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Stereoselective *N*-Demethylation of Chlorpheniramine by Rat-liver Microsomes and the Involvement of Cytochrome P450 Isozymes

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Abstract

Previous studies have suggested that degradation of the two stereoisomers of chlorpheniramine in the liver might be catalysed by different types of cytochrome P450. Stereoselective *N*-demethylation of chlorpheniramine and the involvement of cytochrome P450 (CYP) isozymes have, therefore, been investigated in the liver microsomes of eight-week-old male rats.

Incubation of racemic chlorpheniramine with liver microsomes from the male rat resulted in the formation of both enantiomers of monodesmethylchlorpheniramine (DMChp). Further metabolism of DMChp to didesmethylchlorpheniramine (DDMChp) did not, however, occur. The *S/R* enantiomeric ratio for intrinsic clearance (V_{max}/K_m) was approximately 2·0, suggesting that the *N*-demethylation was stereoselective for *S*-(+)-chlorpheniramine. On the other hand, although the V_{max}/K_m value for the formation of *S*-(+)- and *R*-(−)-DMChp increased with phenobarbitone-inducible rat-liver microsomes, there was no difference between the rates of *N*-demethylation of the enantiomers. In contrast, 3-methylcholanthrene reduced the intrinsic clearance of *S*-(+)-chlorpheniramine by *N*-demethylation and increased its value for *R*-(−)-chlorpheniramine, showing no stereoselectivity for the *N*-demethylation of chlorpheniramine. The difference between the intrinsic clearance of the two enantiomers by *N*-demethylation was because of differences in affinity for the catalysing enzyme. This is indicative of stereoselective involvement of the main enzyme concerned in the *N*-demethylation of the enantiomers, considered to be CYP 2C11. Anti-CYP 2C11 also partially inhibited the *N*-demethylation of racemic chlorpheniramine in rat-liver microsomes exposed to phenobarbitone and 3-methylcholanthrene. That CYP 2B1 was involved in the *N*-demethylation of both enantiomers was also supported by results from an experiment using phenobarbitone-inducible rat-liver microsomes. CYP1A1 did not, however, catalyse the *N*-demethylation of either enantiomer.

These results indicate that *N*-demethylation of the *S*-(+)-enantiomer of chlorpheniramine occurs preferentially in the microsomes, demonstrating the stereoselective contribution of CYP2C11. Immunoinhibition studies suggest, moreover, that the *N*-demethylation of both chlorpheniramine enantiomers is catalysed by CYP2B1, but not by CYP1A1.

Chlorpheniramine is a potent histamine H_1 receptor antagonist widely used as a racemic mixture of the *S*-(+)- and *R*-(−)-enantiomers. In-vivo *S*-(+)-chlorpheniramine is about 200 times more effective than its enantiomer in protecting the guinea-pig against histamine. Microdialysis of rat blood in-vivo indicated there is stereospecificity in the effect of chlorpheniramine on histamine release and studies of the inhibition of [^{3}H]mepyramine binding in the guinea-pig brain, both in-vitro and in-vivo, showed that *S*-(+)-chlorpheniramine was 100-fold more active than the *R*-(−) isomer. Although there is a difference between the potencies of H_1 blockade of the two enantiomers, it has been reported that there is no difference between their effects at reducing the 3,4-dihydroxyphenylacetic acid level, suggesting a lack of stereospecific inhibition of dopamine uptake in regions of the rat brain. These studies have prompted considerable interest in differences between the disposition of the two optical isomers of chlorpheniramine.

We recently demonstrated that *R*-(−)-chlorpheniramine bound more strongly than *S*-(+)-chlorpheniramine to rat plasma protein, suggesting that the stereoselective pharmacokinetics of chlorpheniramine after an intravenous injection of racemic chlorpheniramine is a result of differences in plasma protein binding (Sakurai et al 1992). This difference in disposition suggests, however, that this drug is metabolized stereoselectively, besides binding to the plasma protein.

N-demethylation is a major pathway of chlorpheniramine metabolism both in-vivo and in-vitro (Kabasakalian et al 1968; Kamm et al 1969; Peets et al 1972). Monodesmethylchlorpheniramine (DMChp) and didesmethylchlorpheniramine (DDMChp) are the major by-products of chlorpheniramine metabolism in the liver and skin (Zbaida & Touitou 1988).

