CHLORPROMAZINE-N-OXIDATION, DEMETHYLATION AND PROPERTIES OF CHLORPROMAZINE-N-OXYGENASE

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PROPERTIES OF CHLORPROMAZINE-N-OXYGENASE

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ABBREVIATIONS

CP: Chlorpromazine

nor_1 CP: Monodemethylatedchlorpromazine

nor_2 CP: Didemethylatedchlorpromazine

CPSO: Chlorpromazine sulfoxide

nor_1 CPSO: Monodemethylatedchlorpromazine sulfoxide

nor_2 CPSO: Didemethylatedchlorpromazine sulfoxide

7-OH, CP: 7-Hydroxychlorpromazine

7-OH, CPSO: 7-Hydroxychlorpromazine sulfoxide

7-OH, nor_1 CP: Monodemethylated 7-Hydroxychlorpromazine

7-OH, nor_2 CP: Didemethylated 7-Hydroxychlorpromazine

7-OH, nor_1 CPSO: Monodemethylated 7-Hydroxychlorpromazine sulfoxide

7-OH, nor_2 CPSO: Didemethylated 7-Hydroxychlorpromazine sulfoxide

CPNO: Chlorpromazine-N-oxide

CPNOSO: Chlorpromazine N,S-dioxide

3-OH, CP: 3-Hydroxychlorpromazine

CHAPTER I

INTRODUCTION

The duration of drug action is generally limited by rate of conversion of therapeutic agents to inactive metabolites, since most of the drugs must be metabolized before they are excreted in urine, breath, feces or bile. Without biochemical systems for the metabolism of foreign compounds, much of our present drug therapy would be dangerous and impracticable, since the action of most therapeutic agents would persist for an extended period of time. The metabolism of drugs in the body does not always result in detoxification. Some drugs, like codeine and ephedrine, are converted to metabolites having pharmacologic properties similar to those of the parent drug. Others, like imipramine and prontosil, produce their therapeutic effects only through the formation of pharmacologically active metabolites. Some authors have used the term "toxification" to describe the process of the production of a more pharmacologically active compound by metabolism of the administered drug. The elucidation of the pathways of drug metabolism thus leads to a better understanding of drug action as well as to the mechanisms of drug detoxification.

Tranquilizers are the drugs referred to as ataractic agents. Ataraxia by definition means "not disturbed", perfect peace or calmness of mind (1). From the biochemical point of view, tranquilizers can be divided into two main groups. There are two distinct biochemical

mechanisms (2) whereby these compounds can cause a tranquilizing action: The first group of compounds (e.g. reserpine) are capable of liberating biologically active amines from brain cells, thereby exposing them to attack of metabolizing enzymes, thus decreasing their cerebral concentration; a second group of tranquilizers (e.g. chlorpromazine) are biochemically characterized by their ability to simultaneously inhibit both oxidative phosphorylation and adenosine triphosphatase activity in the brain.

Chlorpromazine is an extremely important drug used in the treatment of mental illness. This tranquilizer has been used to treat some 50 million people the world over, and has been shown to be metabolized by several mammalian species. The major metabolites in higher animals include the sulfoxide, glucuronide derivatives of various hydroxy chlorpromazines, the N-oxide and demethylated metabolites. Urine samples of different species (3-6) showed the following metabolites of CP: CP sulfoxide, nor_1 and nor_2-CP sulfoxides, nor_1 and nor_2-CP, glucuronides of 7-hydroxy, nor_1 and nor_2-7-hydroxy chlorpromazines and CP-N-oxide. Gillette and Kamm (7) observed the conversion of chlorpromazine to chlorpromazine sulfoxide (CPSO) by guinea pig liver microsomes. Chlorpromazine-N-oxide formation by subcellular liver fractions was reported from this laboratory (8,9) and by Beckett and Hewick (10).

This dissertation will report the results of studies on species differences in chlorpromazine-N-oxidation, demethylation and role of CPNO as an intermediate in oxidative demethylation. This thesis will also report results on the isolation, purification and determination of the kinetic properties of chlorpromazine-N-oxygenase from pig liver mocrosomes. Certain other characteristics such as the noninvolvement of

cytochrome P-450, substrate specificity and pH optimum will be reported.

CHAPTER II

LITERATURE REVIEW

Metabolism of Drugs

Drug metabolizing enzymes convert toxic and poorly excreted lipid-soluble foreign compounds to nontoxic, water soluble derivatives that can be eliminated more rapidly by the kidney. A large number of drugs and other compounds, foreign to the body, are metabolized in mammals along a number of metabolic pathways which include sulfoxidation, N-oxidation, dealkylation, deamination, hydroxylation, reduction, hydrolysis and conjugation forming glucuronides, sulfates, etc. Metabolism of drugs has been reviewed in detail by Brodie *et al.* (11) Gillette (12), and Shuster (13). Extensive review on phenothiazines was covered by Gordon (14). The literature review in this thesis will cover the different pathways of drug metabolism, comparative biochemistry of drugs, induction of drug metabolism and a short review of tranquilizers. Pharmacological and biochemical effects and metabolic fate of chlorpromazine *in vivo* and *in vitro* will also be reviewed.

Studies with tissue preparations have revealed that most of the enzyme systems which catalyze these metabolic alterations are present in liver microsomes. It is generally assumed that the intact microsomal system requires only oxygen and NADPH or a generating system for maximum activity. However, Nilson and Johnson (15) claim that in addition to NADPH, NADH may also be required for maximal N-demethylation, O-demethy-

lation, and hydroxylation. A combination of NADH and NADPH was more effective than NADPH alone in the N-demethylation of 3-methyl-4-methylamino benzene (16). According to Waddell et al. (17), the activity of microsomal N-demethylase can be increased considerably by incubation under oxygen instead of air. Of various rabbit tissues tried, namely liver, lung, kidney, heart, muscle and brain, only the microsomes in liver catalyzed the dealkylation of monomethyl-4-aminoantipyrene (18).

The microsomal fraction of liver consists of subcellular components that are generally believed to be derived from the endoplasmic reticulum, a network of tubules which extends into almost all regions of the cytoplasm. The reticulum comprises two major components: A rough-surfaced form consisting of lipoid tubules studded with small dense particles called ribosomes, and a smooth surfaced form consisting of lipoid tubules devoid of ribosomes. On homogenization, the network of tubules is broken and forms small vesicles which can be isolated as 'rough' and 'smooth' microsomes. Fout (19) separated the 'smooth' from the 'rough' microsomes and showed that the drug enzyme systems are associated mainly with smooth microsomes.

Oxidation

Drugs undergo oxidation by a number of metabolic pathways including sulfoxidation, N-oxidation, deamination, dealkylation and hydroxylation. Thio-ethers such as chlorpromazine and 4,4'-diaminodiphenyl sulfide are oxidized to the corresponding sulfoxide derivatives (7). Baker and Chaykin (20) found that trimethylamine is oxidized to trimethylamine oxide. In N-dealkylation, alkyl groups are removed from secondary or tertiary amines to form aldehydes and primary amines. Rat liver micro-

somes contain an enzyme which is relatively specific for tertiary amines. This enzyme demethylates imipramine to desmethylimipramine (21). This enzyme presumably demethylates other compounds including chlorpromazine (22) and amitriptyline (23). In O-dealkylation, alkyl groups of ethers are removed to form aldehydes and phenols. For example, p-ethoxy acetanilide is oxidized to p-hydroxyacetanilide and acetaldehyde (24). Axelrod (25) observed that a number of amines are oxidized to ketones and ammonia by an enzyme system which differs from monoamine oxidase not only in its requirement for NADPH, but also in its ability to deaminate a number of different substrates which are not metabolized by monoamine oxidase. This microsomal system is found in the rabbit liver, but not in hepatic tissues of the dog or rat. Mitoma et al. (26) showed hydroxylation of acetanilide occurred both in the ortho and para positions. Besides aromatic compounds, hydroxylation of alkanes has also been reported. Gillette (27) found that the rabbit liver microsomal system oxidized p-nitrotoluene to p-nitro benzyl alcohol. Toki et al. (28), reported that liver microsomes convert hexobarbital to 3-hydroxy hexobarbital, which is then oxidized to 3-keto hexobarbital by the soluble fraction.

Reduction

Liver microsomes also contain enzyme systems that catalyze the reduction of azo and nitro compounds. Nitro compounds such as chloramphenicol, p-nitrobenzoic acid and nitrobenzene are reduced to primary amines by an enzyme system which can use either NADH or NADPH as its hydrogen donor (29). Nitroso and hydroxylamine derivatives are presumably intermediates in this reaction, since the microsomal enzyme system

reduced both nitrosobenzene and phenylhydroxylamine to aniline more rapidly than it does nitrobenzene (12). Azo-reductase and nitro-reductase are both flavoproteins having FAD as their prosthetic group (30). However, azo-reductase requires NADPH as its hydrogen donor, while nitro-reductase uses either NADPH or NADH (31). In addition to microsmal oxidation, certain foreign compounds (e.g. chloral hydrate) are also metabolized by oxidases, peroxidases and NAD or NADP dependent dehydrogenases (12).

Hydrolysis

A number of enzymes catalyze the hydrolysis of substrates (esters or amides) by the addition of water. Hollunger and Niklasson (32) have succeeded in solubilizing and purifying several esterases from rabbit liver microsomes, and they reported that these esterases differed markedly in their substrate specificity. Bray et al. (33) reported the presence of amidases in rabbit liver homogenates which hydrolyze a number of amides. The rate of hydrolysis of amides is usually slower than the cleavage of esters. For example, procaine amide (34) and salicylamide (35,36) are very slowly deaminated in the body.

Conjugation

The formation of hippuric acid in the body by conjugation of glycine and benzoic acid has been known for 100 years or more. Conjugation reactions include the formation of glucuronides, etheral sulfates, mercapturic acids, amino acid conjugates, acetylated amines and methylated compounds (12). The sythesis of glucuronides generally represents an important pathway of metabolism for phenols, carboxylic acids, long chain

alcohols, primary amines, hydroxylamines and certain thio compounds. Glucuronide formation occurs mainly in liver (37), but it also takes places in the kidney, gastrointestinal tract (38,39) and skin (40). Another important pathway in drug metabolism is sulfate conjugation. In this reaction phenols and alcohols are converted to sulfate esters (41-43) and aromatic amines to sulfamates (44-46). The synthesis of the sulfate derivatives occurs in the soluble fraction of liver homogenates through the formation of adenosine-5'-phosphosulfate and 3'-phosphoadenosine-5'-phosphosulfate. Other pathways which play a minor role in drug metabolism are discussed by Gillette (12).

Comparative Metabolism of Foreign Compounds

Four species of fish and certain types of amphibia, including frogs and salamanders, failed to metabolize monomethyl-4-aminoantipyrine, aminopyrine, N-methylaniline, antipyrine, hexobarbital or p-ethoxyacetanilide (47). These compounds are presumably excreted unchanged through the gills or skin. Reptiles, including alligators and tortoises, metabolize the above drugs. The microsomes of these animals contain an enzyme system for demethylating monomethyl-4-aminoantipyrine that requires NADPH and oxygen. A dealkylating enzyme system with the same requirements is also present in the liver microsomes of birds (chicken, pigeon), the opossum and a large variety of other mammals (rabbit, rat, guinea pig, and mouse) (47,48). Quinn et al. (49) have shown that various strains of rats metabolize antipyrine at markedly different rates. Age and sex differences were also reported (12). New-born mice, rabbits and guinea pigs lack the liver microsomal enzyme systems for the metabolism of many foreign compounds, including aminopyrine, phenacetin

and hexobarbital (50,51). The enzyme systems appear within the first week after birth and increase until the maximum is reached at eight weeks. The action of many drugs persists longer in female rats than in male rats. For example, Holck et al. (52) showed that females, injected with certain oxybarbiturates, sleep considerably longer than males. In accord with this finding, the liver microsomes of male rats metabolized hexobarbital and aminopyrine considerably faster than did those of females (49). The in vitro metabolism of drugs by liver microsomes can be inhibited by prolonging agents (e.g. SKF 525-A) and by a number of other mechanisms discussed by Gillette (12).

Stimulation of Drug Metabolism

Treatment of animals with a wide variety of foreign compounds accelerates the biotransformation of drugs in vivo by increasing the activity of the enzyme systems that catalyze drug metabolism. Studies by Conney (53-55) revealed that barbital and phenobarbital administered orally to rats increases the activity of microsomal drug enzymes which hydroxylate phenylbutazone and 3,4 - benzpyrene, as well as those which metabolize aminopyrine and hexobarbital.

