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THE METABOLISM OF HYDROXYCHLORPROMAZINES BY RAT LIVER MICROSOMES

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Abstract—The metabolism of a series of chlorpromazine derivatives was studied with a fortified preparation of rabbit liver microsomes. All of the monohydroxylated derivatives underwent mono-*N*-demethylation as the principal metabolic pathway. In addition, these hydroxychlorpromazines were shown to undergo further hydroxylation to form ortho-dihydroxychlorpromazines, which were then mono-*O*-methylated. Thus, 7-hydroxychlorpromazine, a major metabolite of chlorpromazine, was apparently converted with this system to a mono-*O*-methylated 7,8-dihydroxychlorpromazine, which represents a new metabolic pathway.

THE IMPORTANCE of the phenothiazine group of tranquilizers in the treatment of mental conditions and the individual variations in response to treatment with these drugs have led to intensive studies on the metabolism of various phenothiazines. The three major metabolic pathways for the most widely employed phenothiazine, chlorpromazine, appear to be *N*-demethylation, sulfoxidation, and ring hydroxylation or a combination of these pathways. Ring hydroxylation is usually followed by conjugation with glucuronic acid or, to a lesser extent, with sulfuric acid. Minor pathways involve the formation of *N*-oxides, loss of the side chain or oxidation of the side chain to a propionic acid derivative.

The hydroxylation of chlorpromazine (Fig. 1) leads principally to the 7-hydroxy derivatives.[1–3] The pharmacology of a series of monohydroxylated and methoxylated chlorpromazines has been reported.[4] In order to determine whether hydroxychlorpromazines are further metabolized to dihydroxychlorpromazines, a study utilizing

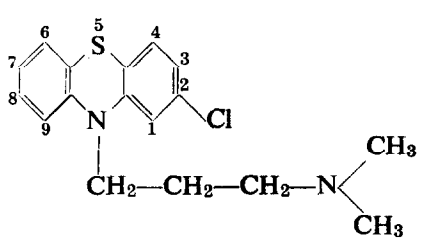

FIG. 1. Chlorpromazine.

liver microsomes was undertaken. Formation of ortho-dihydroxychlorpromazines was assayed by the sensitive radioisotope method of Axelrod *et al.*[5] The formation of noncatecholic dihydroxychlorpromazines is not detected by this method.

MATERIALS

The chlorpromazine derivatives used in this study were synthesized for the Psychopharmacology Research Branch, National Institute of Mental Health, by the Battelle Memorial Institute under Contract No. PH 43-62-162 and by the Research Institute of Temple University under Contract No. SA 43-ph-3748. The authors are indebted to Dr. Julius Axelrod of the National Institute of Mental Health, Bethesda for the S-adenosylmethionine-methyl-^{14}C.

METHODS

Preparation of rabbit liver microsomes. New Zealand, white rabbit livers were homogenized with 5 vol. of ice-cold isotonic potassium chloride solution and centrifuged at 8000 *g* for 30 min to remove cell debris and mitochondria. The supernatant was centrifuged at 100,000 *g* for 1 hr, and the resulting microsome pellet was resuspended in 10 vol. of ice-cold isotonic potassium chloride solution for use in the assay described below.

Standard incubation mixture. The microsomal preparation corresponding to 100 mg liver was incubated at 37° for 30 min in a reaction mixture containing the following components (micromoles): substrate, 0·3; phosphate buffer, pH 7·9, 200; magnesium chloride, 5; NADP, 0·3; glucose 6-phosphate, 1·5; S-adenosylmethionine-methyl-^{14}C (10,000 cpm), 1·5; and glucose 6-phosphate dehydrogenase, 10 μg (C. F. Boehringer and Soehne) in a final vol. of 0·6 ml. Incubations were also carried out without the NADPH-generating system (NADP, glucose 6-phosphate, and glucose 6-phosphate dehydrogenase) to measure simple *O*-methylation of the parent hydroxychlorpromazine. The incubations were terminated by the addition of 0·5 ml of 0·5 M borate buffer (pH 10). The *O*-methylated-^{14}C-metabolites were extracted with 5 ml toluene–isoamyl alcohol (3:2) and the radioactivity was measured in a liquid scintillation counter as described by Axelrod *et al.*[5] On larger (10- to 20-fold) experiments for thin-layer chromatography, the incubations were stopped after 1 hr with solid sodium bicarbonate and the metabolites were extracted with 2 vol. of ethyl acetate. The ethyl acetate was dried with sodium sulphate, concentrated under nitrogen to a small volume, and aliquots were chromatographed in three solvent systems on silica gel thin-layer chromatoplates along with appropriate reference compounds. The thin-layer chromatoplates were scanned for radioactive zones in a Nuclear Chicago thin-layer chromatoplate scanner. Color development of the phenothiazines was carried out with 50% sulfuric acid–ethanol (1:1).