In studies of the effect of substrate configuration on chlorpheniramine *N*-demethylation Thompson & Shioshita (1981) reported that *S*-(+)-chlorpheniramine is *N*-demethylated more rapidly than its optical isomer by rat and rabbit liver microsomes. It was also shown that in eight-week-old male rats the rate of formation of DMChp from the *S*-(+)-enantiomer was about 2·2 times that from the *R*-(−)-enantiomer, whereas the rate of *N*-demethylation of these enantiomers was no different in male rats aged 3 and 24 weeks (Nomura et al 1995). In female rats, on the other hand, the rate of formation of DMChp from both enantiomers of racemic chlorpheniramine was identical, irrespective of age, suggesting a lack of stereoselectivity in the microsomal *N*-demethylation (Nomura et al

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1995). It is, therefore, possible that the conversion of the enantiomers of chlorpheniramine to DMChp or DDMChp in the liver is catalysed by different types of cytochrome P450 (CYP).

To characterize CYP isoform(s) involved in *N*-demethylation of chlorpheniramine enantiomers in rats, we studied the stereoselective metabolism of chlorpheniramine using microsomes from the livers of eight-week-old male rats treated with phenobarbitone or 3-methylcholanthrene, and using anti-rat CYP antibodies (anti-CYP2C11, 2B1, 1A1, 3A2 and 2E1).

Materials and Methods

Materials

RS-(±)-chlorpheniramine maleate (Kowa, Nagoya, Japan), *S*-(+)-chlorpheniramine maleate (Yoshitomi Pharmaceutical Industry, Osaka, Japan), and *R*-(−)-chlorpheniramine maleate, *RS*-(±)-DMChp and *RS*-(±)-DDMChp (Schering Corporation, NJ) were donated by the indicated companies. Glucose 6-phosphate dehydrogenase, NADP, glucose 6-phosphate and 3-methylcholanthrene were obtained from Sigma (St Louis, MO) and sodium phenobarbitone from Wako Pure Chemical Industries (Osaka, Japan). Anti-rat CYP2C11, 2B1, 1A1, 3A2, and 2E1 goat sera and normal goat serum were purchased from Daiichi Pure Chemicals (Tokyo, Japan). All other reagents were of analytical grade or the highest grade available.

Animals

Male, 8-week-old Wistar rats purchased from Japan SLC (Hamamatsu, Japan) were housed at constant temperature (23 ± 1°C) and constant humidity (55 ± 5%); the light cycle was automatically controlled (0700–1900 h). CYP 2B1 and 1A1 were induced in the rats with phenobarbitone and 3-methylcholanthrene, respectively. The animals were injected intraperitoneally with phenobarbitone (80 mg kg^{-1}) in 0·9% NaCl (saline) once daily for three consecutive days, or with 3-methylcholanthrene (20 mg kg^{-1}) in olive oil once daily for four consecutive days.

Preparation of liver microsomes

Rats were killed by decapitation and the livers were quickly removed and freed from blood by infusion with ice-cold 0·9% NaCl. Tissues were homogenized in a 3-fold volume (w/v) of 0·154 M KCl, and the homogenates were centrifuged at 9000 *g* for 20 min. The supernatant liquids were centrifuged twice at 105 000 *g* for 60 min, and the microsomal pellets were resuspended in 0·1 M phosphate buffer (pH 7·4). Protein concentrations were determined by use of the Bio-Rad protein assay kit; bovine serum albumin was used as standard. The microsomes were then rapidly frozen in liquid nitrogen and stored at −80°C until use.

Microsome incubation conditions

Incubation vessels contained microsomes (20 mg mL^{-1}), $MgCl_2$ (25 mM), substrate, glucose-6-phosphate (10 mM) and glucose-6-phosphate dehydrogenase (1 unit mL^{-1}) in a total volume of 2 mL. In the enzyme kinetic studies, racemic chlorpheniramine dissolved in 0·1 M phosphate buffer (pH 7·4) was the substrate at an initial concentration in the range 0·05–3·0 mM (12·5 to 750 mM final concentration of each enantiomer). After addition of NADP (0·5 mM in 0·1 M phosphate buffer) the mixtures were incubated for 30 min at 37°C in a shaking water bath. All reactions were terminated by addition of 1 mL of 0·4 M perchloric acid. After vortex mixing the deproteinized sample was centrifuged at 9000 *g* for 15 min at 4°C and the supernatant was stored at −20°C until assay.

