0·01 N HCl solution in the case of 1 mM pyrithioxine, was added to the controls. The enzyme activity was markedly inhibited by pyrithioxine in the concentration range from 1 mM to 100 μM, but no significant inhibition was observed in the concentration range from 10 μM to 1 μM. The hydroxylation of hexobarbital was also inhibited by pyrithioxine at a concentration of 100 μM. It is evident, therefore, that pyrithioxine inhibits the activities of drug-metabolizing enzymes *in vitro* as well as *in vivo*. Accordingly, the microsomal fraction and the soluble fraction were prepared from 9000 *g* supernatants pre-incubated with or without pyrithioxine, and these preparations were submitted to determinations of enzyme activities in the combinations shown in Table 6. In contrast to

TABLE 6. *N*-DEMETHYLATION OF AMINOPYRINE BY THE RECOMBINATION OF SUBCELLULAR FRACTIONS AFTER THE ADDITION OF PYRITHIOXINE *in vitro**

Fraction		Activity	Difference (%)
Microsomes	Supernatant		
Control	Control	0·125 ± 0·009	
Control	Pyrithioxine	0·073 ± 0·003	−41·6
Pyrithioxine	Control	0·143 ± 0·006	+14·3
Pyrithioxine	Pyrithioxine	0·098 ± 0·002	−21·6

Twelve male mice were used. All livers were combined; 9000 *g* supernatant was divided into two equal parts and preincubated in the presence or absence of pyrithioxine (final concentration: 1×10^{-4} M), then centrifuged at 105,000 *g* for 60 min. The microsomal and supernatant fractions obtained were recombined as indicated. The incubation conditions were described in Methods. The activity was determined in triplicate on each recombined system. Results were expressed as the mean ± S.E. of the metabolites produced, in μmoles per mg of microsomal protein per hr.

the results obtained when pyrithioxine was given *in vivo* (Table 3), no inhibition was observed in the combination of microsomes prepared from the 9000 *g* supernatant treated with pyrithioxine and a decrease of enzyme activities was seen only in the presence of the supernatant of the pyrithioxine-treated group. An evaluation of the concentration of the substrate by the Lineweaver–Burk method[28] showed the inhibition to be formally competitive in type (Fig. 4).

Effects of pyrithioxine metabolites on the hepatic drug-metabolizing enzymes in vivo. Three pyrithioxine metabolites have to date been isolated and identified from rat urine: $R-S-CH_3$, $R-\overset{\overset{O}{\|}}{S}-CH_3$, and $R-\overset{\overset{O}{\|}}{\underset{\underset{O}{\|}}{S}}-CH_3$. In evaluating the effects of these

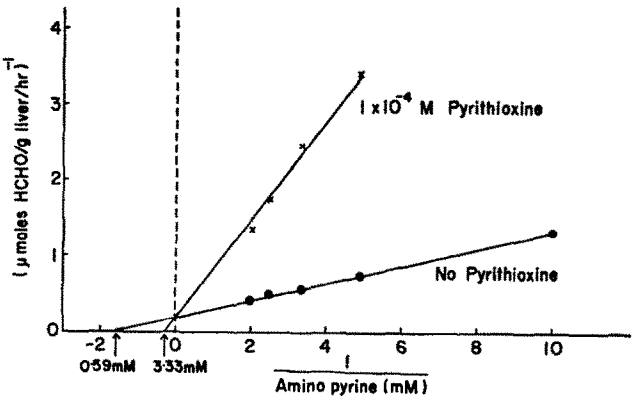

FIG. 4. Lineweaver–Burk plot of inhibition of aminopyrine demethylase by pyrithioxine. Conditions were as described in Methods. Arrows on the abscissa indicate the concentration of aminopyrine at the intercept. Livers of normal male mice were used. The values given are the mean obtained from groups of nine animals, the activity being determined in triplicate on three pairs 9000 *g* supernatants.

