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METABOLISM OF CHLORPROMAZINE:
NON-PHENOTHIAZINE AND
HYDROXY METABOLITES

By

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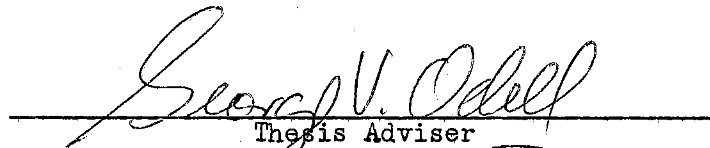

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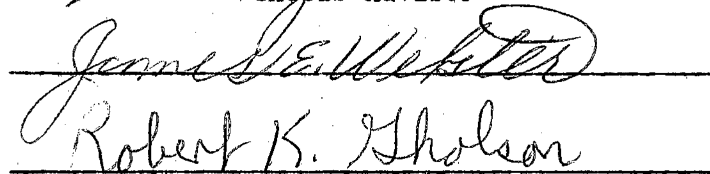

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TABLE OF CONTENTS

Chapter — Page

I. INTRODUCTION. 1

II. REVIEW OF LITERATURE. 3

III. EXPERIMENTAL. 10

Animals and Tissues. 10
Reagents 10
Collection and Preparation of Urine. 11
Preparation of Tissue Subcellular Fractions. 12
Balance Study. 12

Substrate Preparation for Incubation with Liver Microsomal Fraction 12
Incubation of Liver Microsomes with ^{3}H-chlorpromazine 13
Fractionation of Metabolites. 13
Incubation with β-glucuronidase/Aryl Suflatase . 14
Liquid Scintillation Spectrometry 14

Identification of Metabolites. 15

Thin-Layer Chromatography 15
Solvents. 15
Spray Reagents. 15
Preparations of Samples for Detection of Non-Phenothiazine Metabolites 16
Preparations of Samples for Assay of Phenothiazine Metabolites. 17
Identification of Non-Phenothiazine and Phenothiazine Metabolites 18

Quantitative Studies 18

IV. RESULTS AND DISCUSSION. 20

Balance Study. 20
Metabolite Identification Study. 28
Quantitative Study 33

V. SUMMARY 40

LITERATURE CITED 42

LIST OF TABLES

Table Page

I. Radioactivity in Urine Excretion from Rats. 21

II. Percentage of Radioactivity Excreted in Solid Phase of Urine for Each Day. 22

III. Radioactivity of Urine Chloroform Extracts and Residue. . . 24

IV. Chlorpromazine Oxidase Assay System 26

V. Radioactivity of Chloroform Extracts of Pig Liver Microsomes Incubated With ^{3}H-chlorpromazine. 27

VI. Non-phenothiazine Metabolites in Rat Urine: Tests for Aromatic Amines, Orthanilic Acid, Para-chlorobenzenesulfonic Acid 30

VII. Identification of Chlorpromazine Metabolites in Rat Urine and Incubated Subcellular Fractions from Pig Liver. . . . 31

VIII. Quantitative Determination of Unmetabolized Chlorpromazine and 7-Hydroxychlorpromazine from Rat Urine and Pig Liver Subcellular Fractions 37

LIST OF FIGURES

Figure		Page
1.	Structures of Some Reported Metabolites of Chlorpromazine . .	4
2.	Proposed Ring Cleavage of Chlorpromazine With Products That May Result From This Process	29
3.	The Ultraviolet Spectra of Chlorpromazine and 7-Hydroxychlorpromazine	34
4.	The Visible Spectra of Chlorpromazine and 7-Hydroxychlorpromazine	35
5.	Standard Curve of Chlorpromazine and 7-Hydroxychlorpromazine.	36

CHAPTER I

INTRODUCTION

Chlorpromazine, an amine derivative of phenothiazine, was synthesized by Charpentier of the Rhone-Poulenc Research Laboratories in France in 1950 (1). This compound, N,N-dimethylaminopropyl chlorophenothiazine, was found to exert a number of pharmacological effects involving the central and autonomic nervous systems. Its possible pharmacological activities attracted widespread interest and Courvoisier _et al_. (2) published an extensive report on the pharmacological activity of chlorpromazines in animals. The most striking aspect of this drug was its therapeutic value in patients with certain emotional disturbances. This discovery provided the initial stimulus for the development and use of phenothiazine drugs in psychiatric practice.

A large amount of research has been conducted on the metabolism of chlorpromazine. The results present a picture for the site of action of chlorpromazine and the drugs metabolic fate. Domino (3) indicated that in low dosages chlorpromazine appears to have negligible effects on the cerebral cortex and specific relay nuclei of the thalamus, and a slight depressant effect upon the hypothalamus and limbic system. An interesting and apparently unique effect of chlorpromazine is that of increasing the inhibitory effects of the activating system on sensory input. Large doses of chlorpromazine produce very mild mixed depressant and stimulant actions on the cerebral cortex, and also a depressant

effect on the hypothalamus (3). In very large toxic doses, chlorpromazine appears to have a direct stimulant effect upon the limbic system. This does not appear to be essential for its tranquilizing properties, but is related to toxic manifestations. Extensive work has been performed on the relationship of chlorpromazine action to concentration of production of brain amines, oxidative metabolism, ACTH, and cell membranes. What these findings mean in terms of an explanation of the therapeutic action of these agents and their derivatives, is still not clear. Some of these studies reflect a genuinely creative attempt to gain some insight into the mechanism of action of the tranquilizers.

The determination of the complete metabolic fate of chlorpromazine is an unsolved problem. Reports have suggested that more than 20 metabolites are formed, and there is the further complication that the bile is as important a channel of excretion as the urine (4). The important metabolic pathways of chlorpromazine so far reported are sulfoxidation, hydroxylation, and demethylation; any combination of these reactions could occur. No evidence for either thioether cleavage *in vivo* or cleavage of the phenothiazine ring in metabolic studies have been reported (5, 6).

In this study the attempt was made to identify specific non-phenothiazine metabolites of chlorpromazine that are produced *in vivo* and *in vitro*. A study was also made of the conjugation system of hydroxymetabolites of chlorpromazine.

CHAPTER II

REVIEW OF LITERATURE

Consideration of the structure of chlorpromazine suggests several possibilities for this drugs metabolic fate. It has been shown (6) that the phenothiazine nucleus of chlorpromazine is susceptible to sulfoxidation and hydroxylation, while the N,N-dimethylaminopropyl side chain can be either partially or completely demethylated, or converted to the N-oxide. Any combination of these reactions may occur. It is of interest that evidence for cleavage of the phenothiazine ring has not been found (6). Structures of some reported metabolites of chlorpromazine are shown in Figure 1.

Thioethers are not usually cleaved _in vivo_ (5), but are oxidized to the corresponding sulfoxide derivatives. Liver is one of the major tissues which metabolizes the phenothiazines. Salzman _et al_. (7) reported in 1956 that chlorpromazine was metabolized to a sulfoxide in dog and man. The sulfoxide was therefore a major metabolite that could undergo further metabolic change _in vitro_ (8, 9). Sulfoxides have been found to be the principal metabolic products of chlorpromazine in the mouse. Upon administration of ^{35}S-chlorpromazine to mice, the radioactivity in the urine was found in the forms of free sulfate (3-5%), "combined sulfate" (23-48%), and the sulfoxide of chlorpromazine (49-73%) (11). However, using ^{35}S-chlorpromazine in rats, Emmerson _et al_. (10) have reported that the urine contained negligible