Immunoinhibition studies

Five polyclonal antibodies were used for the inhibition study: anti-CYP 1A1, anti-CYP2B1 that cross-reacts with CYP2B2, anti-CYP2C11 that cross-reacts with CYP2B1 and CYP2B2, anti-CYP2E1, and anti-CYP3A2. Each anti-CYP serum (10–50 μL) was incubated with 25 μL untreated rat-liver microsomes (200 μg protein) for 30 min at 37°C. Anti-CYP2B1 and anti-CYP1A1 were also added to rat-liver microsomes treated with phenobarbitone and 3-methylcholanthrene, respectively. Anti-CYP 2C11 was, moreover, also added to phenobarbitone- or 3-methylcholanthrene-inducible rat-liver microsomes. The same volume of normal goat serum instead of anti-CYP serum was incubated with the microsomes to determine non-specific reaction. After this period, 155 μL of the reaction mixture containing 250 μM racemic chlorpheniramine was added to the reaction vessels. All other incubation conditions were as described.

Optical resolution of RS-(±)-*chlorpheniramine,* RS-(±)-*DMChp and* RS-(±)-*DDMChp by HPLC*

Microsomal production was measured as described elsewhere (Sakurai et al 1992; Nomura et al 1995). Briefly, supernatant from microsomal incubations (2 mL) was mixed with sodium hydroxide (5 M; 1 mL) and benzene (7 mL) in a centrifuge tube. The mixture was vigorously shaken for 10 min and centrifuged for 10 min at 800 *g*. The organic layer (5 mL) was evaporated to dryness under reduced pressure. The residues were redissolved in 100–1000 μL of a 9 : 1, v/v, mixture of acetonitrile–0·05 M, pH 5·6 phosphate buffer; 20 μL was injected on to an HPLC-column.

HPLC was performed with a Yanagimoto (Kyoto, Japan) L-5000 apparatus equipped with a UV detector (UV-8 model II, Tosoh) and an ovomucoid-conjugated column (Ultron ES-OVM, 150 × 4·0 mm i.d., Shinwa Kako, Kyoto, Japan). Material was eluted with 9 : 1, v/v, acetonitrile–0·05 M, pH 5·6 phosphate buffer at a flow rate of 1·0 mL min^{-1} at 30°C, and the absorption at 228 nm was measured. The peaks of each enantiomer of authentic chlorpheniramine and its metabolites DMChp and DDMChp were symmetrical and clearly separated from other peaks. The calibration curves for both DMChp and DDMChp enantiomers were linear over the concentration range 0·005–10 μg mL^{-1} and the lower limit for quantitation was 5 ng mL^{-1}. Under our conditions, however, neither enantiomer of DDMChp was detected by incubation with rat-liver microsomes, irrespective of whether or not they were treated with phenobarbitone or 3-methylcholanthrene.

Data analysis

The formation of each enantiomer of DMChp from racemic chlorpheniramine was calculated as nmol formed min^{-1} (mg microsomal protein)$^{-1}$. The kinetic data were fitted to the Michaelis–Menten equation for a one-enzyme system using the non-linear least-squares regression analysis program MULTI, and apparent K_m and V_{max} values were estimated. Values are

presented as mean ± s.e.m. for n experiments and enantiomeric differences were analysed for their significance by use of Student's *t*-test.

Results

Fig. 1 shows Michaelis–Menten plots for the formation of each DMChp enantiomer from racemic chlorpheniramine incubated with untreated liver microsomes from male Wistar rats. The apparent K_m and V_{max} values for *N*-demethylation are listed in Table 1. No difference was found between the K_m values for the *N*-demethylation of each enantiomer, but V_{max} for *S*-(+)-chlorpheniramine *N*-demethylation was about twice as high as that for *R*-(−)-chlorpheniramine. Consequently, the intrinsic clearance (V_{max}/K_m) by *R*-(−)-*N*-demethylation was about half that by *S*-(+)-*N*-demethylation, indicating that *N*-demethylation is stereoselective in untreated rat-liver microsomes. No inhibition of *S*-(+)-chlorpheniramine *N*-demethylation by *R*-(−)-chlorpheniramine or inhibition of *R*-(−)-chlorpheniramine *N*-demethylation by *S*-(+)-chlorpheniramine in the microsomes was observed.