TABLE 7. EFFECT OF PYRITHIOXINE, ITS METABOLITES AND PYRIDOXINE ON THE MICROSOMAL DRUG-METABOLIZING ENZYMES *in vivo**

Substrate	Pretreatment	Enzyme activity	Inhibition (%)
Aminopyrine	Control	1·649 ± 0·023	
	R—S—S—R (Pyrithioxine)	0·877 ± 0·023	47·4
	R—S—CH_3 ‖ O	1·268 ± 0·022	23·1
	R—S—CH_3 ‖ O	1·587 ± 0·011	3·8
	R—S—CH_3 ‖ O	1·473 ± 0·024	10·7
	R—SH	1·272 ± 0·037	22·9
	R—OH (Pyridoxine)	1·453 ± 0·041	11·9
Hexobarbital	Control	0·675 ± 0·028	
	R—S—S—R	0·233 ± 0·009	65·5
	R—S—CH_3 ‖ O	0·277 ± 0·024	58·9
	R—S—CH_3 ‖ O	0·403 ± 0·017	40·9
	R—S—CH_3 ‖ O	0·448 ± 0·054	33·6
	R—SH	0·202 ± 0·021	70·1
	R—OH	0·595 ± 0·010	11·8

* Male mice were given 0·44 m-mole/kg of the drugs intraperitoneally twice, and enzyme activities were determined as described in Methods. Results were expressed as the mean ± S.E. of the metabolites produced or substrate utilized in μmoles per g liver per hr. Groups of nine animals were used, the activity being estimated in duplicate on three paired livers.

TABLE 8. EFFECT OF PYRITHIOXINE, ITS METABOLITES AND PYRIDOXINE ON THE MICROSOMAL DRUG-METABOLIZING ENZYMES *in vitro**

Substrate	Addition	Enzyme activity	Inhibition (%)
Aminopyrine	None	2·569 ± 0·018	
	R—S—S—R (Pyrithioxine)	1·344 ± 0·012	47·4
	R—S—CH_3 (O)	1·740 ± 0·038	34·2
	R—S—CH_3 (=O)	2·287 ± 0·027	10·6
	R—S—CH_3 (=O)	2·303 ± 0·007	10·4
	R—SH	1·919 ± 0·037	25·3
	R—OH (Pyridoxine)	2·092 ± 0·028	17·9
Hexobarbital	None	0·563 ± 0·044	
	R—S—S—R (Pyrithioxine)	0·173 ± 0·029	69·3
	R—S—CH_3 (O)	0·267 ± 0·031	52·6
	R—S—CH_3 (=O)	0·443 ± 0·012	21·3
	R—S—CH_3 (=O)	0·460 ± 0·032	18·3
	R—SH	0·287 ± 0·023	49·1
	R—OH	0·448 ± 0·010	20·4

* The concentration of drugs used was 1×10^{-4} M. Pre-incubation and determination of enzyme activity were performed as in Methods. Groups of nine males were used, the activity being determined in duplicate on three paired livers.

metabolites, R—SH and R—OH (pyridoxine), which have analogous structures, were tested in parallel. The *N*-demethylation of aminopyrine was, as shown in Table 7, inhibited by R—S—CH_3 and R—SH, but to a lesser degree by the other two metabolites. The hydroxylation of hexobarbital was also inhibited by the three metabolites, of which R—S—CH_3 exhibited the strongest inhibitory effects. As with aminopyrine, R—SH proved to be strongly inhibitory.

Effects of pyrithioxine metabolites on drug-metabolizing enzymes in vitro. It is found, as shown in Table 8, that pyrithioxine metabolites inhibit the drug-metabolizing enzymes *in vitro*. Metabolism of both aminopyrine and hexobarbital are inhibited by R—S—CH_3 and R—SH. With R—S—CH_3 (=O) and R—S—CH_3 (=O), the inhibitions are much weaker and were comparable to that seen with pyridoxine.

TABLE 9. EFFECT OF PYRITHIOXINE ON PENTOBARBITAL AND HEXOBARBITAL SLEEPING TIMES IN MICE AND RATS*

Subjects	Treatment	Sleeping time (min)	Difference (%)
	Single administration of pyrithioxine		
Male mice	Control	19·0 ± 3·4 (12)	
	Pyrithioxine	58·4 ± 7·1 (15)	+207·4
Male rats	Control	17·7 ± 2·9 (7)	
	Pyrithioxine	65·3 ± 9·6 (10)	+268·9
Female rats	Control	44·7 ± 6·4 (9)	
	Pyrithioxine	96·7 ± 4·2 (10)	+116·3
	Double administration of pyrithioxine		
Male mice	Control	3·7 ± 1·8 (14)	
	Pyrithioxine	57·6 ± 14·4 (15)	+1456·7
Male rats	Control	6·3 ± 5·3 (6)	
	Pyrithioxine	43·3 ± 8·5 (10)	+587·4
Female rats	Control	35·0 ± 5·5 (9)	
	Pyrithioxine	78·3 ± 7·4 (10)	+123·8

* Thirty min after the single administration of pyrithioxine (200 mg/kg, i.p.), mice and rats injected i.p. with sodium pentobarbital (30 mg/kg) and sodium hexobarbital (80 mg/kg) respectively. In experiments with double administration of pyrithioxine (200 mg/kg, i.p.), mice and rats were given sodium pentobarbital (30 mg/kg i.p.) and sodium hexobarbital (80 mg/kg, i.p.), respectively, 2 hr after the second injection of pyrithioxine. Values are time in min ± S.E. during which righting reflex was absent. The number of animals used is shown in parentheses.