Brodie et al. (11) stated that a drug metabolite is almost invariably more polar (chloral hydrate is an exception since it is reduced to more lipid soluble trichloroethanol in vivo) and, therefore, less toxic than the parent compound because it's decreased lipid solubility prevents it from passing cellular barriers and reaching a potential site of action. However, despite its increased polarity, the derived product may have a pharmacologic action which the parent compound lacks if the conversion should unmask or produce a new functional group. Thus,

prontosil is inactive but is converted to sulfanilamide which is active due to the unmasking of the p-amino group (56). Though most foreign compounds undergo metabolic conversion, certain drugs are excreted virtually unchanged. These are usually strongly ionized compounds like priscoline and hexamethonium, and are chemically unreactive. They are rapidly excreted because their lipid insolubility precludes extensive reabsorption by renal tubules. Other biochemically unreactive drugs like ethyl ether or cyclopropane are lipid soluble but are excreted unchanged by the lungs (57).

Solubilization of Drug Metabolizing Enzymes

Conventional methods of solubilization inactivate many of the drug metabolizing enzymes in liver microsomes. A small number of enzymes that have been shown to retain their activity after solubilization have been summarized by Shuster (13). In several instances the addition of cofactors to the soluble enzymes is required for maximal activity. Imai and Sato (58) found that the activity of lyophilized soluble aniline hydroxylase was destroyed by extraction with methanol-ether. Activity could be restored by addition of an extract of microsomal lipids. Soluble acetanilidehydroxylase also seems to require some heat stable, dializable cofactor for maximal activity (59). Alkylarylamine oxygenase from pork liver microsomes was solubilized using sonication procedure followed by addition of Triton X-100 by Ziegler et al. (60). This enzyme fraction was further purified by ammonium sulfate fractionation. The enzyme preparation thus purified about 1000 fold, was free from dealkylating enzyme, monoamine oxidase and NADPH-cytochrome C reductase. With this preparation, they demonstrated the oxidation of wide variety

of drugs on the basis NADPH oxidation (61).

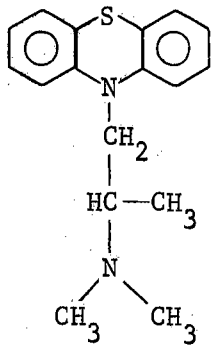
Tranquilizing Drugs

Two agents are responsible for introducing a revolution in psychotherapy. These are reserpine, an alkaloid of Rauwolfia serpentina, and chlorpromazine, a synthetic drug related to the antihistamines. The introduction of Rauwolfia alkaloids (isolated from the whole root) in western medicine began in 1953 and chlorpromazine was introduced in the following year. However, the use of roots and extracts from the Indian Rauwolfia serpentina Benth, a climbing shrub, as tranquilizers goes back for many centuries (62). This activity was first mentioned in western literature by Rumpf (63). Mueller and associates (64) reported the isolation of a new alkaloid reserpine, from Rauwolfia serpentina. Reserpine is essentially antihypertensive as well as being a tranquilizing drug.

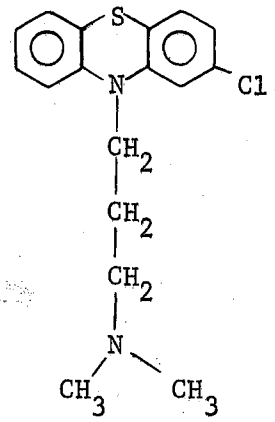
Phenothiazine Derivatives

The development of the alkylated phenothiazines as psychopharmacological agents occurred about the same time as the introduction of reserpine. The former development stemmed from the observation that certain antihistaminic compounds had sedative side effects. In attempts to enhance the sedative effects of these phenothiazines, notably promethazine, chlorpromazine was synthesized. An indication of the qualitative differences between promethazine and the psychopharmacological phenothiazines like chlorpromazine is seen in the fact that promethazine is characterized by pronounced antihistaminic activity and a relative lack of blockage of a conditioned escape response. In contrast chlorpromazine

is a weak antihistaminic but shows pronounced antiemetic and conditioned response blocking activity. It is thus remarkable that small differences in chemical structure produce marked qualitative changes in pharmacological effects (65-68). Pharmacology and metabolism of phenothiazines as a group and chlorpromazine in particular were discussed in detail by Gordon (14). More than 3000 different phenothiazines have been synthesized to date and a detailed review of these has been published by Schenker and Herbst (69).



Promethazine



Chlorpromazine

Pharmacological Effects of Chlorpromazine

The first pharmacological investigations of chlorpromazine were conducted by Courvoisier and colleagues (70). The most important effect of chlorpromazine is its singular sedative effect (71). Animals become quiet and sleepy, remain in unnatural positions (so-called catalepsy) and at higher doses fall asleep. In the brain, chlorpromazine is said to act through the hypothalamus and the limbic system. One of the most important effects is on the central nervous system. Chlorpromazine causes a reduction in motor activity. In mice 1-5 mg/kg of chlorproma-

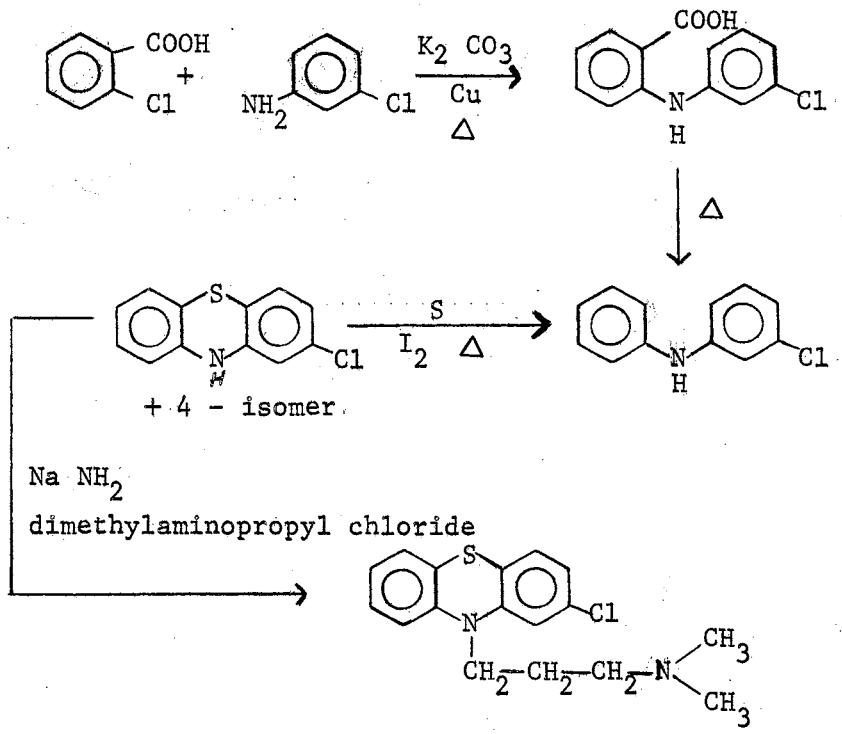
zine, administered orally or subcutaneously, produced a reduction in spontaneous motor activity proportional to the dose (72). Decerebrate and diencephalic cats were more sensitive to the depressant effects of chlorpromazine than normal animals (73). Chlorpromazine antagonizes the emetic effects of certain drugs, irradiation and motion sickness in animals. The antiemetic effect of chlorpromazine is due primarily to depression or inhibition of the medullary chemoreceptor trigger zone. However, there appears to be some inhibitory activity on the vomiting center at elevated dosages. A 50 mg dose of CP gave almost complete protection to a group of human subjects against the emetic effects of one mg of apomorphine administered subcutaneously (74). Effects of chlorpromazine on the activity of other drugs was discussed by Gordon (14). Chlorpromazine lowers body temperature in various laboratory animals (75). Chlorpromazine has been found by Elder and Dille (76) and by Schwartz et al. (77) to antagonize the behavioral effects of LSD, although CP inhibits the metabolism of LSD (78). Pharmacological effects of chlorpromazine on autonomic nervous, cardiovascular, endocrine, gastrointestinal, hepatic and neuromuscular systems were discussed by Gordon (14). Van Woert reported that long term chlorpromazine administration in man is associated with oculocutaneous pigmentation and the accumulation of brown pigment deposits in various organs. These pigment granules were isolated from the liver of a schizophrenic patient treated with chlorpromazine. The ultraviolet absorption maxima were 304 and 254 mμ for one pigment and 261 mμ for the other. Two of the chlorpromazine pigment polymers prepared in vitro by the aerobic irradiation of an aqueous solution of CP HCl by ultraviolet light for 18 hours showed similar ultraviolet and infrared absorption spectra (79).

The approximate 14-day LD_{50} of CP in rats by various routes are intraperitoneal, 75-100 mg/kg; subcutaneous, 540 mg/kg; oral, 492 mg/kg; and intravenous, 25 mg/kg (80). Hollister (81) has stated that "successful suicide with phenothiazine derivatives is virtually unknown despite doses of 10-20 gm; among central nervous system depressants, these drugs are as suicide-proof as any might be."

Biochemical Effects of Chlorpromazine

Chemistry

Chlorpromazine (2-chloro-10-(3-dimethylaminopropyl)phenothiazine) was synthesized by Charpentier of the Rhone-Poulenc Laboratories in France in 1950 (82). CP, like phenothiazine and most of its derivatives, is light sensitive. One method of synthesis for chlorpromazine may be represented as follows (83):



Chlorpromazine

Chlorpromazine hydrochloride in solution exhibits maximum chemical stability when buffered at a pH of 4 to 5 and protected by the presence of antioxidants. It is sensitive to all oxidizing agents including ferric salts, persulfuric acid, permanganates, dichromates, nitric acid and hydrogen peroxide. Characteristic colors are formed and may be used as a spot test for identification purposes. The free base begins to precipitate as the pH of a solution approaches 6. CP has an absorption maximum at 257.5 mμ in the ultraviolet region (83).

Effects of Chlorpromazine on Membrane Permeability

It has been suggested that chlorpromazine alters the permeability of various membranes to substances like norepinephrine and serotonin, and the antipsychotic activity of chlorpromazine has been attributed to its membrane effects (84). Evidence was presented demonstrating a stabilization of the rat liver lysosmal membrane by CP both *in vivo* and *in vitro* (85). Løvtrup observed that DNP and Mg^{++} activated ATPase, succinate dehydrogenase and aspartate transaminase were inhibited by CP. Succinate dehydrogenase was less sensitive when the mitochondria were disrupted by treatment with digitonin, whereas sensitivity of the ATPase activity was significantly increased. Succinate dehydrogenase and certain other enzymes were activated considerably by low concentrations of CP. This activation, inhibition phenomenon is possibly due to interference with the structure of the mitochondrial membrane. Further, he also observed a substantial change in the permeability coefficient of cell membranes in D_2O permeability experiments at low concentrations of CP (86).

Enzyme Effects

Enzyme studies on brain tissue of animals depressed with chlorpromazine revealed that creatine phosphokinase activity and the activities of enzymes catalyzing the dephosphorylation of adenine nucleotides were unaltered by the drug and it was concluded that chlorpromazine in some way depresses the utilization of brain ATP, so that a given stimulus results in less utilization of labile phosphate (87). Lazzalo and Meyer (88) reported that CP both inhibits and potentiates the activity of D-amino acid oxidase. Kurokowa et al. (89) have found that another flavin enzyme, succinic dehyrogenase, was also both inhibited and potentiated by CP. Further, Harris (90) reported that inhibition of CP (final concentration of 4×10^{-4}M) on D-amino acid oxidase in competition with its coenzyme, FAD, was a function of the apooxidase protein concentration. Inhibition was also decreased when albumin was added to the reaction mixture. Therefore, it was suggested that this nonspecific complexing between CP and protein might explain the large number of diverse effects attributed to CP. Chlorpromazine was found to inhibit the activity of a liver enzyme that N-methylates nicotinamide (91). The surface tensions of solutions of CP have been shown to decrease in the presence of ATP due to reversible formation of a complex. The reaction with ATP was specific and probably was a result of salt formation between the phosphate groups of ATP and the quaternary nitrogen of the dimethylamino propyl side chain of CP (92). CP decreases the activity of acetylcholine esterase (93) and acid phosphatase (94). Greenberg et al. (95) reported that CP had little effect on glutamic acid decarboxylase, but did inhibit pyridoxal kinase activity in both normal and B_6-deficient rat brain homogenates. Chlorpromazine, like 2,4 - dinitrophenol, has been shown to produce uncoupling of oxidative phosphrylation in various pre-

parations of brain and liver and also inhibits ATPase activity (96,97). CP inhibited partially purified cytochrome oxidase from rat liver mitochondria (98).