Phenobarbitone did not affect the K_m values, whereas those of the V_{max} for *N*-demethylation of *S*-(+)- and *R*-(−)-chlorpheniramine increased to about 150 and 300% of the control values, respectively (Fig. 1a; Table 1). The (+):(−) ratio for intrinsic clearance was about 1·2, showing that *N*-demethylation was not stereoselective for the *R*-(−)-chlorpheniramine enantiomer. In contrast, in the liver microsomes of rats induced with 3-methylcholanthrene, the V_{max} value for *N*-demethylation of the *S*-(+)-chlorpheniramine enantiomer decreased to 55% of the control level whereas that for the *R*-(−)-chlorpheniramine enantiomer did not change. K_m values for the *N*-demethylation of both enantiomers were, however, essentially identical with those of the control (Fig. 1b; Table 1). Thus, little or no stereoselectivity was observed for *N*-demethylation in liver microsomes from rats given 3-methylcholanthrene.

The effects of anti-CYP antibodies on the *N*-demethylation pathway of *S*-(+)- and *R*-(−)-chlorpheniramine were examined to determine the contribution of CYP isoenzymes in liver microsomes from untreated male Wistar rats (Table 2). Addition of 50 μL anti-CYP2C11 to the reaction mixture containing 1 mg of microsomal protein inhibited the formation of DMChp from both enantiomers to 90 and 77% of the control values for *S*-(+)- and *R*-(−)-chlorpheniramine, respectively. The formation of DMChp of the *S*-(+)- and *R*-(−)-enantiomer was slightly inhibited by anti-CYP2B1, but the enantiomeric dif-

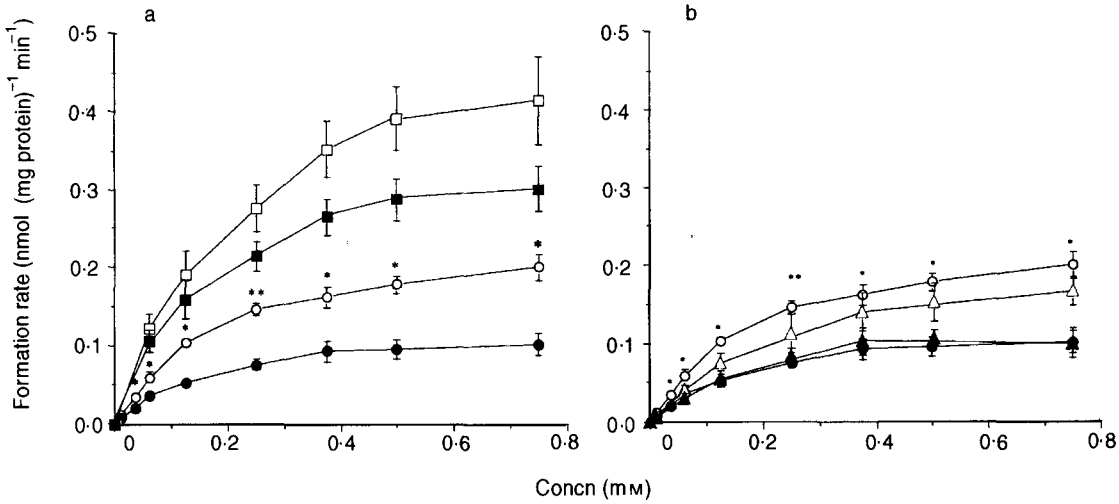

FIG. 1. Michaelis–Menten plots for the formation of each DMChp enantiomer from racemic chlorpheniramine incubated with liver microsomes from untreated (a, b: ○, ●), phenobarbitone-treated (a: □, ■) and 3-methylcholanthrene-treated (b: △, ▲) 8-week-old male rats. The abscissa indicates the final concentration of each chlorpheniramine enantiomer added to the reaction solution. Each value is the mean ± s.e.m. from five rats. ○, □, △, *S*-(+)-DMChp; ●, ■, ▲, *R*-(−)-DMChp. Enantiomer differences: $*P < 0.05$, $**P < 0.01$.

Table 1. Kinetic parameters of *N*-demethylation of racemic chlorpheniramine in male rat-liver microsomes.