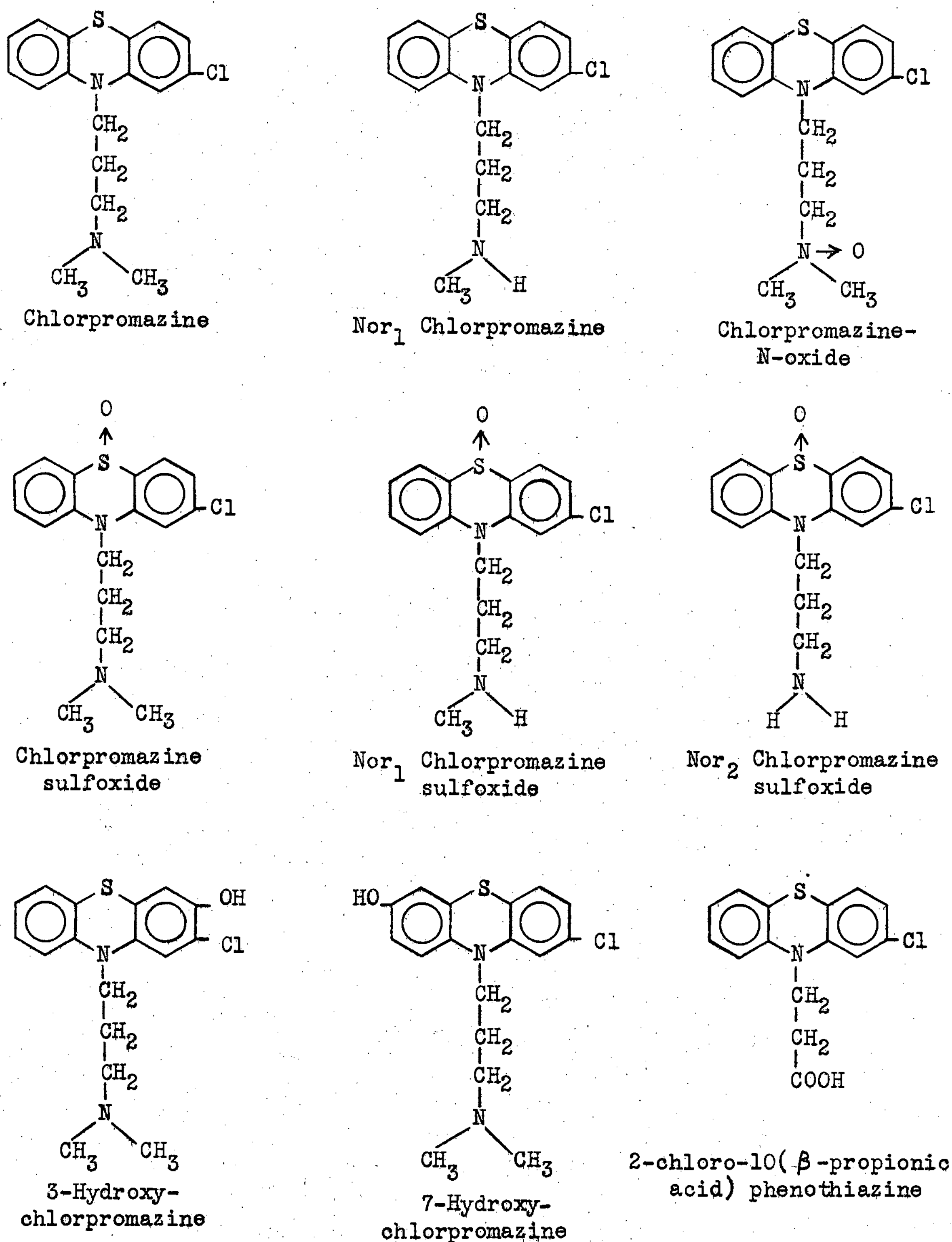

Figure 1. Structures of Some Reported Metabolites of Chlorpromazine

amounts of radioactive inorganic sulfate, and this suggested that, at least as far as the rat is concerned, the ring system in chlorpromazine is biologically stable. The use of ^{35}S-chlorpromazine has shown that in rats 40-50% of single doses are excreted in the urine and an equivalent amount in the feces, the excretion of radioactivity being complete in 72 hours (10). About one half of the urinary radioactivity was made up of four compounds, the main one (12% of the dose) being unmetabolized chlorpromazine. The other three were the sulfoxides of chlorpromazine and its mono-demethylated and di-demethylated derivatives. An enzyme located in a liver microsomal fraction is responsible for the sulfoxidation of phenothiazine (8). Of the various tissues of the guinea pig examined, only liver showed activity. The oxidative conversion of chlorpromazine to sulfoxides requires NADPH and oxygen in addition to the enzyme preparation. Gillette _et al_. (12) showed in 1957 that microsomes contain a NADPH oxidizing system which gave rise to hydrogen peroxide. It was attractive to speculate that the hydrogen peroxide so produced may serve as the source of "active hydroxyl" in the oxidative reaction. The enzyme is probably not a peroxidase. At this time it is not known whether chlorpromazine sulfoxide is further oxidized to the sulfone (6) but Rose and Spinks (13) have shown that methylthioaniline is oxidized to the corresponding sulfone by mice. It has been reported that chlorpromazine sulfoxide but not sulfone is reduced to chlorpromazine by guinea pig liver homogenates under anaerobic conditions, and chlorpromazine sulfone is rapidly metabolized to products of unknown structure by liver mitocondria (9). Moran _et al_. (14) reported that the pharmacological actions of the sulfoxide metabolite differed qualitatively and quantitatively from the parent

drug.

The key reaction of chlorpromazine hydroxylation may be related, by mode of action, to the well established tryptophan hydroxylase in brain (15). Lin *et al*. (16) believe that hydroxylation constitutes an important aspect of chlorpromazine metabolism in man. The hydroxylated compounds (17), referred to as "phenols", are excreted in three identifiable forms, i.e., unconjugated or conjugated to glucuronic acid or to sulfuric acid. A result of this conjugation is the production of more polar compounds than the parent compounds. From chromatographic studies, Posner (18) had proposed and Huang and Kurland (19, 20) also reported, that glucuronides are the major excretory products in man rather than sulfoxides. The degree of glucuronide conjugate formation varies with individuals. A higher degree of conjugation was recognized in patients receiving higher doses of the drug. Dutton *et al*. (21) found that glucuronide synthesis by way of enzymatic transfer of glucuronic acid from UDP-glucuronic acid is not restricted to the liver, but could occur in the renal cortex, gastrointestinal mucosa and skin. Treatment of human urine with β-glucuronidase has been shown to release the hydroxylated metabolites of chlorpromazine (16, 22, 23). Evidence has been obtained to show that glucuronide and hydroxy derivatives can occur with the products of demethylation (24). The situation is further complicated by possible dihydroxylation and hydroxylation of the nucleus at a number of isomeric positions. The major site of hydroxylation was reported to be at the 7 position (17). Hydroxylation reactions perform important roles in many biosynthetic and catabolic processes. Unfortunately, the mechanism of this enzymatic reaction is not known. A monooxygenase, salicylate hydroxylase,

was isolated from a soil bacterium by Katagiric *et al*. (25). This enzyme catalyzes the stoichiometric formation of catechol from salicylate and reduced pyridine nucleotide in the presence of FAD as a specific cofactor. FAD apparently protects the enzyme from inactivation (26).

Chlorpromazine-N-oxide was not a major metabolite of chlorpromazine as it accounted for less than 1% of the dose (27). An alkylarylamine oxygenase is probably involved in the formation of the N-oxide (28).

Demethylation has been proved to be an important process in the rat. Labeled chlorpromazine with ^{14}C in one of the methyl groups showed that as much as 17% of the isotope was exhaled as carbon dioxide in six hours (29). In man both methyl groups may be removed from the dimethylamino group of chlorpromazine; the mono-methyl and the completely demethylated sulfoxide derivatives have been stated to be the major metabolites, but quantitative data are lacking (30). Removal of the entire dimethylaminopropyl group apparently does not occur in dogs (31). Ross *et al*. (29) have shown the mono or complete demethylation of chlorpromazine *in vitro*. Fishman *et al*. (30) noted that demethylated derivatives were found to account for at least two of the sulfoxide metabolites. Additional evidence was given in a later report to substantiate the formation of the desmethyl and desdimethyl sulfoxide metabolites of chlorpromazine (32).

The formation of free radicals from chlorpromazine and its metabolites *in vivo* has been considered to occur. The work of the Forrests *et al*. (33) indicated that free radical intermediates of chlorpromazine appear in the urine of patients under medication, and may be of

pharmacological significance.

The enzyme systems concerned in the metabolic pathways of drugs, i.e., deamination, dealkylation, hydroxylation etc., are located principally in liver microsomes and have the remarkable requirement for both reduced NADP and oxygen (12). In 1955, Axelrod (34) reported that upon fractionations of rabbit whole liver, the microsomes alone could metabolize amphetamine, but a very slow rate. Addition of the soluble fraction greatly increased the microsomal activities. This suggests that the microsomal activities are NADPH dependent, as the soluble fraction contained glucose-6-phosphate and glucose-6-phosphate dehydrogenase which serves as a NADPH generating system. When this system lacks NADPH, the microsomal activities are limited. Gaudette _et al._ (35) have studied the dealkylation of a series of foreign and endogenous alkylamines. They show that only those compounds exhibiting a high chloroform/water partition ratio at physiological pHs are dealkylated by the microsomal system. It is of interest that the endogenous substrates studied exhibit extremely low partition and are not metabolized. This suggests (30) that the microsomal enzymes are protected by a lipid barrier which is impermeable to polar compounds. Bousquet (36) summarizes cofactor requirements for the microsomal enzymes carrying out oxidative reactions as follows; they are quite similar for hydroxylation, ether cleavage, dealkylation, and deamination. The pH optima are also similar, varying only slightly from pH 7.0 to 8.5, depending on the particular substrate. NADPH is a general requirement for all of these reactions, as is molecular oxygen. NADH can not replace NADPH in the oxidative reactions. Nicotinamide is frequently added to homogenates to protect NADP, and Mg^{++} is a required

ion. Glucose-6-phosphate and glucose-6-phosphate dehydrogenase are required to maintain NADP in the reduced state.