Structure-Activity Relationships

Brune et al. observed that progressive demethylation of CP is associated with an increasing loss of pharmacological potency. Conversely, toxicity was found to be highest for nor_2 CP followed by nor_1 CP and CP in decreasing order (99). Thus, it appears that the N,N-dimethyl configuration plays a major role in the pharmacological effectiveness of CP. Replacement of the dimethyl amino propyl chain of CP by the N-methyl-piperazinopropyl chain of prochlorperazine gave an increase in activity and a decrease in acute toxicity (100). Burke showed that substitution in 2-position of phenothiazine seems optimal for activity. The substituents had about the following order of activity (101).

$$CF_2 > Cl > H \approx OCH_3 \approx CONHNH_2$$

Moran and Butler have shown that the sulfoxide produces only slight pharmacological effects in comparison with chlorpromazine (102). Pharmacological and behavioral tests on nor_1 CP and CPNO indicated that these compounds approach CP in activity, whereas CPSO is markedly less active (5,6).

Absorption, Distribution and Metabolism of Chlorpromazine

Absorption and Distribution

Chlorpromazine is rather slowly absorbed from the gastrointestinal

tract and is retained at the site of subcutaneous injection for an extended period of time. Peak blood levels are reached in 20 minutes after intraperitoneal injection. From the blood, CP is readily transferred to the tissues where the concentrations, especially in brain, far transcend those in the blood. Dogs receiving 20 mg of CP/kg intravenously gave tissue/plasma ratios with approximately the following values at one hour after injection: brain, 66:1 to 68:1; lung, 45:1 to 55:1; kidney and spleen 15:1 to 33:1; liver, skeletal muscle, and heart, 7.5:1 to 14:1 and perirenal fat 2.5:1 to 5.0:1. The drug disappears relatively slowly from the tissues. Henrickson et al. reported that small quantities of residual CP hydrochloride were found in the fat, brain, heart, lung and kidney of those animals receiving 0.4 mg/lb body weight when slaughtered 8 hours after injection. Animals held for 72 hours had no detectable residual compound in any of the tissues (103). Man excretes little or no unchanged chlorpromazine in the urine, dogs excrete not more than 1.5%, and rats about 15%, in 5 days (104). The cerebral level of CP is 13 μg/g after the administration of 5 mg/kg of the drug and 85 μg/g after a dose of 20 mg/kg. It's distribution within the brain is not uniform, the hypothalamic areas containing 4 to 5 times as much of the drug as other parts of the brain (2). Intramuscularly and intravenously administered ^{35}S-chlorpromazine in mice accumulated initially in the cerebral cortex, hippocampus and thalamic nuclei of the brain as well as in the adrenal cortex, liver, lung and kidney. Radioactive metabolites later appeared in the lymph nodes, spleen and thymus (105). ^{35}S-activity from ^{35}S-chlorpromazine or its metabolic products was rapidly and widely distributed in the tissues of the mouse. Spleen and brain retained the highest levels of activity up to 5 days after the

administration of ^{35}S-chlorpromazine (106).

After injection of rats and guinea pigs with chlorpromazine (5-10 mg/100 gms body weight), Wechsler et al. (107) determined CP and its metabolites in various organs. The liver and lungs contained in addition to unchanged CP, nor_1 CP, CPSO and nor_1 CPSO. In the kidney nor_1 CP was missing and only CP was found in brain. In addition, in guinea pig liver exclusively, a relatively polar hydroxylated CP compound with the same Rf as 7-hydroxy CP and 8-hydroxy CP was consistently observed. Ninety minutes after i.p.injection, it was found that nor_1 CP accumulates in the brain to a greater extent than CP, nor_2 CP or CPSO. The enhanced accumulation of nor_1 CP in brain cannot be completely accounted for in terms of a blood-brain barrier phenomenon (108).

Metabolic Fate of Chlorpromazine

Salzman and Brodie showed chlorpromazine sulfoxide to be a metabolite of chlorpromazine in the dog and man (109). Hydrolysis of dog and human urine by acid, alkali or β-glucuronidase treatment increases the amount of phenothiazine-like extractable compounds (110). These conjugated compounds represent a large fraction of the chlorpromazine metabolites excreted in the urine. Further, compounds were isolated from the urine of patients on chlorpromazine therapy and were characterized as glucuronic acid conjugated chlorpromazine metabolites (111). Fishman and Goldenberg (112) have studied an organic extractable fraction from the urine of patients administered chlorpromazine. Following organic extraction of alkalinized human urine, chromatographic studies revealed the presence of ten compounds which produce a Dragendorff color

reaction. Six of these appear to be sulfoxides, including CPSO which was a minor product. Two major metabolites had chromatographic behavior indistinguishable from nor_1 CPSO and nor_2 CPSO. A third major metabolite remains unidentified. It was found that the residual urine after extraction contained numerous additional polar metabolies.

The metabolic fate of ^{35}S-chlorpromazine was studied in the rat (4). Four excretion products were determined qualitatively by chromatography and quantitatively as a percent of the dose: CP(12.3%); CPSO (5%); nor_1 CPSO(5.2%); and nor_2 CPSO(2.3%). The $BaSO_4$ precipitates from the urines contained only insignificant amounts of radioactivity. Goldenberg and Fishman (3,113) identified the following metabolites of chlorpromazine by comparison with reference compounds: 7-hydroxy CP, 7-hydroxy CPSO, nor_1 7-hydroxy CP and nor_2 7-hydroxy CP. These compounds were excreted both in the free form and as glucuronic acid conjugates. Huang *et al.* (114) have speculated, on the basis of potential oxidizability, that hydroxylation *in vivo* takes place at the 3 - or 7 - positions. They have found in man that glucuronides are major metabolites. Dogs excrete two series of phenolic derivatives of chlorpromazine. One is hydroxylated at position 7(major site), the other at position 3. The human phenolic metabolites are principally of the 7-hydroxy series (115). Fishman *et al.* (6) and Posner *et al.* (5) reported the isolation and identification of chlorpromazine-N-oxide from human urine. This compound, although not a major human metabolite, is excreted in somewhat larger amounts than CPSO. Dogs surpass humans in their output of the N-oxide. The metabolism of chlorpromazine was compared in dogs and man by Goldenberg and Fishman (116). Humans tend to favor excretion of polar

derivatives, together with one or two major nonpolar metabolites, nor_1 and nor_2 CPSO. The human output of chlorpormazine and its sulfoxide was trivial. Dogs excreted less polar material, less nor_2 CPSO, more nor_1 CPSO, and substantial amounts of both chlorpromazine and its sulfoxide. Human polar fractions contained persulfate-blue as well as persulfate-lavender staining metabolites; the blue series was absent from dog urine. Three new metabolites of chlorpromazine have been isolated from the urine of mental patients by Johnson et al. (117). These were identified as 2-chlorophenothiazine, 2-chlorophenothiazine sulfoxide and 2-chlorophenothiazine-10-propionic acid. The first two metabolites, of interest because the complete loss of the side chain, were isolated by extracting alkaline urine with dichloromethane, and the acidic compound was extracted from urine at pH 3 with chloroform. Extraction of urine at acidic pH's removed at least three other acidic phenothiazines in addition to the propionic acid metabolite. The identity of these are as yet unconfirmed. Fishman and Goldenberg (115) also reported that chlorpromazine undergoes side-chain cleavage as a minor route of metabolism. In addition to 2-chlorophenothiazine sulfoxide and 2-chlorophenothiazine, they observed 2-chloro-7-hydroxy-phenothiazine, 2-chloro-phenothiazone and 2-chlorothionol. Thus, by different combinations of hydroxylation, demethylation, sulfoxidation, N-oxidation and side chain cleavage, chlorpromazine is metabolized into more than 20 metabolites. Extensive work on the urinary analysis of CP metabolites has been done on human and dog (3-6). In summary the concentration of major urinary metabolites might be ranked in the following manner in the species shown.

Man:

Polar metabolites: nor_1 and nor_2 7-OH CP> 7-OH CPSO > 7-OH CP

Non polar metabolites: nor_2 CPSO > nor_1 CPSO > CPNO > CPSO > CP

Dog:

Polar metabolites: 7-OH CP series > 3-OH CP series

Non polar metabolites: nor_1 CPSO = CPSO > CPNO = CP > nor_2 CPSO

Rat:

Non polar metabolites: CP> nor_1 CPSO > CPSO > nor_2 CPSO

The metabolic fate of chlorpromazine in mammalian species is shown in Figure 1.

Analytical Methods for Identification and Determination of Chlorpromazine Metabolites

The detection and estimation of chlorpromazine metabolites continues to receive much attention as new metabolites are being discovered. No satisfactory comprehensive systematic procedures have yet been developed. Paper, thin layer and now gas chromatography have become increasingly popular. Dubost and Pascal (118,119) have found that sulfuric acid develops a red color with phenothiazines and have reported that this color is stabilized by the addition of ethanol or formaldehyde. Various other oxidative reagents were also used to detect phenothiazines (14). Detection of chlorpromazine in body fluids (120) and a spectrophotometric method for chlorpromazine determination in biological material has been reported (109,121). Van Loon developed a spectrophotometric method for the determination of chlorpromazine and chlorpromazine sulfoxide in biological fluids based on the ultraviolet spectra of these compounds (122). A new extraction method using dichloromethane

Figure 1. Metabolic Fate of Chlorpromazine in Mammalian Species.

CP = Chlorpromazine;

CPSO = Chlorpromazine sulfoxide;

CPNO = Chlorpromazine-N-Oxide;

nor_1 = Monodemethylated;

nor_2 = Didemethylated;

-OH = Hydroxy

A = 7-OH, CP;

B = 7-OH, CPSO;

C = 7-OH, nor_1 CP;

D = 7-OH, nor_1 CPSO;

E = 7-OH, nor_2 CP

F = 7-OH, nor_2 CPSO

G = 2-Chlorophenothiazine sulfoxide;

H = 2-Chloro-7-OH, phenothiazine;

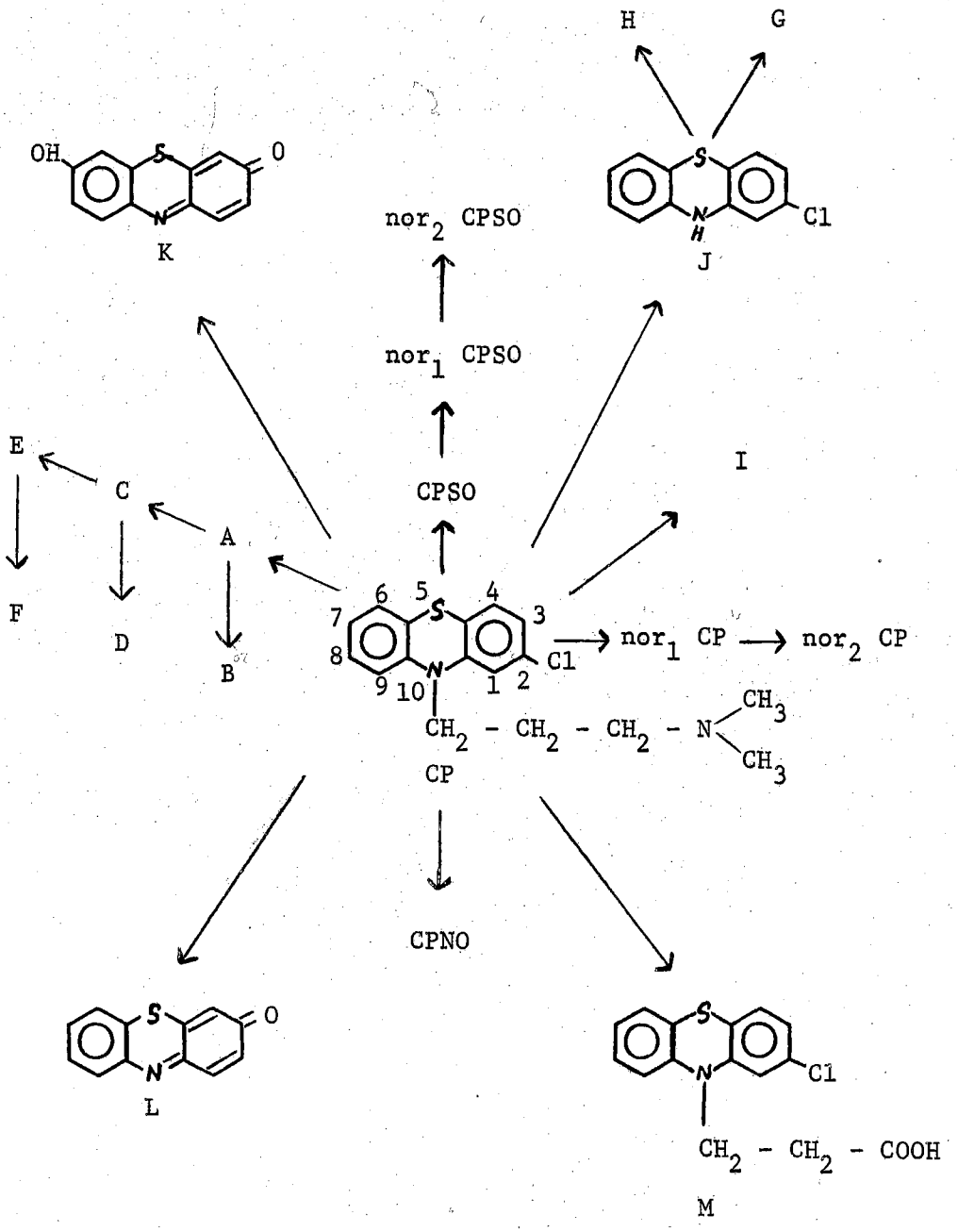
I = 3-OH, CP;

J = 2-Chlorophenothiazine;

K = Thionol;

L = Phenothiazone;

M = 2-Chlorophenothiazine-10-propionic acid.