	K_m (mM)	V_{max} (nmol (mg protein)$^{-1}$ min^{-1})	V_{max}/K_m (μL (mg protein)$^{-1}$ min^{-1})
No treatment			
S-(+)-chlorpheniramine	0·18 ± 0·07	0·29 ± 0·04*	1·73 ± 0·28*
R-(−)-chlorpheniramine	0·16 ± 0·07	0·13 ± 0·03	0·87 ± 0·12
Phenobarbitone-treatment			
S-(+)-chlorpheniramine	0·15 ± 0·04	0·43 ± 0·07	2·56 ± 0·45
R-(−)-chlorpheniramine	0·17 ± 0·03	0·38 ± 0·04	2·24 ± 0·41
3-Methylcholanthrene-treatment			
S-(+)-chlorpheniramine	0·14 ± 0·01	0·16 ± 0·07	1·34 ± 0·18
R-(−)-chlorpheniramine	0·11 ± 0·02	0·13 ± 0·03	1·08 ± 0·15

Each value is expressed as the mean ± s.e.m. of five experiments. $*P < 0.05$, significantly different between enantiomers.

Table 2. Immunoinhibition of the *N*-demethylation of racemic chlorpheniramine by liver microsomes from untreated male rats.

Antibody	Amount (μL)	*S*-(+)-Chlorpheniramine (% of control)	*R*-(−)-Chlorpheniramine (% of control)
Control	10	100·0	100·0
	30	100·0	99·5
	50	100·0	100·0
1A1	10	100·0	100·0
	30	100·0	100·0
	50	94·7	100·0
2B1	10	96·5	97·9
	30	91·0	90·3
	50	80·6	74·7
2C11	10	73·5	80·6
	30	26·1	43·0
	50	11·6	22·5
2E1	10	95·7	100·0
	30	89·0	100·0
	50	95·8	89·2
3A2	10	86·3	95·4
	30	72·7	72·8
	50	60·1	59·9

Liver microsomal protein (200 μg) was combined with 10–50 μL each of various anti-rat P450 sera and normal goat serum and incubated at 37°C for 30 min before adding the reaction mixture containing 250 μM racemic chlorpheniramine. Values for the antibody-treated group are expressed as a percentage of those for the control group.

ference in the inhibition by anti-CYP2B1 was not evident in the male rats. The addition of anti-CYP3A2 (50 μL) also caused 40% inhibition of the formation of DMChp from the *S*-(+)- and *R*-(−)-enantiomers. Anti-CYP1A1 and anti-CYP2E1 had no inhibitory effects on the rates of microsomal *N*-demethylation of either *S*-(+)- or *R*-(−)-enantiomer.

In the liver microsomes of male rats treated with phenobarbitone, anti-CYP 2B1 serum (50 μL) inhibited the *N*-demethylation of both enantiomers (Fig. 2a) suggesting a lack of stereoselectivity. Anti-CYP2C11 also partially suppressed the *N*-demethylation of chlorpheniramine in phenobarbitone-induced rat-liver microsomes (Fig. 2b). On the other hand, although anti-CYP1A1 had little effect on microsomal *N*-demethylation of either chlorpheniramine enantiomer in the livers from rats given 3-methylcholanthrene (Fig. 3a), anti-CYP2C11 partially inhibited the *N*-demethylation of racemic chlorpheniramine (Fig. 3b).

Discussion

N-demethylation is a major pathway of chlorpheniramine metabolism in-vivo and in-vitro (Kabasakalian et al 1968; Kamm et al 1969; Peets et al 1972; Zbaida & Touitou 1988; Thompson & Shioshita 1981). In this study, we investigated the contribution of CYP isoenzymes involved in the *N*-demethylation of chlorpheniramine enantiomers by hepatic microsomes from 8-week-old male Wistar rats using two classical inducers (phenobarbitone and 3-methylcholanthrene) and five polyclonal anti-CYP antibodies, to clarify the cause of the stereoselective metabolism.