Consideration has been given to the literature associated with the metabolism of chlorpromazine. Several important metabolic pathways have been elucidated for this type drug. A particularly characteristic reaction is sulfoxidation. Others include hydroxylation, demethylation, N-oxide formation, and conjugation of the phenols with glucuronic acid. Evidence indicates that aromatic hydroxylation followed by conjugation with glucuronic acid is the dominant metabolic pathway for the phenothiazine drugs in man, and does not necessarily result in loss of pharmacological activity. In view of the number of known and theoretical metabolites of chlorpromazine, and the inherent lability of the phenothiazine nucleus, one should conclude that the progress in elucidating the metabolic fate of chlorpromazine has been quite remarkable. However, much work remains to be done, since no single metabolite identified to date accounts for more than 8-10% of the dose (1).

CHAPTER III

EXPERIMENTAL

Animals and Tissues

Male albino rats (300 to 460 gm) obtained from Holtzman Company were used for the *in vivo* experiments. The rats were not fasted before experiments, and following the intraperitoneal administration of ^{3}H-chlorpromazine, or saline solution for control, the rats were placed in urine collection cages. They received water for the duration of the experiment.

Fresh pig livers were purchased from Ralph's Packing Co., Perkins, Oklahoma. As soon as they were removed from pig, they were sectioned and chilled with ice.

Reagents

^{3}H-chlorpromazine: 20 mg dissolved in 2 ml saline solution (9 gm/l.). Source: Randomly labeled by the Wilzbach method (37) Biochemistry Dept., Oklahoma State University, Stillwater, Oklahoma.

Chlorpromazine hydrochloride. RSO#66503, Smith, Kline and French Labs., Philadelphia, Pennsylvania.

Orthanilic acid. K and K Laboratories, Inc., Plainview, New York.

Eastman Chromagram Sheet, type K301R2. Silica Gel, without fluorescent indicator. Eastman Kodak Co., Rochester, New York.

Silica Gel G. acc. to Stahl. Brinkman Instruments Inc., Cantiague Road Westbury, L.I., New York.

POPOP (1,4-bis-2-5-phenyloxazolyl-benzene), PPO (2,5-diphenyloxazole). Scintillation Grade, Packard Instrument Company, Inc., Box 428, LaGrange, Illinois.

β-Glucuronidase/Aryl Sulfatase. B grade, Calbiochem, Los Angeles, California.

Glucose-6-phosphate dehydrogenase, type VI. Sigma Chemical Company, 3500 Dekalb St., St. Louis 18, Missouri.

Ethylene glycol. Fisher Scientific Company, 4102 Greenbriar Drive, Houston, Texas.

Diethylamine. Matheson Coleman and Bell, East Rutherford, New Jersey.

Toluene, para-chlorobenzenesulfonic acid, naphthalene. Eastman Organic Chemicals, Rochester 3, New York.

Chloroform, ceric ammonium nitrate, ethyl acetate, methanol. J. T. Baker Chemical Co., Phillipsburg, New Jersey.

Collection and Preparation of Urine

After the intraperitoneal administration of ^{3}H-chlorpromazine, or saline solution for control, each rat's urine was collected in an Erlenmeyer flask containing 5 ml of toluene at each period of 24 hours (23). Any feces were filtered out, and the filtrate was acidified to pH 2 with 6 N HCl, and separated into a volatile and solid portion with a rotary evaporator. Both portions separated were used as part of a balance study and for the identification of specific metabolites.

Preparation of Tissue Subcellular Fractions

Subcellular fractions were prepared according to the method described by Gillette and co-workers (12). Four hundred gms of liver were sectioned into small pieces, then 1200 ml of 1.15% isotonic KCl solution containing 8.0 ml of 0.02 M K_2CO_3 was added. This mixture was immediately homogenized for 2 minutes in a large size Waring blender at full speed to obtain the homogenate. Four ml of the homogenate was equivalent to 1 gm of liver. The homogenate was centrifuged in a refrigerated centrifuge, Model RC-2, Ivan Sorvall, Inc., at 10,000 X G (7,500-8,000 rpm) for 30 minutes. A volume of 1200 ml of supernatant was obtained by filtration through glass wool. This served as the microsomal and soluble fractions. This supernatant was recentrifuged to separate the microsomal fraction. A Beckman Model-L Ultracentrifuge was used at 27,000 rpm for one and half hours. The supernatant was decanted and the microsomal fraction was suspended in isotonic KCl solution to make a final volume of 400 ml. One ml of liver microsomal fraction suspension was equivalent to 1 gm of liver. All operations were conducted in a cold room, and all cell fractions prepared were kept frozen for up to 6 months without appreciable loss of the activities of N-oxidase and N-demethylase enzymes.

Balance Study

Substrate Preparation for Incubation with Liver Microsomal Fraction

Five μmoles of ^{3}H-chlorpromazine were used in the incubation mixture in the *in vitro* balance study. ^{3}H-chlorpromazine was dissolved in 95% ethanol to make a 0.02 M solution and then diluted to 0.01 M with

0.2 M phosphate buffer pH 7.4.

Incubation of Liver Microsomes with ^{3}H-chlorpromazine

The assay system described by Mueller *et al.* (38) and Shuster *et al.* (39) was followed. Two ml portions of liver microsomal fraction, 1 ml equivalent to 1 gm of liver, was incubated with 0.5 ml of 0.01 M ^{3}H-chlorpromazine, 0.2 ml of 0.004 M NADP, 0.2 ml of 0.1 M magnesium chloride, 0.5 ml of 0.1 M nicotinamide, 0.5 ml of 0.05 M dipotassium glucose-6-phosphate, 0.2 ml of glucose-6-phosphate dehydrogenase (2 units), 0.1 ml of 0.25 M neutralized semicarbazide, and 1.0 ml of 0.2 M phosphate buffer pH 7.4, to make a final volume of 5.3 ml. The mixture was then incubated in a shaking water bath at 37°C under an oxygen atmosphere. After one hour the mixture was heated to 70°C for 5 minutes to denature the enzyme and the denatured protein was removed by centrifugation. This incubated solution was then treated in the same manner as the urine collected from the *in vivo* experiments.

Fractionation of Metabolites

Both urine samples and microsomal fractions (incubated solution) were adjusted to pH 13 with 1 N NaOH. Chlorpromazine N-oxide, sulfoxide, dealkylated products, and unmetabolized chlorpromazine were removed by extraction of the pH 13 solution twice with three volumes of chloroform. The residual solution was titrated to pH 9.5 with 1 N HCl and the "free" phenolic metabolites of chlorpromazine were extracted from this solution with two extractions of three volumes of chloroform. The residue solution was titrated again to a pH of 4.5 and after incubation with β-glucuronidase/aryl sulfatase, the

liberated phenolic metabolites were extracted twice with three volumes of chloroform at pH 9.5. Each chloroform solution was concentrated to a volume of about 3 ml.

Incubation with β-glucuronidase/Aryl Sulfatase

A procedure developed by Talalay *et al.* (40) in 1946 for β-glucuronidase/aryl sulfatase release of products formed by conjugation of phenolic metabolites and aryl sulfonic acid was used. Two ml of pH 4.5 metabolic fraction and urine were mixed with equal volumes of acetate buffer pH 4.5. After addition of 0.05-0.1 ml of β-glucuronidase/aryl sulfatase (10,000 units/ml), the mixture was incubated in a shaking water bath for 18 hours at 37°C.