H
G
OH
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nor2 CPSO
nor1 CPSO
CPSO
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Cl
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6
5
4
3
7
8
9
10
1
2
nor1 CP
nor2 CP
CH2 - CH2 - CH2 - N
CH3
CH3
CP
CPNO
L
M
CH2 - CH2 - COOH

and tetrahydrofuran was recently used for isolation of chlorpromazine metabolites from urine by Johnson et al. (123). A method for the determination of urinary chlorpromazine and its metabolites employing absorption on Dowex 50 W-X4 cation exchange resin was described by Forrest et al. (124). Chlorpromazine and chlorpromazine sulfoxide were separated by paper chromatography. The chromatograms were dried and sprayed with 50% H_2SO_4. Approximately one inch squares including the spots of CP and CPSO were cut out, extracted with 3.5 ml of 50% H_2SO_4 and filtered. Thus, the compounds were determined quantitatively, by measuring the acid extracts at 525 mμ in "Spectronic 20" (125). Eisdorfer and Ellenbogen (126) described a paper chromatographic procedure for separation of CP, nor_1 and nor_2 CP; CPSO, nor_1 and nor_2 CPSO, CP sulfone; nor_1 and nor_2 CP sulfone. Posner et al. separated nonphenolic and phenolic metabolites of chlorpromazine from human urine by paper chromatography. The chromatograms were sprayed with sulfuric acid-H_2O-ethanol (1:1:8) reagent and then strips were scanned with the Spinco model RB Analytrol using the 550 mμ filter, B-2 cam and a 1.5 mm slit width (5). Gas chromatographic methods for quantitative analysis of chlorpromazine metabolities in human urine, were developed by Johnson et al. (127) and Driscoll et al. (128,129). Curry and Brodie (130) reported a method for estimation of nanogram quantities of CP in plasma using gas-liquid chromatography with an electron-capture detector. Using a combination of gas chromatography and mass spectrometry by which 1×10^{-11} gram or less can be separated and analyzed, Holmstedt discovered the first chlorpromazine metabolite in human blood (131). Recently Ziegler et al. (61) determined CP-N-oxidation, on the basis of NADPH oxidation, by measuring

a decrease at 340 mμ .

In Vitro Studies on Chlorpromazine Metabolism

It has been demonstrated that the demethylation of chlorpromazine takes place in vivo in the rat, using labelled chlorpromazine (N-methyl-^{14}C), by the oral or intravenous route. In this experiment the methyl group of chlorpromazine was oxidized to carbon dioxide - ^{14}C (132). Later Young et al. (133) also demonstrated that this N-demethylation takes place in vitro in rat and rabbit liver homogenates. Ross et al. (134) have studied the metabolism of ^{14}C - or ^{35}S-labelled chlorpromazine in rat and rabbit liver homogenates. Metabolic products identified were $^{14}CO_2$, ^{14}C-labelled formaldehyde, and the N-demethyl derivative of chlorpromazine. Gillette and Kamm (7) demonstrated conversion of chlorpromazine to chlorpromazine sulfoxide by guinea pig liver microsomes in the presence of reduced NADP and oxygen. Shuster and Hannam (135) showed two to three fold increase in the activity of chlorpromazine-N-demethylase in the livers of mice treated with chlorpromazine. Demethylation, sulfoxidation and hydroxylation of chlorpromazine was shown by Robinson (136) using 9000 X g supernatant fraction containing microsomal and soluble fraction from livers of male rats. Chlorpromazine was converted to CPSO (trace), nor_1-CP, nor_2-CP, hydroxy CP, and hydroxy-nor_1-CP. CPSO was transformed to CP (trace). nor_1-CP was transformed to nor_1-CPSO and hydroxy-nor_1-CP. nor_1-CPSO, nor_2-CPSO were not metabolized. Beckett and Hewick (10) and Harinath and Odell (8) showed chlorpromazine-N-oxide formation from chlorpromazine by liver microsomes of rat, mouse and dog. Further chlorpromazine-N-oxygenase from pork liver

microsomes was solubilized, purified and then pH and temperature characteristics were studied (9). Ziegler et al. (61) demonstrated oxidation of chlorpromazine and a wide variety of other drugs, using 1000 fold purified enzyme from pig liver. They reported a Km value of 2.6 x 10^{-5} for chlorpromazine, on the basis of NADPH oxidation. The metabolic pathways by which chlorpromazine and its metabolites are transformed in vitro by rat and human liver microsomal enzyme systems were studied in detail by Coccia and Westerfeld (137). By thin layer chromatography and radio chemical quantification, the following pathways have been demonstrated: demethylation of tertiary and secondary amine metabolites, oxidative deamination of primary amines, N-oxidation of tertiary amines, N-oxide reduction, sulfoxidation and aromatic ring hydroxylation of non-sulfoxidized metabolites at position 7 and to a lesser extent at position 3. Daly and Manian (138) studied the metabolism of a series of chlorpromazine derivatives 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-dihydroxy-chlorpromazines, which were then mono-o-methylated. Thus, 7-hydroxy chlorpromazine, a major metabolite of chlorpromazine, was apparently converted with this system to a mono-o-methylated 7,8-dihydroxy chlorpromazine, which represents a new metabolic pathway.

CHAPTER III

CHLORPROMAZINE-N-OXIDATION AND DEMETHYLATION

Experimental Procedure

Chemicals

Chlorpromazine (CP); nor_1 and nor_2 CP; CPSO; nor_1 and nor_2 CPSO; CPNO and CPSONO were obtained from Smith Kline and French Laboratories and from Psychopharmacology Service Center, National Institute of Mental Health, Bethesda, Maryland. Glucose-6-phosphate, NADP, NADPH, nicotinamide and G-6-P dehydrogenase were obtained from Sigma Chemical Co., and xanthine oxidase from Mann Research Laboratories.

Thin layer chromatogram sheets (Type K301R2, Silicagel without fluorescent indicator) were obtained from Eastman Kodak Company. Diethylamine was obtained from Matheson Coleman and Bell and ethyl acetate, methanol and other solvents from Fisher Scientific Company. Ethanol was obtained from U.S. Industrial Chemicals Co., all other chemicals used were of reagent grade.

Methods

Separations of Nonpolar Chlorpromazine Metabolites by Thin Layer Chromatography

Solutions of reference compounds of non-polar (non phenolic) chlorpromazine metabolites were prepared by dissolving 1.0 mg in 1.0 ml of

95% alcohol. A mixture consisting of all the compounds (shown in the Table I) was also prepared. Aliquots of 25 µl containing 25 µg of each compound were spotted on Eastman Kodak TLC sheet (Type K301R2·Silicagel without fluorescent indicator) with Lang-Levy pipettes. The chromatograms were developed in the three different solvent systems. 1. Acetone, isopropyl alcohol, 1% ammonia (9:7:4); 2. Butanol, Acetic acid, Water, (88:5:7); and 3. Ethylacetate, Methanol, Diethylamine (14:4:5). After development (about 90 min.), the chromatogram was sprayed with a color development reagent consisting of H_2SO_4: water: 95% ethanol (1:1:8) (5). Nonpolar metabolites of chlorpromazine produce pink color on the thin layer chromatogram when sprayed with the sulfuric acid reagent. Rf values for the reference compounds in the different solvent systems were measured and recorded in Table I.

Enzymatic Assay for Chlorpromazine-N-Oxygenase

Assay System for CP-N-Oxygenase

The incubation mixture (135) consisted of microsomes or other fraction equivalent to 500 mg fresh liver; chlorpromazine, 5 µmoles; neutralized semicarbazide, 25 µmoles; nicotinamide, 50 µmoles; magnesium chloride, 20 µmoles; dipotassium glucose-6-phosphate, 25 µmoles; NADP, 0.8 µmole; glucose-6-phosphate dehydrogenase 2 units; 0.1 M phosphate buffer pH 7.4, diluted to a total volume of 6.8 ml. This preparation was incubated in a shaking water bath at 37° for 30 minutes under an oxygen atmosphere. After incubation, the reaction was stopped by the addition of 1.1 ml of a 20% solution of $ZnSO_4 \cdot 7H_2O$ and 2.1 ml of saturated $Ba(OH)_2$. The resulting precipitate was removed by centrifugation

and the supernatant was used for identification and quantitative determination of CPNO. Since glucose-6-phosphate dehydrogenase was present in the soluble fraction, it was not added to the homogenate or 10,000 x g fractions as part of the incubation mixture. The control consisted of the liver fraction, chlorpromazine, semicarbazide, and phosphate buffer diluted to 6.8 ml.

Separation of CPNO

A five ml aliquot of the supernatant was made alkaline by addition of 1.0 ml of 10% sodium hydroxide and then extracted two times with 15 ml portions of chloroform. The chloroform layers were removed with a separatory funnel, pooled and evaporated to dryness in a rotary evaporator and the residue was dissolved in 1.0 ml of 95% alcohol. One hundred µl aliquots of the concentrates were spotted on an Eastman Kodak thin layer chromatography sheet (Type K301R2, Silica gel, without fluorescent indicator) with Lang-Levy pipettes. The chromatograms were developed in a solvent system consisting of ethyl acetate: methanol: diethylamine (14:4:5), by an ascending technique (113). Authentic CPNO was spotted on the same sheet for reference. After development, the chromatogram was sprayed with a color development reagent consisting of H_2SO_4: water: 95% ethanol (1:1:8).

Colorimetric Assay for Determination of CPNO and Formaldehyde

Determination of CPNO

CPNO produces a pink color on the thin layer chromatogram when sprayed with the sulfuric acid reagent. The chromatogram section con-

taining the spot (Rf value was 0.26) was cut into small pieces and extracted with 3.5 ml of 50% H_2SO_4 (139) and then allowed to stand for one hour with occasional shaking. The polythene support was removed and then the sample was centrifuged to remove the silia gel. The absorbancy of the colored acid extracts were determined at 525 mμ in a Beckman D.U. Spectrophotometer. The control was used as the blank. Figure 2 shows the standard curve for determination of chlorpromazine-N-oxide by the colorimetric method. In general, recovery of standard through the complete process showed a variation of 0.017 $\pm$ 0.002 O.D. units for one microgram of CPNO per ml of solution read.

Determination of Formaldehyde

Formaldehyde was determined according to method of Nash (140). One ml of reagent (containing 15.0 gm ammonium acetate, 0.2 ml acetylacetone in 50 ml of water) was added to 4.0 ml of deproteinized supernatant (described under CP-N-oxygenase assay) and held in a water bath at 60°C for 30 minutes. The yellow color produced, due to the formation of diacetyldihydrolutidine, was read using filter #42 in a Klett-Summerson colorimeter.