A simple method for the chiral stationary-phase liquid chromatographic resolution of racemic chlorpheniramine and its metabolites, DMChp and DDMChp has been established using an ovomucoid-conjugated column. The procedure was used for enzyme kinetic studies. The formation of DMChp enantiomers in mixtures with *RS*-(±)-chlorpheniramine in microsomes could then be measured. Our data demonstrated that *S*-(+)-chlorpheniramine was *N*-demethylated more rapidly than the *R*-(−) isomer. The K_m values for the enantiomers were the same. Among Michaelis–Menten kinetic parameters, the V_{max}/K_m ratio is useful for estimating in-vivo clearance rates. These results showed, therefore, that the intrinsic clearance by *R*-(−)-*N*-demethylation was significantly lower than that by *S*-(+)-*N*-demethylation, suggesting high stereoselectivity for the microsomal formation of DMChp in the rat liver. This is in good agreement with the findings of Thompson and Shioshita (1981), obtained using a combination of deuterium labelling and GC-MS. They also reported that although further metabolism of DMChp to DDMChp occurs stereoselectively in New Zealand White rabbit liver microsomes, favouring the *S*-(+)-enantiomer, DDMChp was not formed in the liver microsomes of the rat and the mouse. Our HPLC analysis detected no measurable quantities of either DDMChp enantiomer in rat-liver microsomes and in 30-min post-reaction mixtures of racemic chlorpheniramine neither DDMChp enantiomer was detected in the liver microsomes of rats given phenobarbitone or 3-methylcholanthrene.

Immunoinhibition studies suggest that CYP2C11 is the major CYP isoenzyme involved in *N*-demethylation of *S*-(+)- and *R*-(−)-chlorpheniramine in male rats. CYP2B1 and

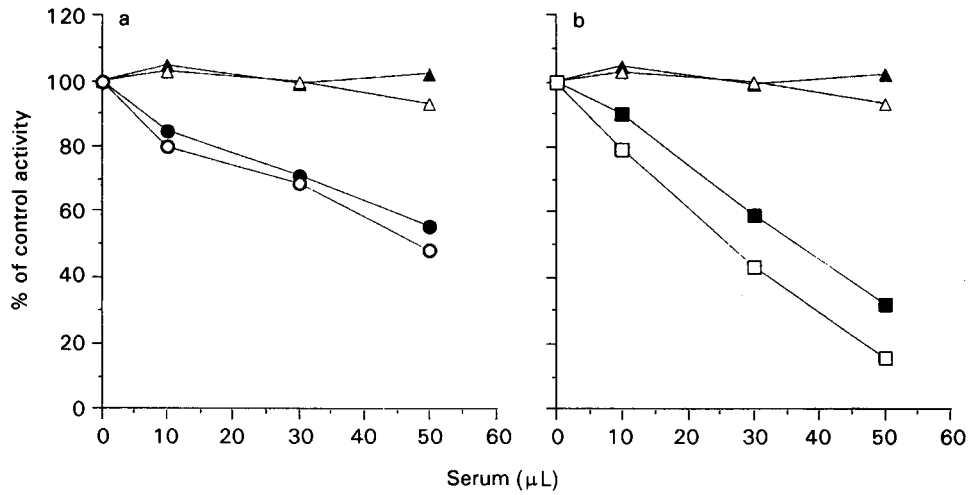

FIG. 2. Immunoinhibition of the *N*-demethylation of racemic chlorpheniramine by liver microsomes from phenobarbitone-treated male rats. Liver microsomal protein (200 μg) was combined with 10–50 μL each of anti-rat CYP2B1 (a: ○, ●) and CYP2C11 (b: □, ■) sera and normal goat serum (control: △, ▲) and incubated at 37°C for 30 min before adding the reaction mixture containing 250 μM racemic chlorpheniramine. Values for the antibody-treated group are expressed as a percentage of the activity of the control group. ○, □, △, *S*-(+)-chlorpheniramine; ●, ■, ▲, *R*-(−)-chlorpheniramine.