Liquid Scintillation Spectrometry

The total radioactivity of each fraction of either chloroform soluble phase or aqueous phase, which was obtained as described above, was determined in a Liquid Scintillation Spectrometer, Tri-Carb Model 527, Packard Instrument Co. A scintillation solvent system developed by Bray (41) was used in this study. One tenth or one ml of sample was added to 15 ml of Bray solvent in the liquid scintillation counting. The Bray solvent was prepared by dissolving 60 gm of naphthalene, 4 gm of PPO and 200 mg of POPOP in 100 ml of methanol, 20 ml of ethylene glycol and para-dioxane to make 1 liter. A quenching correction factor was determined by recounting all samples after adding 1 ml of ^{3}H-chlorpromazine solution which contained 2.5 mg of ^{3}H-chlorpromazine in 100 ml of Bray solvent. This solution was added so that the radioactivity of the blank was increased to about 1,000 cpm. In this study,

the urine collected from control rats was used as a blank.

Identification of Metabolites

An attempt was made to determine whether there was a cleavage at the position of the ring N and/or the S. If this process occurred, it was proposed that there would be orthanilic acid, aromatic amines and their derivatives, and sulfonic acids present as metabolites. A quantitative determination was also devised for unmetabolized chlorpromazine and hydroxylated chlorpromazine.

Thin-Layer Chromatography

In this study, the thin-layer plates were prepared by adding 61 ml of water to 30 gm of Silica Gel G. The layer thickness was 50 μ. Eastman Kodak thin-layer chromagram sheets were also used in this study.

Solvents

Chromatograms were developed in two solvent systems by an ascending technique. Other solvent systems were used, but these two systems were the best. Solvent 1: ethylacetate:methanol:diethylamine (14:4:5) (17). Solvent 2: n-butanol:acetic acid:water (40:10:50). Solvent 1 was adequate for the separation of non-polar metabolites, while aromatic amines and other compounds gave an acceptable separation in solvent 2.

Spray Reagents

Chlorpromazine and its derivatives can be detected by a spray reagent consisting of sulfuric acid :water : 95% ethanol (1:1:8) (23); the spots were a pink or purple color.

Aromatic amines can be detected at concentrations as low as 0.1 µmoles in a test tube color reaction by a method described by Hearn *et al*. (42). This reagent can also be used as a spray and the sensitivity is still very good for detecting concentrations as low as 0.01 µmoles on thin-layer chromatograms. One other advantage of this reagent is that each amine has its own characteristic color, and this changes to a final stable color after warming from 30 seconds to 1 minute. This reagent was prepared by dissolving 10 gm of ammonium ceric nitrate in 100 ml of 5% nitric acid. Other reagents were tried, but none were as sensitive and simple as this reagent. A dichromate-sulfuric acid solution, in which 5 gm potassium dichromate was dissolved in 100 ml of 40% aqueous sulfuric acid, was also valuable for identifying primary, secondary, and tertiary amines by various colors (43). Primary amines gave a blue color immediately, secondary amines gave a yellow color which changed to blue after 10 minutes, while tertiary amines gave light yellow colors. Sulfoxides, sulfones, and sulfonic acids were detected as purple spots on thin-layer chromatograms under ultraviolet light. No suitable spray reagent was found for sulfoxides and sulfonic acids. However sulfones can be detected by an iodine spray solution (44). This reagent was prepared by dissolving 0.3 gm of iodine in 100 ml of aqueous 5% potassium iodide solution.

Preparations of Samples for Detection of Non-Phenothiazine Metabolites

The urine collected on the first and second days from three rats administrated ^{3}H-chlorpromazine was pooled for each rat and concentrated with a rotary evaporator until a volume of about 3 ml was obtained.

Two extractions with 3 volumes of chloroform at pH 11 (fraction A) were made. Fraction A would contain free aromatic amines. Concentrated HCl was added to the aqueous phase (fraction B) until a concentration of 20% HCl was obtained, and then hydrolysis with refluxing was conducted for one and half hours. The resulting solution was evaporated to dryness under reduced pressure and 50 ml of deionized water was added. This was evaporated to dryness again to remove most of the HCl. The residue was dissolved and transferred, with a small amount of water, into a beaker. After adjustment of the pH to 11, the liberated aromatic amines (fraction C) from conjugation was extracted twice with 3 volumes of chloroform. This residue (fraction D) was adjusted to pH 5. All three fractions were then dried and 0.2 ml of methanol was added to fractions A and C and 0.5 ml of H_2O to fractions B and D. A system of color tests was arranged and thin-layer chromatography was utilized for qualitative identification of metabolites.

Preparations of Samples for Assay of Phenothiazine Metabolites

Both urine which was collected on the first, second, and third days and liver microsomal incubated solutions were used for the identification study of phenothiazine metabolites. The procedures were the same as that described in the balance study. Samples of phenothiazine metabolites were obtained in the three chloroform extracts at pH 13, at pH 9.5 and at pH 9.5 after incubation with β-glucuronidase/aryl sulfatase. The chloroform extracts of samples were evaporated to dryness by passing dry air over the liquid surface. Two tenths ml of $CHCl_3$ was added to each sample residue and these extracts were used for qualitative identification and quantitative studies.

Identification of Non-Phenothiazine and Phenothiazine Metabolites

Non-phenothiazine metabolites were identified by color tests in test tube reactions and by thin-layer chromatographic techniques. In the thin-layer chromatography studies, orthanilic acid, chlorobenzenesulfonic acid, and other aromatic amines which were considered to be possible metabolites were used as references. On the other hand, in the phenothiazine identification studies, the compounds used for references were the 7-hydroxychlorpromazine, chlorpromazine, and chlorpromazine N-oxide. Since the interest in this study was on the hydroxylated metabolites and the non-phenothiazine metabolites other phenothiazine unknown spots were not identified.

Quantitative Studies

Street (45) found that the colored solution formed by chlorpromazine in 50% sulfuric acid exhibited a characteristic ultraviolet absorption spectra. Based on this observation Kurland *et al*. (46) developed a quantitative assay for chlorpromazine. In this study, their method was followed and a standard curve was prepared for chlorpromazine and 7-hydroxychlorpromazine, then the unchanged chlorpromazine and the 7-hydroxychlorpromazine formed was determined. The procedure for this study was: all the samples were dried, a volume of 0.1 ml of chloroform was added and 25-50 λ of each sample was spotted on thin-layer chromatographic sheets. The sheets were developed in a solvent system consisting of ethylacetate : methanol : diethylamine (14 : 4 : 5) by an ascending technique. Chlorpromazine and 7-hydroxychlorpromazine were detected by spraying with the sulfuric acid

reagent, and they produced a pink and purple color respectively. All the colored area of this spot was removed and the color was extracted with 3.5 ml of 50% sulfuric acid. Centrifugation was used to remove the silica gel. The absorbancy of the colored acid extract was determined in a Beckmann D. U. Spectrophotometer. A solution of 50% sulfuric acid was used as the blank. The Cary 14 Recording Spectrophotometer was used to determine the absorption spectra of reference compounds and isolated metabolites.

CHAPTER IV

RESULTS AND DISCUSSION

Balance Study

As has been indicated in the literature review the metabolic modifications of chlorpromazine are manifold and include sulfoxidation, demethylation, ring hydroxylation and conjugation, and oxidation at the terminal nitrogen of the side chain. Since any combination of these reactions may occur, a theoretically large number of metabolites could result. No single metabolite identified to date accounts for more than 8-10% of the dose (1). Emmerson _et al_. (10) have shown that approximately 40% of the administered radioactivity was excreted by the rat in the urine when ^{35}S-chlorpromazine was administered. In this study, when the rat was administered ^{3}H-chlorpromazine, results similar to those reported by Emmerson _et al_. were obtained. About 35 to 40% of the administered radioactivity appeared in the urine within five days (Table I). It is shown in Table I and II that the major portion of administered radioactivity was in the solids of urine, while the volatile phase, which might contain $^{3}H_2O$, R-$CH^{3}O$, etc. accounted for only 0.1% of the ^{3}H-chlorpromazine. As stated the major part of the radioactivity was excreted by the rat during the first day, while after the fourth day less than 2% was excreted. It is of interest that in this study, although half of the dose was administered on the first day and the remainder on the next day, an accumulation of radioactivity on the

TABLE I

RADIOACTIVITY IN URINE EXCRETION FROM RATS

Rat[a]	Body Weight	CPZ[b] Dose	Activity Observed: Day Collected	1	2	3	4	5	Total Activity Observed	Recovery
	gms	mμc		mμc	mμc	mμc	mμc	mμc	mμc	%
1	452	690	Solid	158.0	34.3	32.9	9.6	5.6	242.8	35.2
			Liquid	0.7	0.6	0.4	0.4	0.4		
2	457	764.6	Solid	199.8	80.9	15.9	2.7	3.3	305.4	40.0
			Liquid	0.6	0.6	0.6	0.5	0.5		
3	350	298	Solid	73.7	20.7	13.6	2.6		112.0	37.6
			Liquid	0.7	0.3	0.2	0.2			
4	340	298	Solid	70.2	19.5	9.2	1.4		101.5	34.1
			Liquid	0.7	0.3	0.1	0.1			
5	360	410	Solid	105.7	32.3	12.0	6.2		157.2	38.4
			Liquid	0.4	0.4	0.1	0.1			

[a]Rat 1, 2, were injected with half of the dose on the first day, and the remainder on the next day.