Preparation of Subcellular Liver Fractions

A modified method of Schneider and Hogeboom (141) was followed in the preparation of Subcellular fractions. Liver tissue from the mammalian species (rat, mouse, dog, bovine and pig) was washed in 0.25M sucrose at 4°C. Then the tissue was gently blotted between filter papers and weighed. Fifty gms of the tissue in 25.0 ml of 0.25 M sucrose was minced with a pair of scissors and then homogenized in Potter-Elvehjem

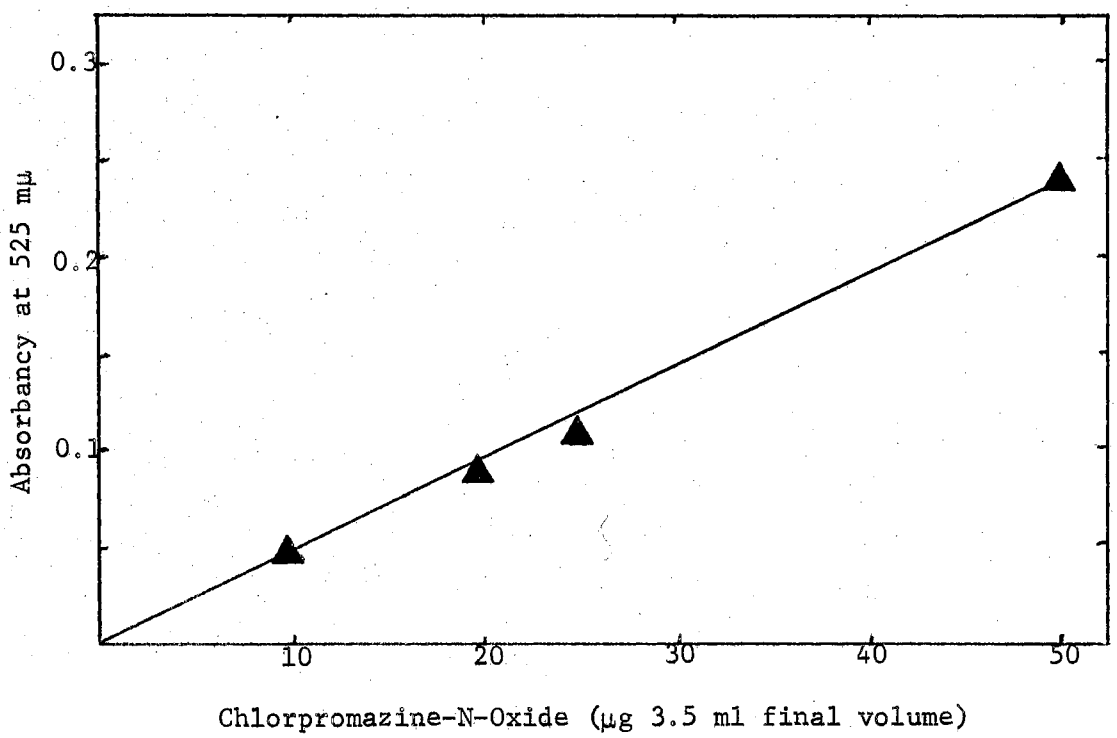


Figure 2. Standard Curve for Determination of CPNO.

Known amounts of reference CPNO were spotted on TLC, developed and eluted as described in the experimental procedure. The acid extracts were read at 525 mμ in a Beckman DU spectrophotometer using one ml quartz cells.

homogenizer for 2 min. The resulting suspension was centrifuged at 700 x g for 10 minutes. The precipitate was washed with 10.0 ml of 0.25 M sucrose and recentrifuged at 700 x g. The precipitate obtained (nuclear fraction) was suspended in 0.25 M sucrose and freed from large pieces of connective tissue by filtering the suspension through four layers of cheesecloth. The combined 700 x g supernatant solutions were centrifuged for 10 min. at 5,000 x g. The precipitate, after washing in 0.25 M sucrose was the mitochondrial fraction. The 5,000 x g supernatant solution was further centrifuged for 60 min. at 105,000 x g. The microsomal pellet was resuspended in 0.25 M sucrose and both this microsomal suspension and the soluble fraction were recentrifuged for 90 min. at 105,000 x g.

Spectrophotometric and Electrophoretic Procedures

The Cary 14 recording spectrophotometer was used to determine the absorption spectra of reference compounds and isolated metabolites. Twenty five µg of reference compounds were dissolved in 3.0 ml of 95% ethanol. These solutions were used against control solutions containing 95% ethanol in quartz cells of 1 cm light path, for measuring absorption spectrum in the ultraviolet region. Similar amounts were spotted on thin layer chromatograms developed and eluted as described earlier. The absorption spectrums of the sulfuric acid extracts were measured against control solutions containing 50% sulfuric acid.

High voltage paper electrophoresis was conducted on a Precision Measurements Co., Instrument to confirm CP-N-oxide formation. A 100 µl sample of reference CPNO and isolated metabolite solutions were spotted for comparison on Whatman #3 mm paper (45 cm x 56 cm). The paper was

dipped on both sides in 0.05 M borate solution in such a manner that the buffer wetted all the paper except the part of the paper near origin. Wetting of the paper near the origin was done carefully by allowing the wetting to occur by capillary action. The excess of the buffer was removed by carefully blotting between two sheets of filter paper avoiding the removal of sample from the paper. Then the paper was placed in the electrophoresis tank and a voltage of 2500 volts was applied for two hours. The paper was removed, air dried and sprayed with sulfuric acid reagent. CP-N-oxide moved towards the cathode. The distances were measured for reference and isolated compounds.

Results

Separation of Nonpolar Chlorpromazine Metabolites

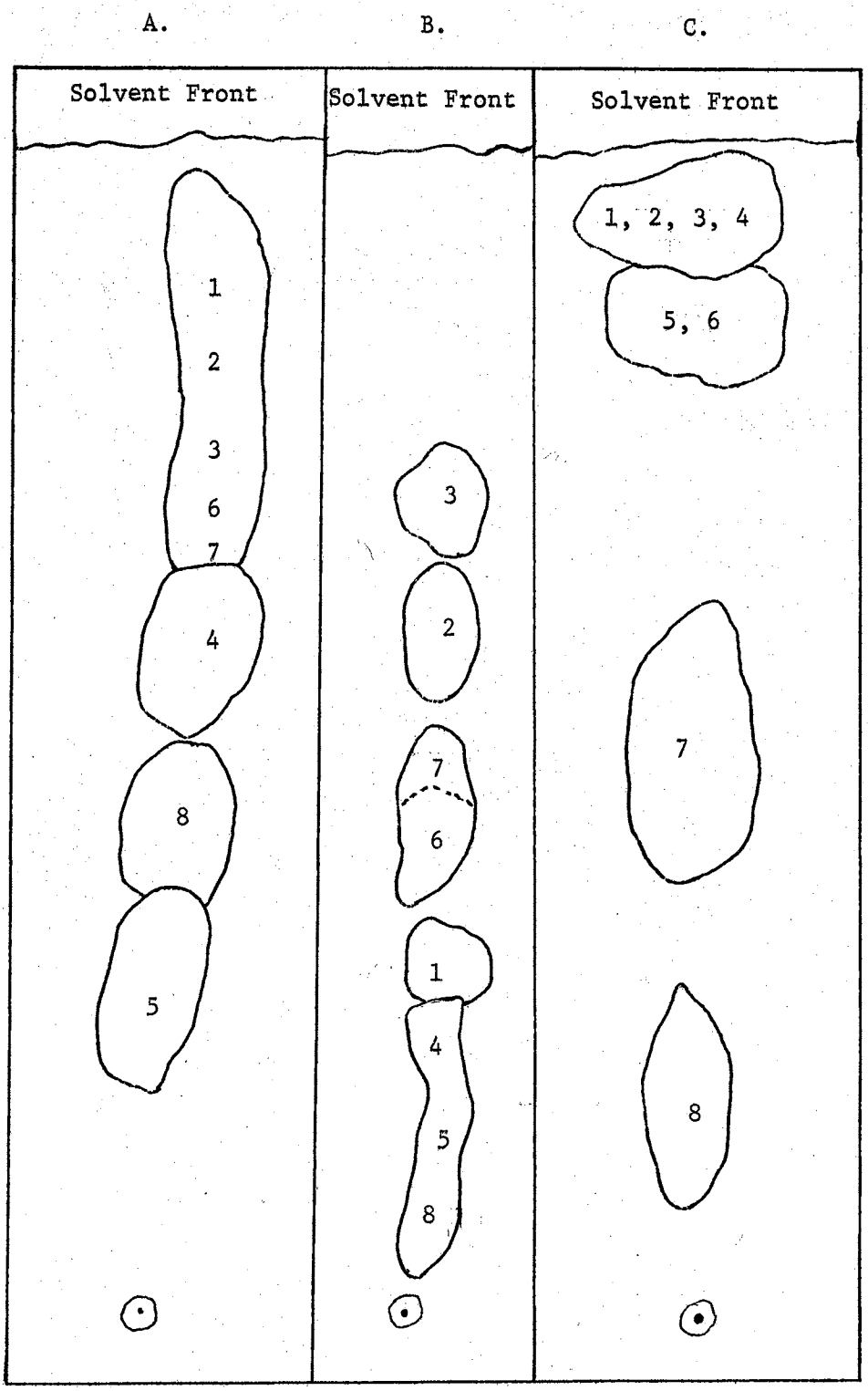
Several solvent systems were described by other workers (3,5,125, 126) for separation of chlorpromazine metabolites by paper chromatography. Thin layer chromatography was used for separation of hydroxy chlorpromazines (3). Most of the solvent systems developed were used for separation of groups of chlorpromazine metabolites such as sulfoxides, hydroxy chlorpromazines and demethylated chlorpromazines. There was no comprehensive method for separation of major nonpolar chlorpromazine metabolites by thin layer chromatography. Since absorption maxima for related metabolites (Table II) appear similar, it is necessary to separate and quantitatively determine the desired metabolite in order to develop an enzymic assay. About fifteen solvent systmes described by other workers were tried (3,5,125,126). Three were found to be suitable for separation of major nonpolar CP metabolites by thin layer chromatog-

raphy (Figure 3). A mixture containing eight reference compounds shown in Table I, presents comparative results on the basis of Rf values of reference compounds in different solvent systems. Each can be separated from this mixture. CPSO, CPSONO and nor_1 CPSO were separated in solvent A; CP, nor_1 and nor_2 CP in solvent B; CPNO, CPSONO and a mixture of nor_1 CPSO and nor_2 CPSO in solvent C. This mixture of nor_1 and nor_2 CPSO was eluted into methanol from an unsprayed chromatogram and respotted and developed in solvent A for separation of nor_1 and nor_2 CPSO. Thus, all the eight nonpolar metabolites can be separated and determined quantitatively from enzyme incubated mixture, as reported in experimental for chlorpromazine-N-oxide. Solvent C was routinely used to separate CPNO from the assay system.

Absorption maximum (λmax) for certain related metabolites in the ultraviolet region were shown in Table II. Twenty-five μg of chlorpromazine and other compounds in 3.0 ml of 95% ethanol were used for recording ultraviolet absorption spectra. A similar amount was spotted on TLC and developed in solvent C (shown in Table I). After spraying with sulfuric acid reagent, the pink area of each of the compounds on the TLC sheet were eluted with aqueous (50%) sulfuric acid. These acid extracts were used for recording the ultraviolet absorption spectra. From Table II it may be seen that CP and CPNO have absorption maxima at 256 mμ , whereas for CPSONO it is at 239, 276 and 300 mμ . Sulfuric acid extracts of all the three compounds have λmax at 269 and 277 mμ. In addition to chlorpromazine-N-oxide, an additional metabolite with a closely related Rf value in solvent C (shown in Table I) was obtained from incubation mixtures. An orange color was developed when the TLC

Figure 3. Separation of Nonpolar Chlorpromazine Metabolites by Thin Layer Chromatography.

Developing solvent systems and spraying reagent were as described in Table I. 1 = CP, 2 = nor_1 CP, 3 = nor_2 CP, 4 = CPSO, 5 = nor_1 CPSO, 6 = nor_2 CPSO, 7 = CPNO, 8 = CPNOSO. Solvent C was routinely used for isolation of CPNO.


A.
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1, 2, 3, 4
5, 6
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8

TABLE I

SEPARATION OF NONPOLAR CHLORPROMAZINE METABOLITES

Compound	Rf Values		
	Solvent A[1]	Solvent B[2]	Solvent C[3]
Chlorpromazine (CP)	0.79	0.31	0.94
nor_1 CP	0.79	0.59	0.94
nor_2 CP	0.79	0.70	0.94
CPSO	0.56	0.17	0.94
nor_1 CPSO	0.27	0.17	0.84
nor_2 CPSO	0.79	0.44	0.84
CPNO	0.79	0.44	0.49
CPSONO	0.40	0.17	0.18

[1]Acetone, Isopropyl alcohol, 1% Ammonium hydroxide (9:7:4).