Barbiturate potentiation by pyrithioxine. Pyrithioxine increases pentobarbital and hexobarbital sleeping times in mice and rats (Table 9). It appears that male mice and rats sleep for a significantly longer period after a double administration of pyrithioxin than after a single administration. However, in female rats, there is no difference between the 2 experimental results.

DISCUSSION

These experiments indicate that pyrithioxine exerts significant inhibitory effects on the drug-metabolizing mixed-function oxygenase of hepatic microsomes of male mice and rats, although its potency is considerably less than that of SKF 525-A.[29] Its effect is exerted on the microsomes themselves and not by means of NADPH-generating enzymes such as G-6-P dehydrogenase. The quantity of cytochrome P-450 showed no change after pyrithioxine administration, and the compound was found to accelerate, rather than to inhibit, the uptake of ^{14}C-amino acids into acid-insoluble protein. The fact that pyrithioxine inhibits not only reactions involving cytochrome P-450 but also esterase activity as seen in its inhibition of procaine hydrolysis, may suggest a direct action on the drug-metabolizing enzymes rather than on the terminal oxidase. Pyrithioxine inhibits the drug-metabolizing enzymes *in vitro* as well as *in vivo*. *In vitro*, the inhibition exerted by pyrithioxine appears to be formally competitive in type.

Sex differences were observed in the inhibitory action of pyrithioxine on the hepatic drug-metabolizing enzymes in rats. The *N*-demethylation of aminopyrine was remarkably inhibited by pyrithioxine in female rats, but the hydroxylation of hexobarbital

was not inhibited. However, pyrithioxine was found to prolong the hexobarbital sleeping time in female as well as male rats.

It appears from the latter data of Table 9 that male mice and rats sleep for a significantly longer period after a double administration of pyrithioxine than after a single administration. Since double administration of pyrithioxine at intervals of 2 hr provokes a marked decrease in hepatic drug metabolism, the inhibition of hexobarbital metabolism plays an important role in barbiturate potentiation. In the case of female rats, although pyrithioxine does not affect the hydroxylation of hexobarbital in the liver, hexobarbital sleeping times are increased with both single and double doses of pyrithioxine; in addition, although a single administration of pyrithioxine does not affect drug metabolism within 30 min, pentobarbital sleeping times in male mice are already increased markedly by a single dose. These results indicate that barbiturate potentiation by pyrithioxine cannot be solely because of inhibition of hepatic drug-metabolizing enzymes. These sex differences in the inhibitory action of pyrithioxine on hepatic drug-metabolizing enzymes and the other function on barbiturate potentiation are presently under investigation in our laboratory.

Although the details of pyrithioxine metabolism are not fully elucidated, three metabolites have to date been isolated from rat urine. Of these three metabolites, R—S—CH_3 exerts inhibitory actions on the drug-metabolizing enzymes both *in vivo* and *in vitro*. Therefore, it may well be that since pyrithioxine forms active metabolites *in vivo* the inhibitory effects of pyrithioxine on the drug-metabolizing enzymes result both from the actions of the original compound and of its metabolites. Since R—SH has inhibitory effects comparable to those of R—S—CH_3, it may be surmised that the structure R—S— is involved in the inhibition. As for the metabolism of compounds having disulfide linkages, a cleavage reaction of the —S—S— structure has been reported for 2,2′-dithiopyridine.[30] Since R—S—CH_3 is presently thought to be formed by methylation of R—SH, a series of reactions such as

$$\mathrm{R{-}S{-}S{-}R \rightarrow R{-}SH \rightarrow R{-}S{-}CH_3 \rightarrow R{-}\overset{\overset{\mathrm{O}}{\|}}{\mathrm{S}}{-}CH_3 \rightarrow R{-}\overset{\overset{\mathrm{O}}{\|}}{\underset{\underset{\mathrm{O}}{\|}}{\mathrm{S}}}{-}CH_3}$$

may be justifiable from the data accumulated to date. The results showing R—SH and R—S—CH_3 to be as inhibitory to enzymes as pyrithioxine *in vitro* support the above-suggested series of reactions.

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