[b]Rat 3, 4, 5, were given the entire dose on first day.

TABLE II

PERCENTAGE OF RADIOACTIVITY[a] EXCRETED IN SOLID PHASE OF URINE FOR EACH DAY

Rat	Total Radioactivity Observed	Day 1	Day 2	Day 3	Day 4	Day 5
	mμc	%	%	%	%	%
1	242.8	65.2	14.1	13.5	4.0	2.3
2	305.4	65.5	26.5	5.2	0.9	0.1
3	112.0	65.6	18.5	12.0	2.3	
4	101.5	69.2	19.2	9.1	1.4	
5	157.2	67.2	20.5	7.6	3.9	

[a]Based on total radioactivity observed.

second day in excreted urine was not observed (Table II).

Posner (18) had observed that glucuronides were the major excretory products in man rather than sulfoxides. In this study with ^{3}H-chlorpromazine, it may be seen from Table III that this is not the case with the rat. Fractionation of urine from rats receiving chlorpromazine, based on radioactivity of each day observed, showed over 50% of the administered radioactivity was in the chloroform extracts of urine at pH 13, while less than 7% was in the chloroform extracts of urine at pH 9.5 before and after incubation with β-glucuronidase/aryl sulfatase. As shown in Table III, the amount of "free" and conjugated phenolic metabolites were increased on the second day, although the radioactivity in this fraction was low. In spite of the small amount of "free" and conjugated phenolic metabolites, it is of interest that both fractions are almost equal, and the degree of glucuronide conjugation was independent of the day after drug administration. This suggests that there may exist an equilibrium between "free" and conjugated metabolites, and the equilibrium constant is close to unity.

If we look at the radioactivity of the aqueous urine residue (Table III), which is obtained after incubating with β-glucuronidase /aryl sulfatase, the activity of ^{3}H was almost negligible. These results are quite different from that of mice receiving ^{35}S-chlorpromazine. Which Christensen <u>et al.</u> (11) found with mice 3-5% administrated radioactivity was in the form of free sulfate, and 23-48% in the "combined sulfate" form. This indicates that the phenothiazine ring in chlorpromazine is possibly more stable in rats than in mice.

As described in the literature review, Gillette <u>et al.</u> (12) observed that liver microsomes had many enzyme systems concerned with

TABLE III

RADIOACTIVITY OF URINE CHLOROFORM EXTRACTS AND RESIDUE

			Distribution of Radioactivity			
					Enzyme[a] Incubated	
Rat	Day Collected	Total Activity	pH 13 $CHCl_3$ Extracts	pH 9.5 $CHCl_3$ Extracts	pH 9.5 $CHCl_3$ Extracts	Water Residue
		mμc	mμc	mμc	mμc	mμc
1	1	158.0	109.6	7.0	10.1	1.4
	2	34.3	16.5	5.3	7.4	1.9
	3	32.9	17.4	3.1	2.0	0.8
2	1	199.8	139.0	13.9	14.1	5.0
	2	80.9	48.3	6.5	8.4	5.7
	3	15.9	9.3	1.1	2.0	0.5

Distribution[b] of Radioactivity as Percent of Total Per Day

Rat	Day Collected		pH 13 $CHCl_3$ Extracts	pH 9.5 $CHCl_3$ Extracts	pH 9.5 $CHCl_3$ Extracts (Enzyme Incubated)	Water Residue
			%	%	%	%
1	1		69.4	4.9	6.3	0.9
	2		48.1	15.5	21.6	5.5
	3		53.0	9.4	6.1	2.4
2	1		69.5	6.9	7.0	2.5
	2		59.9	8.0	10.4	7.1
	3		58.5	6.9	12.6	3.1

[a] β-Glucuronidase/aryl sulfatase.

[b] Calculation based on total radioactivity excreted of each day observed.

the metabolism of drugs, and these enzyme systems had the remarkable requirement for both reduced NADP and oxygen. In the *in vitro* experiments in this study, glucose-6-phosphate and glucose-6-phosphate dehydrogenase were added to microsomal fractions to serve as a NADPH generating system. The NADPH generating system was not required with the 10,000 X G supernatant and the homogenate. As in the method described by Gillette *et al.* (12), nicotinamide was added to protect NADP and Mg^{++} was a required ion. The enzyme assay system for chlorpromazine oxidase is shown in Table IV.

When ^{3}H-chlorpromazine was incubated with pig liver microsomes, the radioactivity distribution from the fractionation of the incubated solution was similar to that of urine, except with the conjugated metabolites. As shown in Table V, chloroform extracts at pH 13 still contained the major portion of the administered radioactivity. The radioactivity in the aqueous phase after incubation with β-glucuronidase/aryl sulfatase was negligible. With the pig liver microsomal fraction, the degree of glucuronide conjugation was greatly decreased and was approximately one tenth of that of free phenolic metabolites. It might be possible that there exists a slow step in the conjugation reaction so that a one hour incubation was not enough time for a complete reaction. There might also be a lack of the enzyme system or cofactors for this reaction in pig liver microsomes, or the reaction may occur in another tissue. Comparing Table V with Table III, it is evident that the sum of free and conjugated phenolic metabolites in the *in vitro* study with pig liver and the first days rat urine are similar. Although the degree of glucuronide conjugation was different between these two experiments, the rate of hydroxylation might be the

TABLE IV

CHLORPROMAZINE OXIDASE[a] ASSAY SYSTEM

Item	Homogenate[b]	Supernatant[b] 10,000 x G	Microsome[b]
	μmoles	μmoles	μmoles
Chlorpromazine	5	5	5
NADP	0.8	0.8	0.8
$MgCl_2$	20	20	20
Nicotinamide	50	50	50
Glucose-6-phosphate	25	25	25
Glucose-6-phosphate dehydrogenase			2 units
Neutralized semicarbazide	25	25	25
0.2 M phosphate buffer pH 7.4	1 ml	1 ml	1 ml

[a]Ring hydroxylation at the 7-position of the phenothiazine nucleus.

[b]A volume of tissue preparation equivalent to 0.5 g of fresh liver was added, a volume of water added to each mixture to make the volume up to 6.8 ml.

TABLE V

RADIOACTIVITY OF CHLOROFORM EXTRACTS OF PIG LIVER MICROSOMES INCUBATED WITH ^{3}H-CHLORPROMAZINE

				Residue Incubated With Enzyme[a]	
	Total Activity	pH 13 $CHCl_3$ Extracts	pH 9.5 $CHCl_3$ Extracts	pH 9.5 $CHCl_3$ Extracts	Residue Aqueous Phase
	mμc	mμc	mμc	mμc	mμc
Radioactive Distribution	242	181.3	29.8	3.0	4.2
		%	%	%	%
Distribution		75	12.3	1.2	1.7

[a] β-Glucuronidase/aryl sulfatase.

same. The comparison of rat urine metabolites with pig liver microsome metabolites may not be valid.

Metabolite Identification Study

Although no evidence has been obtained that there is a cleavage of the phenothiazine ring in chlorpromazine metabolism, this might occur as shown in Figure 2. In Table I, II, and III, there is only about 1.2% of administered radioactivity left in the aqueous urine residue. In other words, when three rat urines were pooled (Table I), there was an amount of compound in the water residue equivalent to 1 μmole or about 0.36 mg of chlorpromazine. These concentrations are within the detectable range of color tests and thin-layer chromatography. However, as shown in Table VI, the suspected compounds were not detected. The aromatic amines, orthanilic acids, chlorobenzenesulfonic acid and other sulfonic acids tests were negative. These results do not prove that ring cleavage does not occur as a high salt concentration in the samples and other technique problems were present. On the other hand, there may be so many different kinds of compounds present that each compound could only be present in a concentration to low for detection.