[2]Butanol, Acetic acid, Water (88:5:7)

[3]Ethyl acetate, Methanol, Diethyl amine (14:4:5).

Spray reagent: Concentrated H_2SO_4, H_2O, 95% Ethanol (1:1:8).

TABLE II

ABSORPTION MAXIMA OF CHLORPROMAZINE
AND RELATED METABOLITES

Compound	Solvent	Absorption Maxima
		mμ
CP	Ethanol	256
CP	Sulfuric Acid	269,277
CPNO	Ethanol	256
CPNO	Sulfuric Acid	269,277
CPNOSO	Ethanol	239,276,300
CPNOSO	Sulfuric Acid	269,277
Unknown Metabolite (Orange)	Sulfuric Acid	273.5

plate was sprayed with the sulfuric acid spray reagent. This acid extract had an absorption maximum at 273.5 mμ . The ultraviolet absorption spectra of this metabolite shows that it is different from CPNO and CPSONO.

Chromatographic and Electrophoretic Identification of Chlorpromazine-N-Oxide

In the separation and elution of CPNO from the incubation mixture, 2.0 ml of the concentrate was spotted on the chromatogram sheet in a horizontal line. After development in solvent C (shown in Table I), part of the chromatogram was sprayed. A corresponding section of the chromatogram from the unsprayed portion of the chromatogram was cut into small pieces and extracted with 25.0 ml methanol. The methanol layer was removed after centrifugation, evaporated to dryness in a rotary evaporator and taken up in 2.0 ml of 95% alcohol. Aliquots of 100 μl are spotted along with authentic CPNO and developed in different solvent systems (see Table III) to confirm the identification of CPNO. It may be seen that reference CPNO and a product from the incubation mixture have the same Rf value in 4 solvent systems with a thin layer chromatographic technique.

Figure 4 shows the ultraviolet absorption spectra of CP, reference CPNO and CPNO produced by action of mouse liver microsomes on CP. The reference CPNO and the microsomal metabolite have essentially the same spectra. High voltage electrophoresis of reference and microsomal CPNO shows a migration of 7 and 7.3 cm. respectively, under the conditions described in experimental. A CP reference migrated 0 cm under the same conditions. From these data it may be concluded CP was con-

TABLE III

CHROMATOGRAPHIC IDENTIFICATION OF CHLORPROMAZINE-N-OXIDE

Thin Layer Solvent System[1]	Reference CPNO	Microsomal Metabolite
1. Ethylacetate, methanol, diethylamine (14:4:5)	0.27	0.26
2. Acetone, diethylamine (9:1)	on base line	on base line
3. Acetone, isopropanol, 1% NH_4OH (9:7:4)	0.81	0.82
4. nButanol, acetic acid, water (50:12:50)[2]	0.72	0.74

[1]Eastman Kodak Thin Layer Chromatographic Sheet (Type K301R2, Silica gel on polythene backing).

[2]Organic phase used.

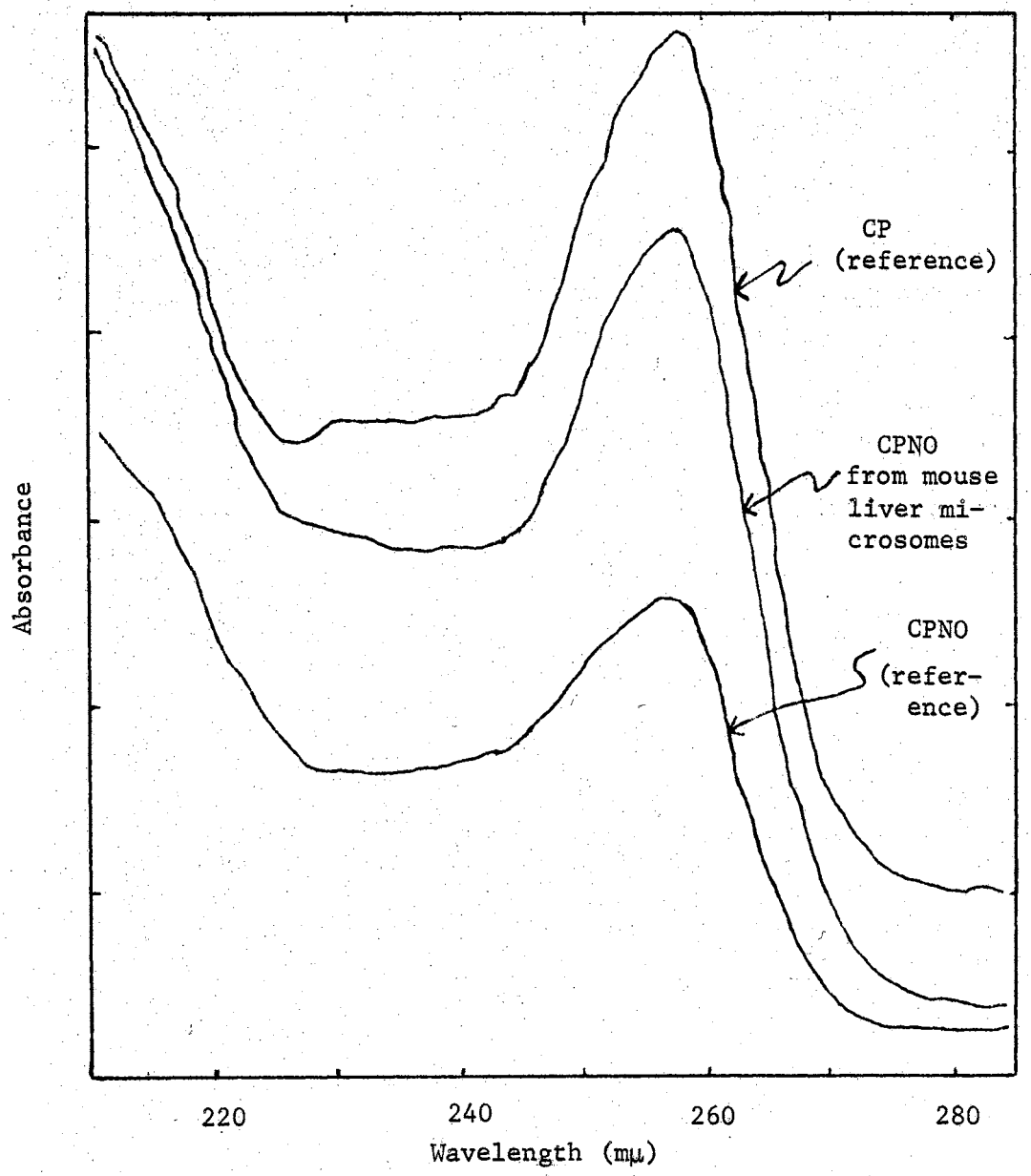


Figure 4. Absorption Spectra of Chlorpromazine and the Metabolite Chlorpromazine-N-oxide.

One cm light path, fused quartz cells were used with 25 μg of the reference compound in 3.0 ml of 95% ethanol. The ultraviolet absorption spectra were determined in a Cary 14 recording spectrophotometer.

verted to CPNO by a mouse liver microsomal preparation. This established the earlier identification of CPNO in the urine of dog (6).

Formation of Chlorpromazine-N-Oxide by Subcellular Liver Fractions

Table IV shows an example of the distribution of CP-N-oxygenase activity in cellular fractions from mouse liver. In general, higher activities were observed in the microsomal preparations. This agrees with the general concept that drug metabolizing enzymes are located in microsomes.

Species Differences in N-Oxidation and Demethylation of Chlorpromazine

Table V shows the relative activities of CP-N-oxygenase and demethylase in the liver tissues of mouse, rat, dog, pig and bovine. CP-N-oxygenase activity at the microsomal level in different mammalian species shows the following pattern: dog = mouse > pig > rat > bovine. Dog and man were shown to excrete CPNO as an urinary product. The liver microsomal demethylase activities of dog, mouse and rat were similar, whereas pig showed decreased demethylase activity.

Table VI presents data obtained with the mouse liver fractions for the formation of CPNO and formaldehyde. The demethylase reaction apparently proceeds at a slower rate than the CPNO reaction. This evidence alone would not exclude CPNO as an intermediate in demethylation as both compounds are found as metabolic products in the urine.

Table IV and VI show less N-oxide formation by homogenates than by microsomes. Possible explanations for these data are as follows: 1)

TABLE IV

FORMATION OF CHLORPROMAZINE-N-OXIDE BY SUB-CELLULAR LIVER FRACTIONS FROM THE MOUSE

Liver Fraction	CPNO Formed μmoles CPNO/gm liver/hr.		
	Mouse 1	Mouse 2	Mouse 3
Homogenate	4.76	0.10	0.20
Nuclei (700 x g)	3.50	0.27	0.36
Mitochondria (5000 x g)	3.78	0.00	0.45
Microsomes (5000-105,000 x g)	6.65	2.16	2.79
Soluble Supernatant	0.00	0.00	0.00

TABLE V

SPECIES DIFFERENCES IN N-OXIDATION AND DEMETHYLATION OF CHLORPROMAZINE BY LIVER MICROSOMES

Animals		CPNO Formed	Formaldehyde Formed
		μmoles/gm liver/hr.	μmoles/gm liver/hr.
Mouse	1	2.70	1.13
	2	3.24	1.19
	3	4.50	0.60
Rat	1	1.57	1.19
	2	1.33	0.60
	3	1.26	1.01
Dog	1	4.05	1.49
	2	4.73	0.98
Pig	1	2.31	0.35
	2	1.33	0.34
	3	3.94	0.19
Bovine		0.15	-

TABLE VI

CHLORPROMAZINE-N-OXIDE AND FORMALDEHYDE FORMATION FROM CHLORPROMAZINE BY MOUSE LIVER FRACTIONS

Fraction	CPNO Formed µmoles/gm liver/hr.		Formaldehyde Formed µmoles/gm liver/hr.	
	Mouse 1	Mouse 2[1]	Mouse 1	Mouse 2[1]
Homogenate	0.81	1.49	1.76	1.97
Supernatant (10,000 x g)	3.2	4.46	1.58	2.02
Microsomes	4.5	6.48	0.60	0.76

[1]C3H strain mouse used in this experiment.

the CPN-oxide formed by microsomes in the homogenate may have been demethylated (N-demethylase was found in the mitochondrial and light mitochondrial fractions), 2) CP-N-oxide may be reduced by xanthine oxidase (as a source of oxygen) (142) and 3) some inhibitor may be present in the homogenate.

Requirement for NADPH for Demethylation of Chlorpromazine and Chlorpromazine-N-Oxide

The NADPH requirement of the CP-N-oxygenase is established by the data shown in Table VII. CP was not converted to the N-oxide and was not demethylated to an appreciable extent in the absence of NADPH. Ziegler et al. (146) reported that NADPH was not required for oxidative demethylation of N,N-dimethylaniline-N-oxide by pig liver microsomes. However, from this experiment NADPH seems to be essential for N-oxide formation from CP and for oxidative demethylation of CP or CPNO. It has been observed that hydrogen peroxide may be utilized by non-specific enzymes in microsomes to catalyze various drug reactions (18). Hydrogen peroxide could not substitute for NADPH in the N-oxidation of CP. This is in agreement with the observation made by Cooper and Brodie (143) in the oxidation of hexobarbital.

It was also observed that the demethylation of CP was greater than that of CPNO in the presence of NADPH. This would indicate that CPNO is not an intermediate in oxidative demethylation of CP.