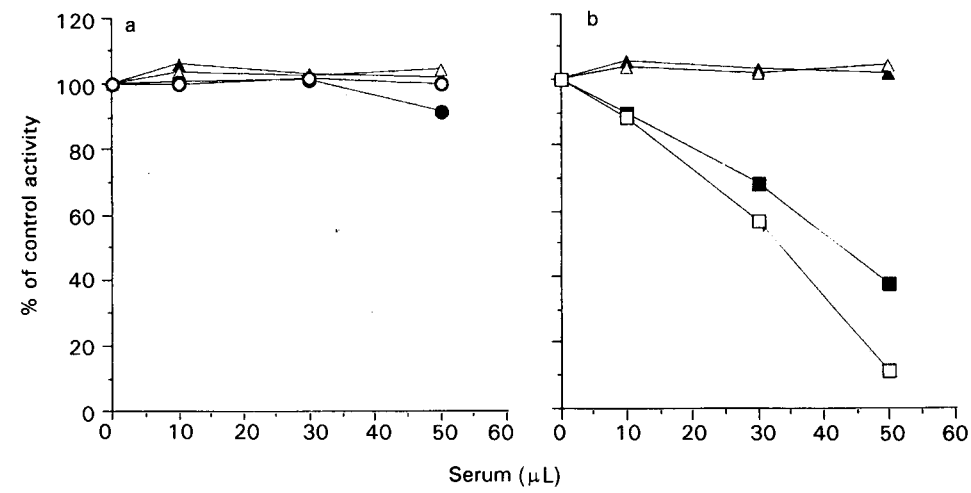

FIG. 3. Immunoinhibition of the *N*-demethylation of racemic chlorpheniramine by microsomes from the livers of 3-methylcholanthrene-treated male rats. Liver microsomal protein (200 μg) was combined with 10–50 μL each of anti-rat CYP1A1 (a: ○, ●) and CYP2C11 (b: □, ■) sera and normal goat serum (control: △, ▲) and incubated at 37°C for 30 min before adding the reaction mixture containing 250 μM racemic chlorpheniramine. Values for the antibody-treated group are expressed as a percentage of the activity of the control group. ○, □, △, *S*-(+)-chlorpheniramine; ●, ■, ▲, *R*-(−)-chlorpheniramine.

CYP2B2 are also present in the hepatic microsomes of untreated male rats at low levels (Guengerich et al 1982; Christou et al 1987; Yamazoe et al 1987; Imaoka et al 1989). Anti-CYP2B1, which cross-reacts with CYP2B2, slightly inhibited the microsomal *N*-demethylation of both enantiomers in untreated rats and enzymatic activity for the *N*-demethylation of both enantiomers was induced without stereoselectivity by treating rats with phenobarbitone. Because phenobarbitone potently induces the two major CYP isozymes, CYP2B1 and CYP2B2 (Guengerich et al 1982; Christou et al 1987; Yamazoe et al 1987; Imaoka et al 1989), these CYP isoenzymes are probably involved in the *N*-demethylation. In fact, addition of anti-CYP2B1 to the liver microsomes of phenobarbitone-treated male rats inhibited *N*-demethylation, but there was no apparent stereoselective contribution of rat CYP2B1. In addition, experiments using anti-CYP2C11 showed that *S*-(+)- and *R*-(−)-chlorpheniramine *N*-demethylation were partially inhibited in phenobarbitone-induced rat-liver microsomes. These findings suggest that CYP2C11 is still present at a moderate level in liver microsomes from phenobarbitone-treated male rats. Anti-CYP1A1, on the other hand, did not clearly inhibit microsomal *N*-demethylation of the chlorpheniramine enantiomers in untreated male rats.

The two major CYP isoenzymes, CYP1A1 and CYP1A2 are potently induced by 3-methylcholanthrene (Degawa et al 1988; Juedes & Kupfer 1990). In the liver microsomes of rats given 3-methylcholanthrene, the intrinsic clearance of *S*-(+)-chlorpheniramine by *N*-demethylation decreased, but that of *R*-(−)-

chlorpheniramine increased, showing no stereoselectivity for the *N*-demethylation of chlorpheniramine. The results suggested, however, that the contribution of CYP1A1 was excluded from the antibody inhibition experiments. Because *N*-demethylation by anti-CYP2C11 was partially inhibitory in liver microsomes treated with 3-methylcholanthrene, other CYP1A isozymes are probably not involved in *N*-demethylation of *S*-(+)- and *R*-(−)-chlorpheniramine in the presence of this inducer. Further investigation will be necessary for immunoinhibition studies using various anti-CYP isoforms.

In conclusion, this study indicated that the *N*-demethylation occurs preferentially for the *S*-(+)-enantiomer of chlorpheniramine in the microsomes of eight-week-old male rats, demonstrating the stereoselective contribution of CYP2C11. Our results also suggest that the *N*-demethylation of both chlorpheniramine enantiomers is also catalysed by CPY2B1, but not by CYP1A1.

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