7-Hydroxychlorpromazine and unmetabolized chlorpromazine were identified in both rat urine and incubated pig liver subcellular fractions, but as shown in Table VII, the pattern for other metabolites appears to be different. In chloroform extracts at pH 13, there was a pink unidentified spot at R_f 0.72 in rat urine, while the solution incubated with pig liver subcellular fractions failed to show this metabolite, but did show a pink spot of chlorpromazine N-oxide at R_f

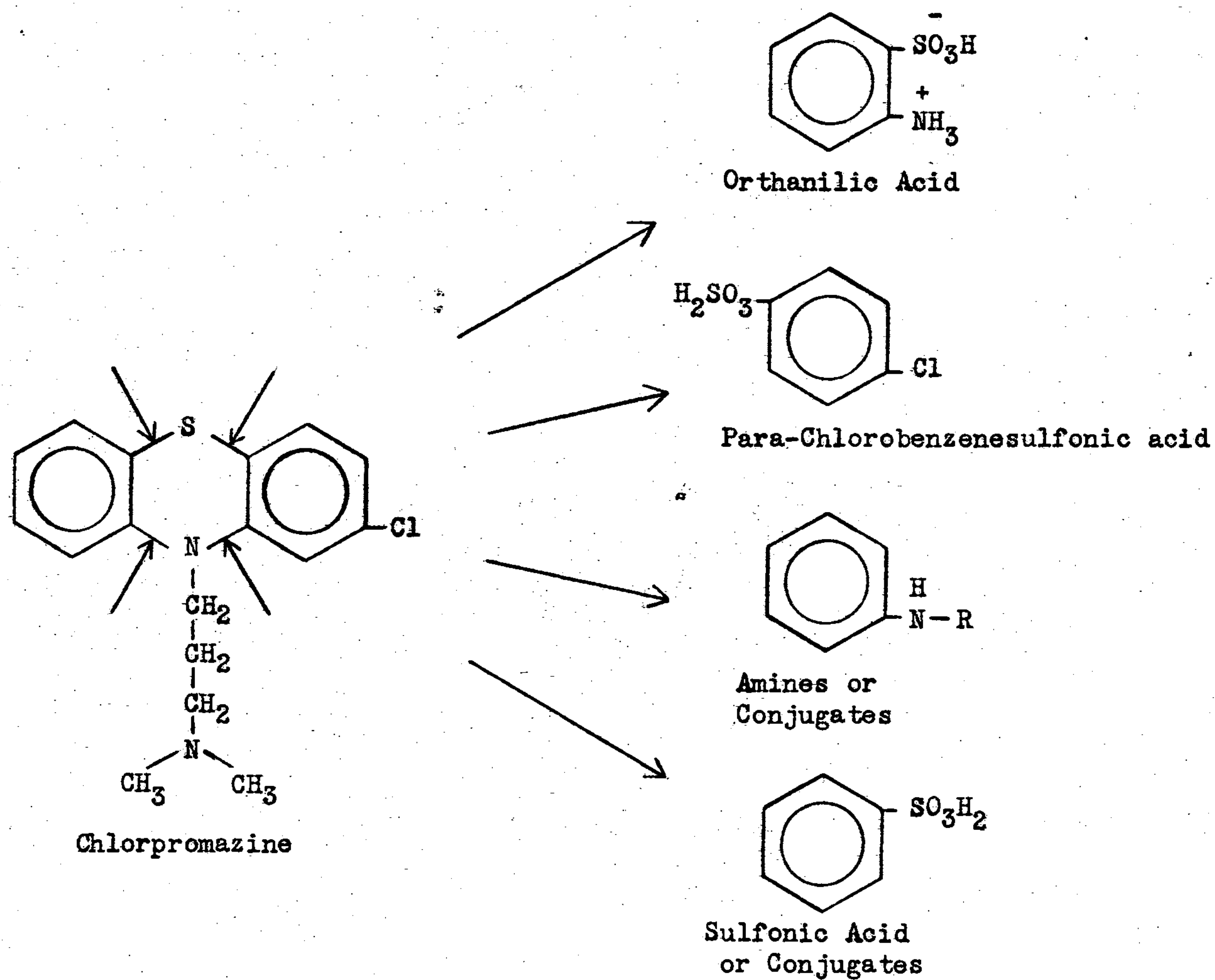

Figure 2. Proposed Ring Cleavage of Chlorpromazine With Products That May Result From This Process

TABLE VI

NON-PHENOTHIAZINE METABOLITES IN RAT URINE: TESTS FOR AROMATIC AMINES, ORTHANILIC ACID, PARA-CHLOROBENZENESULFONIC ACID

	pH 11 Chloroform Extracts	pH 11 Aqueous Phase	pH Chloroform Extracts After Acid Hydrolysis	pH 11 Aqueous Phase After Hydrolysis	R_f Value of Reference compd	
					Solvent I[a]	Solvent II[b]
Aromatic Amine	Negative	Negative	Negative	Negative		
Orthanilic Acid[c]	Negative	Negative	Negative	Negative	0.15	0.27
Para-chlorobenzene-Sulfonic Acid[d]	Negative	Negative	Negative	Negative	0.33	0.44

[a]Solvent I: ethylacetate : methanol : diethylamine (14:4:5).

[b]Solvent II: n-butanol : acetic acid : H_2O (40:10:50).

[c]Detected by spraying with ammoniun ceric nitrate in 5% nitric acid, pale orange-brown (final color).

[d]Detected under ultraviolet light (short frequency).

TABLE VII

IDENTIFICATION OF CHLORPROMAZINE METABOLITES IN RAT URINE AND INCUBATED SUBCELLULAR FRACTIONS FROM PIG LIVER

	R_f[a]	Color[b] With H_2SO_4 Reagent	Probable Identity[c]
First Chloroform Extract at pH 13			
Urine (1st. day)	0.82	pink	CPZ[e]
	0.72	pink	unknown
Subcellular[d] Fractions	0.81	pink	CPZ
	0.26	pink	CPZ-N-oxide
	0.1	pink	unknown
Second Chloroform Extract at pH 9.5 Before Incubation With β-Glucuronidase/Aryl Sulfatase			
Urine first day	0.85	pink	unknown
	0.67	purple	7-hydroxy CPZ
second day	0.86	pink	unknown
	0.66	purple	7-hydroxy CPZ
	0.21	pink	unknown
	0.09	pink	unknown
Microsomes	0.67	purple	7-hydroxy CPZ
	0.58	yellow	unknown
	0.21	pink	unknown
	0.10	pink	unknown

TABLE VII (CONTINUED)

Third Chloroform Extract at pH 9.5 After Incubation With β-Glucuronidase/Aryl Sulfatase			
	R_f	Color With H_2SO_4 Reagent	Probable Identity
Urine			
first day	0.80	purple	unknown
	0.66	purple	7-hydroxy CPZ
	0.10	pink	unknown
second day	0.82	purple	unknown
	0.66	purple	7-hydroxy CPZ
	0.10	pink	unknown
Microsome	0.66	yellow/purple	suspected 7-hydroxy CPZ
	0.10	pink	unknown

[a]Eastman Chromagram Sheets were used, developed in ethylacetate: methanol:diethylamine (14:4:5).

[b]H_2SO_4:water:95% ethanol (1:1:8).

[c]Chlorpromazine and 7-hydroxychlorpromazine as references.

[d]Microsomes, homogenate, and supernatant (10,000XG) all show the same pattern.

[e]CPZ = chlorpromazine.

0.26 and an unidentified pink spot at R_f 0.10. A more complicated pattern was observed in chloroform extracts in the second days urine at pH 9.5. Two metabolites in the first days urine in the nonconjugated form, and three metabolites in the conjugated form were observed. Both extracts before and after incubation, contained 7-hydroxychlorpromazine, the other unknown metabolites showed a different color and R_f value. The second days urine showed two more unknown metabolites with pink colors in nonconjugated forms and the same for conjugated metabolites. However microsomes showed a similar result relative to the second days urine sample with nonconjugated metabolites, but showed only two spots for conjugated metabolites. It is of interest that microsomes did not show the unknown spot with R_f 0.8-0.86 as seen in urine with nonconjugated and conjugated metabolites. 7-Hydroxychlorpromazine was positively identified in rat urine and in the solution incubated with pig liver subcellular fractions in both nonconjugated and conjugated. This suggests that 7-hydroxychlorpromazine is an important metabolite and may be the major one.

Quantitative Study

Ultraviolet and visible spectra were made for chlorpromazine and 7-hydroxychlorpromazine in 50% sulfuric acid (see Experimental), which were shown in Figures 3 and 4. The wave length of maximum absorption was determined. As shown in Figure 5, the standard curve was made at 560 millimicrons for the quantitative determination of both unmetabolized chlorpromazine and 7-hydroxychlorpromazine.