Ability of Xanthine Oxidase to Catalyze N-Oxidation of Chlorpromazine

Oxidation of nicotinamide to nicotinamide-N-oxide has been demon-

TABLE VII

REQUIREMENT FOR NADPH OF MOUSE LIVER MICROSOMES[1]
IN THE DEMETHYLATION OF CHLORPROMAZINE
AND CHLORPROMAZINE-N-OXIDE

Additions to Liver Microsomal Preparation	CPNO Produced	Formaldehyde Produced
	μ moles/gm liver/hr.	μmoles/gm liver/hr.
CP + NADPH Generating System (Complete)	2.16	1.85
CP + NADPH Generating System (Complete)	2.79	1.31
CP	0.00	0.12
CP	0.00	0.14
CP + Hydrogen Peroxide	Trace	0.23
CP + Hydrogen Peroxide	Trace	0.35
CPNO + NADPH Generating System	-----	0.79
CPNO + NADPH Generating System	-----	0.44
CPNO	-----	0.28
CPNO	-----	0.19

[1]All experiments were not conducted with the same microsomal preparation. Five moles of CPNO were used in the last 4 assays in place of CP.

strated in vitro with use of liver microsomes from rats, mice and rabbits (144). Murray and Chaykin (142) reported that xanthine oxidase catalyzed the transfer of ^{18}O from nicotinamide ^{18}O-N-oxide to xanthine in the course of the formation of uric acid. Further, they suggested a possibility that heterocyclic N-oxides may be capable of acting as general biological oxygenating agents. Studies were made on commercially available xanthine oxidase (from Mann) to see whether this enzyme can catalyze N-oxidation of chlorpromazine or utilize CP-N-oxide for oxidation of xanthine under anaerobic conditions. In the presence or absence of NADPH generating system, xanthine oxidase did not produce any significant amount of CP-N-oxide. Further xanthine was not oxidized to uric acid (no detectable increase in absorption at 295 mμ) in the presence of CP-N-oxide, with this enzyme under anaerobic conditions. However, when xanthine oxidase was incubated with chlorpromazine and Tris chloride buffer pH 8.0, a very small amount of a purple metabolite (when sprayed with sulfuric acid reagent) was infrequently observed indicating a hydroxylated metabolite of chlorpromazine.

CHAPTER IV

PARTIAL PURIFICATION AND PROPERTIES OF CHLORPROMAZINE-N-OXYGENASE FROM PIG LIVER

Experimental Procedure

Chemicals

Calcium phosphate gel was prepared according to the method of Keilin and Hartree (145). DEAE cellulose, CM cellulose, alumina C_γ, protamine sulfate and PCMB were obtained from Sigma Chemical Co. Agarose gel (10%) and thyroglobulin were obtained from Mann Research Laboratories. Bio-gel from Bio-Rad Laboratories and catalse from Worthington Biochemical Corporation. EDTA and sodium dithionite were obtained from J.T. Baker Chemical Co., and sodium azide from Matheson Coleman and Bell. Potassium cyanide and ammonium sulfate were obtained from Mallinckrodt Chemical works and carbon monoxide gas from Matheson Co., Inc. All other chemicals used were the same as described in Chapter III. Fresh pig liver tissue was obtained from Ralph's Packing Company, Perkins, Oklahoma.

Methods

Enzymatic Assay for Chlorpromazine-N-Oxygenase

The procedure was the same as described in Chapter III with a slight

modification in the assay system and conditions. This consisted of enzyme protein 1-2 mg; chlorpromazine, 50 μmoles; glucose-6-phosphate 25 μmoles; NADP, 0.9 μmole; nicotinamide, 100 μmoles; $MgCl_2$, 20 μmoles; G-6-P dehydrogenase, 2 units; Tris chloride buffer, pH 8.5, 200 μmoles and dilution with water up to 5.0 ml. With certain inhibitors instead of NADPH generating system, 6 μmoles of NADPH were added. The control contained heat inactivated enzyme. These preparations were incubated in open erlenmeyer fasks in a metabolic shaker at 37° for 30 minutes.

Enzymatic Assay for N,N-Dimethylaniline Oxygenase

The incubation mixture was similar to that of chlorpromazine-N-oxygenase, with the following modifications. In this assay N,N-dimethylaniline 10-40 μmoles and potassium phosphate buffer pH 7.5 (200 μmoles) were used. Incubation was conducted in open erlenmeyer flasks at 37°C for 15 minutes. N,N-dimethylaniline-N-oxide was determined according to the method of Ziegler et al. (146). Each reaction mixture was deproteinized with 0.3 ml of 6N perchloric acid. The deproteinized supernatant solution was adjusted to pH 9.4 with 10% sodium hydroxide and extracted three times with diethylether to remove all of N,N-dimethylaniline. After extraction, the pH of the aqueous solution was adjusted to 2.5 with 3M trichloroacetic acid and 0.1 M sodium nitrite solution was added to attain a final concentration of 0.009 M of nitrite. This solution was then heated for 5 minutes at 60°C, and then read at 420 mμ against water blank. The assay is based on the observation that at pH 2.5 the N-oxide is quantitatively reduced by nitrous acid to N,N-dimethylaniline which readily forms the yellow p-nitroso derivative.

Enzymatic Assay for Methanol Oxidase

The assay mixture was similar to that of N,N-dimethylaniline oxidase, except that the substrate consisted of 250 μmoles of methanol instead of N,N-dimethylaniline. The deproteinized solution was used for determination of formaldehyde as described in Chapter III.

Spectrophotometric Measurement of Cytochrome P-450

The carbon monoxide difference spectra of liver microsomes and protamine sulfate supernatant were measured according to the method of Omura and Sato (147). Difference spectra were measured in a Cary model 14 spectrophotometer with cuvettes of 1-cm optical path. Microsomal and protamine sulfate supernatant preparations, usually containing 1.5 mg of protein per ml of 0.1 M phosphate buffer (pH 7.0), were placed in both the sample and reference cells. Reduction of samples was effected with a few milligrams of solid sodium dithionite ($Na_2S_2O_4$). After recording the base line, carbon monoxide was carefully bubbled through the sample for about 30 seconds which was sufficient to saturate the sample with the gas. The difference spectrum was measured after several minutes. All spectrophotometric measurements were made at room temperature.

Purification of Chlorpromazine-N-Oxygenase from Pork Liver

Protein was determined by the method of Lowry *et al.* (148). Unless otherwise stated all procedures were carried out at 0-4°C.

Preparation of Pork Liver Microsomes

Liver microsomes were prepared following the procedure of Gillette

et al. (18). The livers were collected on the kill floor of the slaughter house within 10-15 minutes after the animals had been killed. These organs were immediately cut into slices about one inch thickness and placed into large polyethylene bags which were stacked into an ice chest between layers of crushed ice. The chilled tissue slices were washed twice with approximately equal volumes of 1.15 percent KCl solution and homogenized for 2 minutes in a waring blendor at low speed, with three volumes of 1.15 percent KCl solution. The homogenate was centrifuged at 10,000 x g for thirty minutes to remove unbroken cells, nuclei and mitochondria. The supernatant fraction, containing microsomes and soluble supernatant, was filtered through glasswool and centrifuged in a Spinco preparative ultracentrifuge at 85,000 x g for one hour. The microsomes were separated, washed once with the KCl solution and recentrifuged for 90 min. The microsomes were then suspended in KCl solution and stored at -20°.

Solubilization of Microsomes

Initial attempts using trypsin were unsuccessful. Then microsomes were solubilized according to the method of Ziegler et al. (60). Microsomal suspensions in 0.2M glycine buffer pH 8.9 were subjected to sonic vibration with a Bronson 20KC sonifier, model No. S125 set at position 8, for 75 sec. at 4 intervals. Triton X-100 was added to a final concentration of 0.5 mg detergent per mg protein and then held at 0° for 15 minutes. The suspension was then centrifuged at 105,000 x g for six hours and the sonication supernatant was collected.

Further Purification of Chlorpromazine-N-Oxygenase

The enzyme from 800 ml of sonication supernatant was precipitated by the addition of solid ammonium sulfate (194.5g) to approximately 40% saturation, allowed to stand for 20 minutes and then centrifuged at 27, 750 x g for 15 minutes. The precipitate was suspended into 240 ml of 0.025 M phosphate buffer. At this stage the preparation can be stored at -20°C for several weeks with no significant loss in CP-N-oxygenase activity. The 0-40 fraction was then dialyzed for four hours against 10 liters of 0.01 M phosphate buffer. The dialyzed fraction was further treated with neutralized protamine sulfate solution (0.2 ml of 2% protamine sulfate solution was added for each ml of dialyzed fraction), allowed to stand for 20 minutes and then centrifuged at 27,750 x g for 10 minutes. The supernatant obtained was labelled as protamine sulfate supernatant.

Addition of either Alumina Cγ or calcium phosphate gel at different concentrations to protamine sulfate supernatant did not yield further purification. DEAE cellulose column (2.5 x 26 cm^2) chromatography at different pH (7.6, 8.0, 8.5, and 8.9) and CM cellulose column chromatography (at pH 8.0) were not successful in increasing the purification. This enzyme was excluded along with blue dextran 2,000 on Sephadex G-75 (50 x 1 cm^2) (Figure 5) and 10% Agarose gel (1 x 40 cm^2) (Figure 6) columns which were previously equilibrated with 2M glycine buffer pH 8.9 and 0.025 M phosphate buffer pH 8 respectively and then subsequently eluted with the same buffers. This indicates that the enzyme has a molecular weight around 400,000.

Bio-Gel A-5M, 200-400 mesh (exclusion limit, 5 x 10^6) was suspended in 0.05 M phosphate buffer, pH 8 containing .1M KCl and all entrained air was removed by placing the slurry in a vacuum flask and deaerated

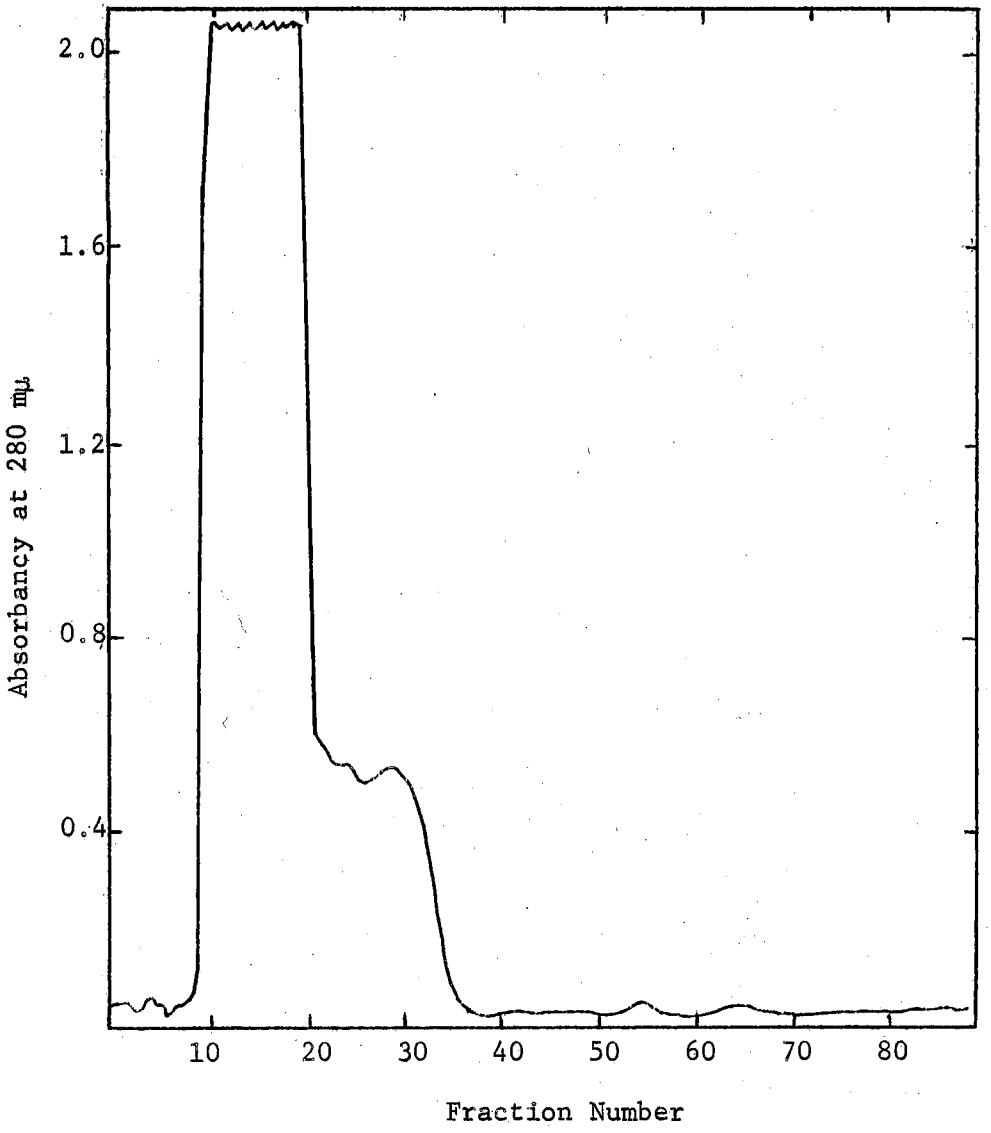


Figure 5. Elution of CP-N-Oxygenase From a Sephadex G-75 Column.