RESULTS

In Table 1 are presented the data on the formation of *O*-methylated-(^{14}C)-dihydroxychlorpromazines. As reference compounds, phenol and 4-hydroxyacetophenone were also assayed.[6] When the NADPH-generating system (NADP, glucose 6-phosphate, and glucose 6-phosphate dehydrogenase) required for enzymatic hydroxylation is omitted, only *O*-methylation of the phenolic group can occur (Table 1, column A).

Phenol and certain other simple phenols undergo methylation by the enzyme phenol-*O*-methyltransferase found in rabbit liver microsomes,[7] whereas 4-hydroxyacetophenone and all of the hydroxychlorpromazines tested, with the possible exception of 1-hydroxychlorpromazine, do not methylate with this enzyme. When the NADPH-generating system is present (Table 1, column B), hydroxylation occurs and the

TABLE 1. ORTHO-HYDROXYLATION AND *O*-METHYLATION OF PHENOTHIAZINE DERIVATIVES*

Substrate	Activity mμ mole/g liver/hr	
	A	B
Phenol (reference)	10	23
4-Hydroxyacetophenone (reference)	0	62
1-Hydroxychlorpromazine	1	5
3-Hydroxychlorpromazine	0	25
6-Hydroxychlorpromazine	0	12
7-Hydroxychlorpromazine	0	40
7-Hydroxydesmethylchlorpromazine	0	12
7-Hydroxydidesmethylchlorpromazine	0	2
7-Hydroxychlorpromazine sulfoxide	0	0
8-Hydroxychlorpromazine	0	38
9-Hydroxychlorpromazine	0	9
2-Hydroxypromazine	0	27
3-Hydroxypromazine	0	11
Chlorpromazine	0	0
3-Methoxychlorpromazine	0	0
7-Methoxychlorpromazine	0	0

* Substrate (0·3 μmole) incubated with microsomal preparation for 30 min; (A) without NADPH-generating system; (B) with NADPH-generating system. Values are the average of 4 determinations

catechols formed are rapidly methylated by the active catechol-*O*-methyltransferase found in rabbit liver microsomes.[8] All of the monohydroxychlorpromazines formed *O*-methylated catechols with this system, but none of the nonphenolic phenothiazines, such as chlorpromazine, desmethylchlorpromazine, didesmethylchlorpromazine, chlorpromazine sulfoxide, desmethyl- and didesmethylchlorpromazine sulfoxides, chlorpromazine-*N*-oxide, chlorpromazine-*N*,S-dioxide, 2-chlorophenothiazine, and 3- and 7-methoxychlorpromazine, showed any activity in this assay. Certain hydroxychlorpromazines, such as the 7-hydroxy and the 8-hydroxy, were excellent substrates for microsomal hydroxylation (for comparison, see Daly *et al.*[6]).

In Table 2 the chromatographic data on the radioactive methoxy-(^{14}C)-hydroxychlorpromazines are presented. The 3-hydroxychlorpromazine is probably converted by hydroxylation and *O*-methylation to the *O*-methylated 3,4-dihydroxychlorpromazine. Similarly, the 6-hydroxy derivative probably forms an *O*-methylated 6,7-dihydroxychlorpromazine and the 9-hydroxy derivative probably forms an *O*-methylated 8,9-dihydroxychlorpromazine. The products from the 6- and 9-hydroxychlorpromazines appear to be different in chromatographic behavior (Table 2) from the products formed on hydroxylation and *O*-methylation of either 7-hydroxy- or 8-hydroxychlorpromazine. The product from these latter phenothiazines appears identical in chromatographic behavior and is therefore probably the *O*-methylated

7,8-dihydroxychlorpromazine. Formation of 3,7-dihydroxychlorpromazine would not be detected by this system, since it would not be methylated by catechol-*O*-methyltransferase.