The results of a quantitative determination are shown in Table VIII. To confirm these quantitative results, it is necessary to compare

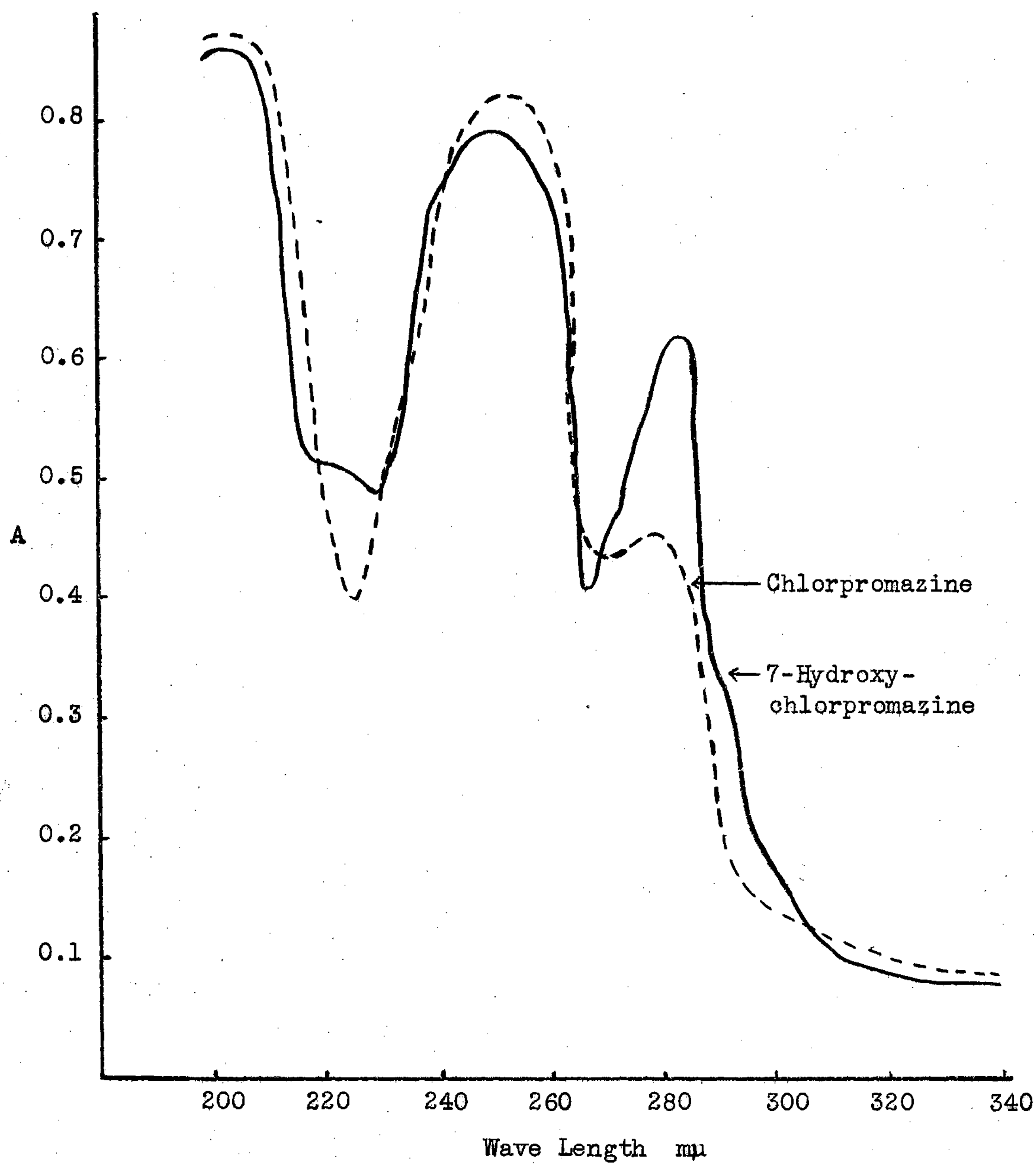

Figure 3. The Ultraviolet Spectra of Chlorpromazine and 7-Hydroxychlorpromazine

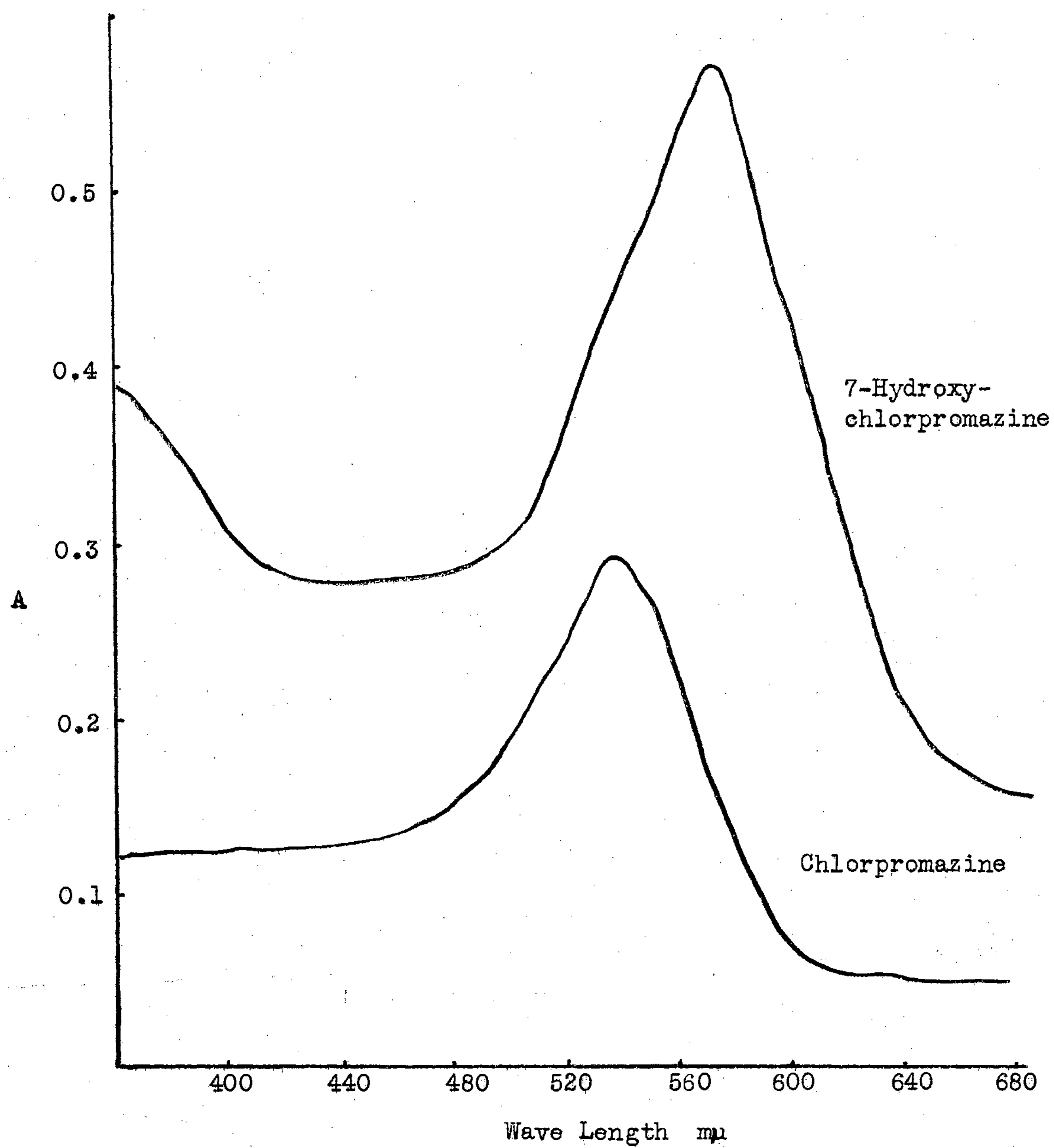

Figure 4. The Visible Spectra of Chlorpromazine and 7-Hydroxychlorpromazine

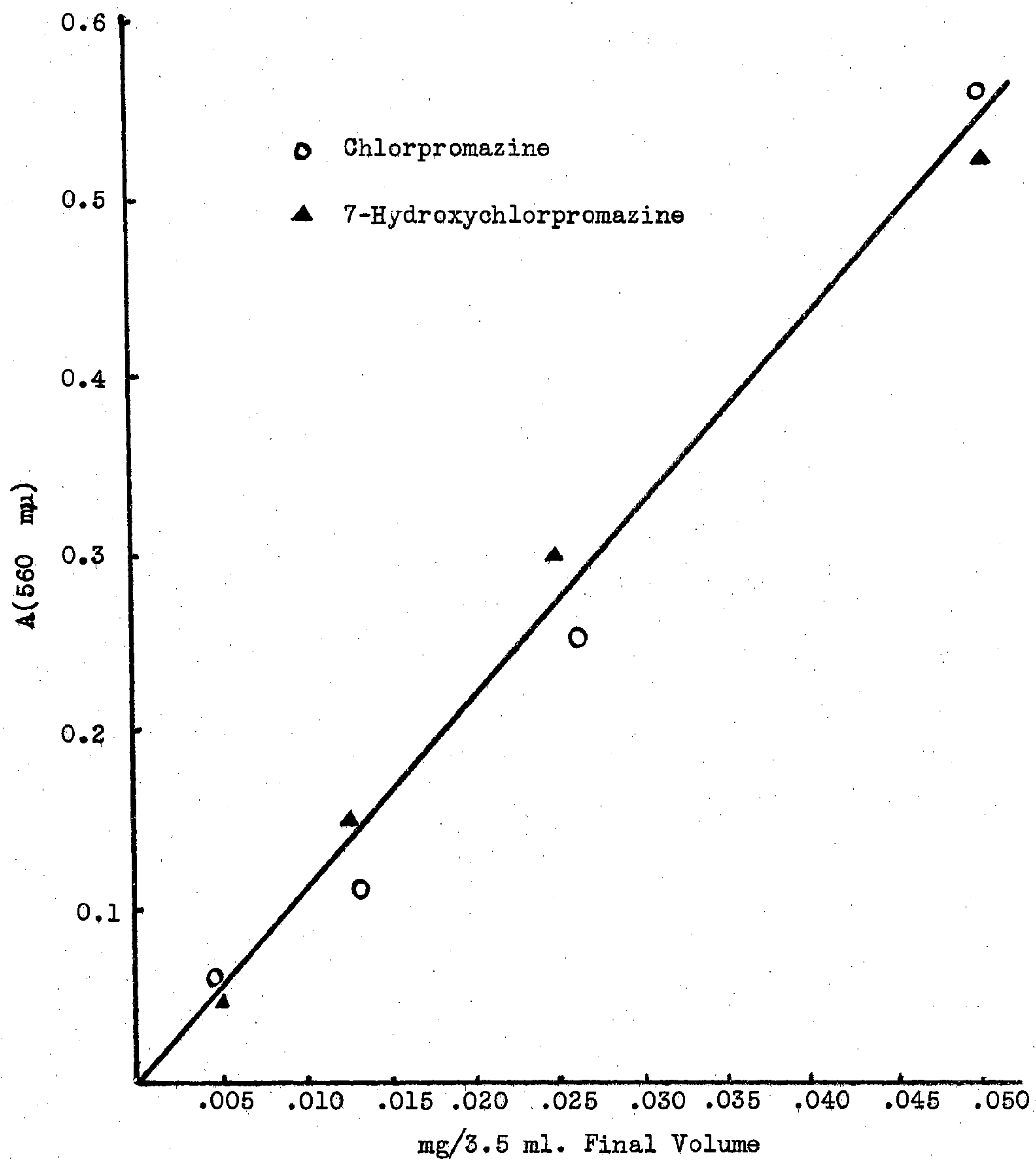

Figure 5. Standard Curve of Chlorpromazine and 7-Hydroxychlorpromazine

TABLE VIII

QUANTITATIVE DETERMINATION OF UNMETABOLIZED CHLORPROMAZINE AND 7-HYDROXYCHLORPROMAZINE FROM RAT URINE AND PIG LIVER SUBCELLULAR FRACTIONS

	Unmetabolized Chlorpromazine[a]	7-Hydroxy-Chlorpromazine[b]	
	%		%
Urine[c]			
first day	10.9	A[d]	0.38
		B[d]	0.19
second day	trace	A	0.14
		B	0.11
third day	undetectable	A	undetectable
		B	undetectable
Homogenate[e]	45.0		-
Supernatant[e] (10,000XG)	46.6		-
Microsomes[e]	67.0	A	1.5
		B	undetectable

[a]First chloroform extracts at pH 13 were examined.

[b]Second and third chloroform extracts at pH 9.5.

[c]9-10 mg of chlorpromazine was injected to rat, an average value was taken from two rats.

[d]A and B represent the chloroform extracts at pH 9.5 before and after incubated with β-glucuronidase/aryl sulfatase, respectively.

[e]1.78 mg of chlorpromazine was incubated for 1 hour.

this data in Table VIII with Table III and V. The unmetabolized chlorpromazine accounted for 45-67% of the metabolites from pig liver subcellular fractions. Since the metabolism of chlorpromazine seems to be incomplete for microsomes this suggests that added glucose-6-phosphate and glucose-6-phosphate dehydrogenase were not sufficient to replace the soluble fraction cofactors. Also, the one-hour incubation may not permit complete reaction, since with the in vivo study in the rat (Table VIII), unmetabolized chlorpromazine represented only 11% of the administered dose.

As shown in Table VIII, the results of the quantitative determination agree with the distribution of administered radioactivity in chloroform extracts at pH 13 in the balance studies (Tables III and V), especially for microsomes. These results suggests that there are some important metabolites other than unmetabolized chlorpromazine in the chloroform extracts at pH 13, especially with rat urine.

There was a higher concentration of 7-hydroxychlorpromazine in the free form than in the conjugated form in the first days urine. However, in the balance study conjugated metabolites had about the same radioactivity as that of free metabolites. This indicates that there were other important metabolites for conjugation. The thin-layer chromatographic results provided evidence for this point (Table VII). The percentages of 7-hydroxychlorpromazine found in the second day urine agrees with the results shown in Table III and VII (Table VIII, line 2).

When ^{35}S-chlorpromazine was given to the rat, Emmerson et al. (10) were able to account for 12% of the dose in urine as unmetabolized drug. In this study, it was shown that 11% of the unmetabolized chlorpromazine was excreted in the first days urine while only traces were

observed in the second day, and it was undetectable in the third day.

CHAPTER V

SUMMARY

The metabolic fate of administered ^{3}H-chlorpromazine has been studied in the rat and pig. Urinary metabolites from the rat were examined to establish the relative importance of the 7-hydroxychlorpromazine metabolic pathway. These samples were also analyzed for metabolites which would result from cleavage of the phenothiazine ring. *In vitro* studies included incubation of the tritium labeled drug with rat and pig liver homogenates and pig liver microsomes. Glucuronide complexes of chlorpromazine metabolites were isolated as a group after incubation of urine samples with β-glucuronidase. An assay system for the enzyme which hydroxylates the phenothiazine ring of chlorpromazine has been devised.

From 35-40% of administered radioactivity appeared in the urine within 5 days. On the basis of total excreted ^{3}H, 65% was excreted on the first day while after the fourth less than 2% was observed. The solids of urine contained the major portion of the radioactivity while the volatile ^{3}H metabolites contained less than 1% of the total activity. Extraction of urine samples at adjusted pH values gave the following results: First extraction with chloroform at pH 13 showed 56-69% of the total ^{3}H was present as unchanged chlorpromazine, chlorpromazine-N-oxide and the sulfoxide, chloroform extraction at pH 9.5 before and after incubation with β-glucuronidase (at pH 4.5) showed

the nonconjugated and conjugated phenolic metabolites were about equal in concentration for each days urine sample; the activity remaining in the urine residue after the three extractions described above was negligible. 7-Hydroxychlorpromazine was identified as the phenolic metabolite in rat urine. It was present in both conjugate and nonconjugate forms.

Pig liver microsomes showed most of the activity in the chloroform extracts from samples adjusted to pH 13. Thin layer chromatography of the pH 9.5 chloroform extracts of this same sample revealed the presence of hydroxychlorpromazine. These samples contained less than 2% of the drug as the conjugated derivative. The aqueous residue of the microsomal fraction after the extractions described above contained about 2% of the ^3H activity.

No evidence was obtained to support the existance of a phenothiazine ring cleavage metabolic system in the rat. The tests for aromatic amines and their conjugates, sulfonic acids and orthanilic acid were negative.

An assay system for the microsomal enzyme which catalyes the ring hydroxylation of chlorpromazine has been developed by adaptation of a method proposed by other workers for the N-oxidase enzyme.

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