7,8-Dimethylmethylenedioxychlorpromazine on treatment with 2 N HCl for 1 hr at 70° yields 7,8-dihydroxychlorpromazine, an unstable material which in air rapidly forms purple to blue pigments. This material was *O*-methylated with microsomes and S-adenosylmethionine-^{14}C, and the chromatographic properties of the *O*-methylated product were determined (Table 2). The R_f values are nearly the same as those of the

TABLE 2. R_f VALUES OF METHOXY-[^{14}C]-HYDROXYCHLORPROMAZINES FORMED ENZYMATICALLY FROM HYDROXYCHLORPROMAZINES

Substrate	R_f* of [^{14}C]-methoxyhydroxychlorpromazine		
	Solvent 1	Solvent 2	Solvent 3
3-Hydroxychlorpromazine	0·32	0·09	0·02
6-Hydroxychlorpromazine	0·36	0·05	0·05
7-Hydroxychlorpromazine	0·45	0·17	0·02
8-Hydroxychlorpromazine	0·45	0·15	0·02
9-Hydroxychlorpromazine	0·38	0·10	0·09
7,8-Dihydroxychlorpromazine	0·49	0·20	0·04

* Thin-layer chromatography (SiO_2). Solvents: (1) acetone, isopropanol, 1% ammonium hydroxide (9:7:4); (2) ethyl acetate, acetone, methanol, diethylamine (68:2:20:15); (3) benzene, ethanol, methanol, triethanolamine (70:20:10:0·25). Only R_f of major radioactive product is reported but 3-, 7-, 8-, and 9-hydroxychlorpromazines also gave minor radioactive products.

O-methylation product formed enzymatically from hydroxylated 7-hydroxy- or 8-hydroxychlorpromazine, thus providing further evidence for the formation of 7,8-dihydroxychlorpromazine from these compounds. The position of *O*-methylation in such compounds is at this time unknown. *O*-methylation of the 7,8-dihydroxychlorpromazine might occur either on the 7-hydroxy or the 8-hydroxy group, or a mixture of isomers might be formed.[9, 10]

The color development of the various hydroxychlorpromazines with 50% sulfuric acid–ethanol (1:1) was quite characteristic (Table 3). The results usually indicated formation of one major and one very minor metabolite with the same color reactions as the tested hydroxychlorpromazine derivative. Where standards were available (chlorpromazine and 7-hydroxychlorpromazine), these were identified by R_f values as the desmethyl (major metabolite) and didesmethyl (minor metabolite) derivatives. It appeared from chromatographic behaviour and color reactions that *N*-demethylation was also the principal route of metabolism for the other hydroxy- and methoxychlorpromazines. Chlorpromazine formed a trace amount (< 2 per cent) of a hydroxychlorpromazine with R_f values and color reactions corresponding to 7-hydroxychlorpromazine. The amounts of *O*-methylated dihydroxychlorpromazines formed were too small to detect by color reactions. The hydroxylation values given in Table 3 are, therefore, based on the data from Table 1. 3-Hydroxychlorpromazine appeared to form trace amounts of a dihydroxy derivative which gave a green reaction with sulfuric acid, had very low R_f values, and was not coincident with the radioactive

O-methylated 3,4-dihydroxychlorpromazine. The enzymatically *O*-methylated 7,8-dihydroxychlorpromazine gave a blue-purple color with the sulfuric acid reagent as did the 7,8-dihydroxychlorpromazine.

TABLE 3. METABOLISM OF CHLORPROMAZINE AND DERIVATIVES BY RABBIT LIVER MICROSOMES

Substrate	Color with 50% H_2SO_4–ethanol (1:1)	Per cent conversion to products*		
		Desmethyl	Didesmethyl	Hydroxylated
Chlorpromazine	pink	15–20	Trace	Trace
Desmethylchlorpromazine	pink		0	0†
1-Hydroxychlorpromazine	pink	trace	0	0·1†
3-Hydroxychlorpromazine	blue	15–20	trace	0·8†
6-Hydroxychlorpromazine	red-purple	5–10	0	0·4†
7-Hydroxychlorpromazine	purple	20–30	trace	1·3†
7-Hydroxydesmethylchlorpromazine	purple		trace	0·4†
8-Hydroxychlorpromazine	blue-green	25–35	trace	1·2†
9-Hydroxychlorpromazine	pink-purple	trace	0	0·3†
3-Methoxychlorpromazine	blue	10–15	trace	0†
7-Methoxychlorpromazine	purple	10–15	trace	0†

* Values for percent conversion to desmethyl and didesmethyl based on thin-layer chromatography in three systems (Table 2) and color development.
† Values calculated from the data of Table 1.

DISCUSSION

Previously Robinson[11, 12] has shown that liver microsomes metabolize chlorpromazine principally to the desmethyl derivatives and to a hydroxy derivative, probably 7-hydroxychlorpromazine. Trace amounts of chlorpromazine sulfoxide and 7-hydroxydesmethylchlorpromazine are also formed. In the present study with rabbit liver microsomes, the principal metabolic pathway for the various hydroxy- and methoxychlorpromazines studied was found to be *N*-demethylation. Great variation in the rate of demethylation was found for the various hydroxychlorpromazines (Table 3).

A new pathway for the metabolism of hydroxychlorpromazines has been demonstrated by radioisotope techniques. The technique involves the assay of catechols formed from the hydroxychlorpromazines, through *O*-methylation of the catechol, with catechol-*O*-methyltransferase and S-adenosylmethionine-^{14}C as the methyl donor.[5] The radioactive *O*-methylated dihydroxychlorpromazine is then extracted and assayed by scintillation counting. 7-Hydroxychlorpromazine, a major hydroxylated metabolite of chlorpromazine, was an excellent substrate for this hydroxylation–methylation system and apparently formed an *O*-methylated-7,8-dihydroxychlorpromazine. The same *O*-methylated compound appeared to be formed from 8-hydroxychlorpromazine and from 7,8-dihydroxychlorpromazine. It is of interest that chlorpromazine or 7-hydroxychlorpromazine is a better substrate for the hydroxylating enzymes than their desmethyl derivatives[12] and that the 7-hydroxychlorpromazine sulfoxide was not a substrate at all. This is in agreement with the finding that the chlorpromazine sulfoxide is not metabolized by liver microsomes.[12] The rate-limiting step in the formation of these *O*-methylated dihydroxychlorpromazines from chlorpromazine certainly appears to be monohydroxylation of chlorpromazine.

The 1-hydroxychlorpromazine was a very poor substrate for the hydroxylation reaction and probably forms a 1,2-dihydroxypromazine derivative prior to *O*-methylation. Either the chlorine is lost in this hydroxylation or it may undergo a migration to the 3-position in analogy to the findings of Guroff *et al.* on the hydroxylation of *p*-chlorophenylalanine.[13]

Dihydroxychlorpromazines have been detected in urine by Posner *et al.*,[14, 15] who postulated that they were 7,8-dioxygenated derivatives based on their color reactions. This conclusion appears to be substantiated by our findings. The formation of these oxygenated chlorpromazines and desmethyl derivatives appears to occur principally in the liver. Rabbit brain microsomes were virtually inactive in carrying out the hydroxylation of 7-hydroxychlorpromazine nor was any demethylation of chlorpromazine or 7-hydroxychlorpromazine observed (J. W. Daly, unpublished results). This is in agreement with other reports on the lack of *N*-demethylation and hydroxylation of drugs by brain microsomes.[16, 17]

For the hydroxylated metabolites of chlorpromazine to exhibit central activity, it would appear that they must be transported into the central nervous system from their peripheral site of formation (liver). The absence of hydroxychlorpromazine[18] or dioxygenated chlorpromazine derivatives in brain has been reported after chlorpromazine administration.

Manian *et al.*[4] have shown that both the 3-hydroxy- and 7-hydroxychlorpromazine demonstrate pharmacological activity similar to chlorpromazine. Perry *et al.*[19] have proposed that 7-hydroxychlorpromazine or a transformation product is responsible for the toxic reactions and purple pigmentation frequently seen in patients receiving high dosages of chlorpromazine. Rapid formation of purple pigments from 7,8-dihydroxychlorpromazine was observed when this compound was incubated with microsomal preparations. Thus, the present studies suggest that the highly reactive 7,8-dioxygenated chlorpromazines should also be considered as potential toxic metabolites of chlorpromazine.

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