3.0 ml of 0-40 enzyme fraction (60 mg protein), blue dextran and 120 mg sucrose were placed on the column. Three ml fractions were collected. Flow rate was 8 ml/hour. After measuring the absorbancy at 280 mμ, fractions 9→15, 16→20, 21→26, 27→30 and 31→34 were pooled and 2.0 ml of each was used to determine CP-N-oxygenase activity as described under experimental procedure. All the activity was found between fractions 9→20. Blue dextran was also observed between 9→20 fractions.

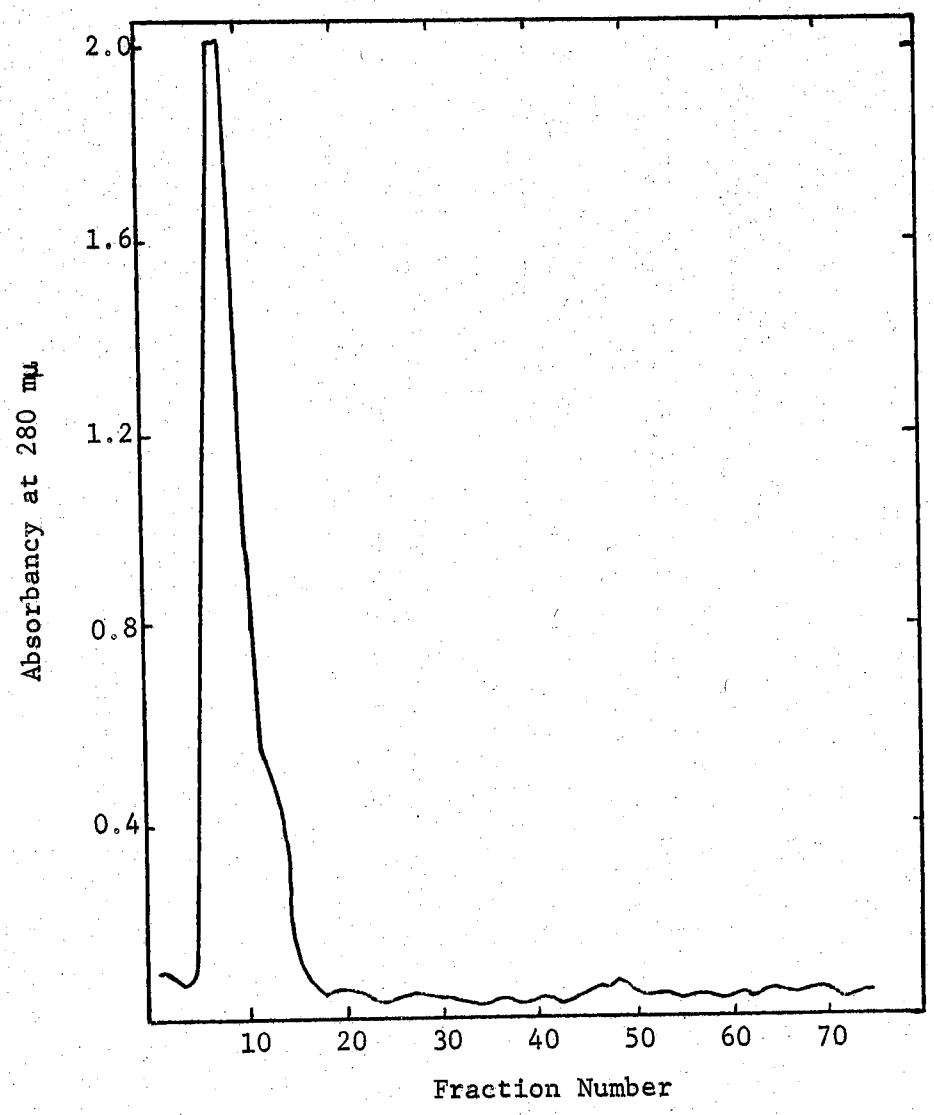


Figure 6. Elution of CP-N-Oxygenase from an Agarose-Gel (10%) Column.

3.0 ml protamine sulfate supernatant (8 mg protein), and blue dextran were placed on the column. 3.5 ml fractions were collected. Flow rate was about 9.0 ml/hour. After reading the absorbancy at 280 mμ , fractions 6→10, 11→14 and 26,27 were pooled and 2.0 ml of each was used to determine CP-N-oxygenase activity as described under experimental procedure. About 50% of the activity was found between fractions 6→10. No activity was observed in other fractions. Blue dextran was distributed between fractions 5→12 and maximum was observed in 7th fraction.

until bubbling ceases. The slurry was allowed to stand at $4^{o}C$ for one day with decantation of the buffer twice. A column (4.5 x 67 cm^2) was packed with the prepared Bio-Gel and equilibrated for two or three days. The column was calibrated using thyroglobulin (molecular weight 670,000) and catalase (molecular weight, 244,000). Eight mg of thyroglobulin, 150 µl of catalase and 80 mg of sucrose were dissolved in 2.0 ml of 0.05 M phosphate buffer, pH 8 containing 0.1 M KCl and placed on the column and eluted with the same buffer. The column was washed for two days and then 3.0 ml of protamine sulfate supernatant fraction containing about 15 mg of protein and 90 mg of sucrose was placed on the column. Pressure head was 40 cms and rate of flow was 10 ml per hour. Six ml fractions were collected and elution was with 0.05M phosphate buffer, pH 8 containing 0.1 M KCl.

Protein was measured at 280 mµ in Beckman DU spectrophotometer. Two ml of eluant was used to determine catlase activity by DB spectrophotometer. Sample cell contained 1.0 ml of H_2O_2 solution containing 0.3 ml of 30% H_2O_2 in 100 ml of 0.05 M phosphate buffer, pH 7 and 2.0 ml of each fraction from column. Reference cell contained water instead of H_2O_2 solution. Catalase activity was measured by following decrease at 240 mµ for fifty seconds (149).

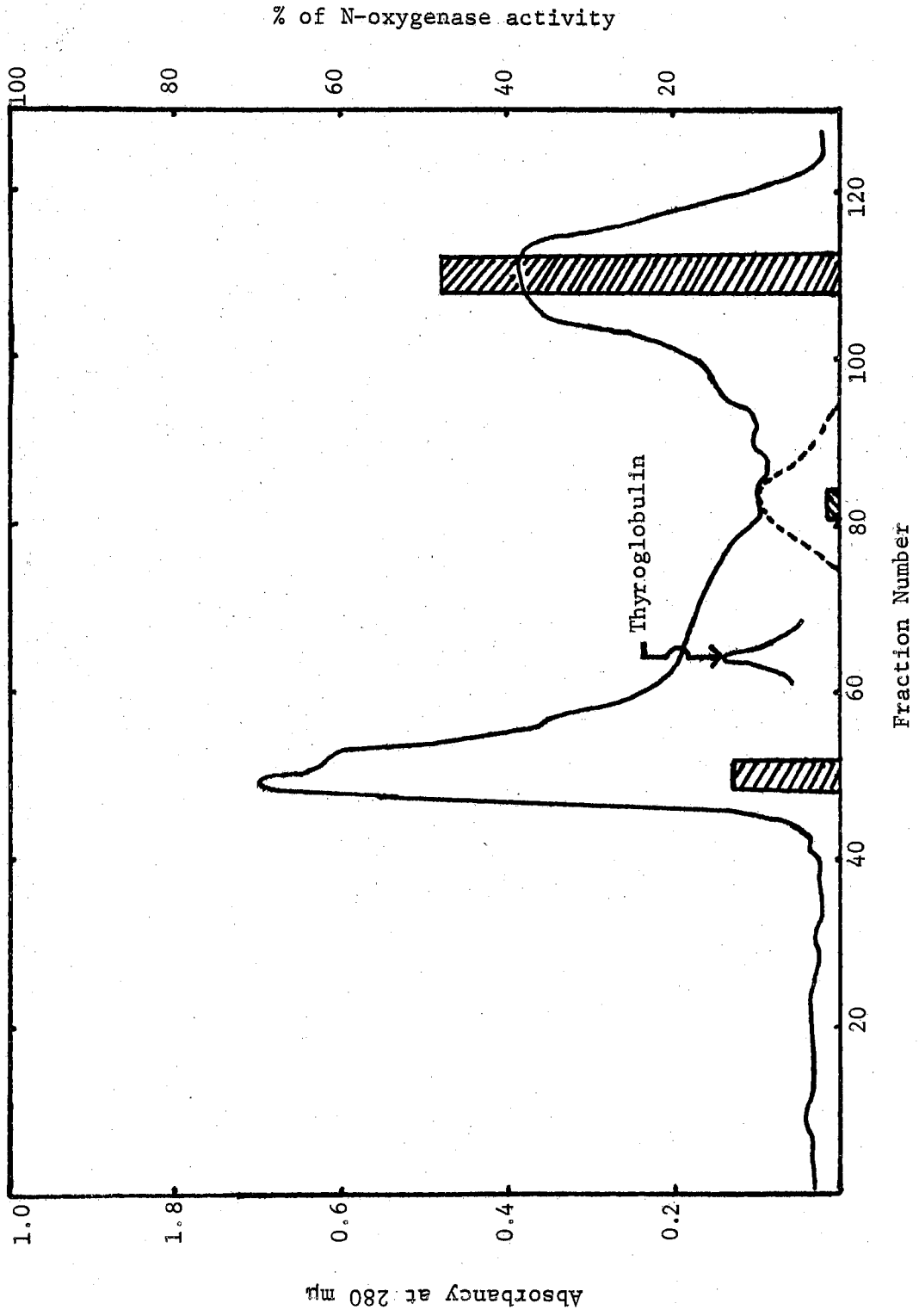
Fractions 48-51, 81-84 and 109-113 were pooled, lyophilized and the residues were suspended in 2.0 ml of water (Figure 7). One ml of each was used for determination of CP-N-oxygenase activity, as described under experimental procedure. The enzyme activity appears to be distributed between 48 - 51 and 109 - 113 fractions; about 13% of the activity between 48-51 and 48% of the activity, between 109-113 fractions.

Figure 7. Elution of Thyroglobulin, Catalase and CP-N-Oxygenase From a Bio-Gel A-5m Column.

_____ = absorbance at 280 mμ ,

= CP-N-oxygenase activity,

- - - = catalase activity.


% of N-oxygenase activity
100
80
60
40
20
Thyroglobulin
120
100
80
60
40
20
Fraction Number
1.0
1.8
0.6
0.4
0.2
Absorbancy at 280 mμ

From this pattern, molecular weight of the enzyme could not be decided. The concentration of CPNO was too low for determination by colorimetry. Therefore, the acid extracts were read at 277 mμ, absorption maximum for sulfuric acid extract of CPNO as shown in Table II. CP-N-oxygenase in the fractions 109-113 showed about 10 fold increase in purification compared to protamine sulfate supernatant, whereas the enzyme between 48-51 fractions showed no purification. However, more sensitive techniques, such as using ^{35}S labelled chlorpromazine, will be beneficial in further purification and studies on mechanism of CP-N-oxygenase.

Results

Purification of Chlorpromazine-N-Oxygenase

A summary of purification steps are shown in Table VIII. The purified enzyme from pig liver microsomes shows an 18 fold purification from microsomes or about 900 fold from liver homogenate, with no loss in yield. Protamine sulfate supernatant was used for studying the properties of this enzyme.

Requirements of Chlorpromazine-N-Oxygenase

Table IX shows certain requirements of chlorpromazine-N-oxygenase. The incubation mixture as described in the experimental procedure section, and other reaction mixtures omitting substrate, NADPH generating system, buffers were incubated with 0.4 ml of the enzyme, purified through the protamine sulfate step at 37°C for thirty minutes. From the table the NADPH generating system appears to be essential for maximum enzyme activity.

TABLE VIII

PURIFICATION OF CHLORPROMAZINE-N-OXYGENASE

	Total Volume	Total Protein[1]	Total Units[2]	Percent Yield	Specific Activity[2]	Fold Purification
	ml	mg	mμmoles/min		mμmoles/mg protein/min	
Homogenate	4000	156000	5440	---	.035	---
Microsomes	1000	23200	40800	100	1.76	1
Sonication Supernatant	800	11840	30000	73.6	2.54	1.45
40% Ammonium Sulfate Precipitate	240	3660	72480	177	19.8	11.2
Protamine Sulfate Supernatant	276	1440	47300	116	32.8	18.6 (935)

[1]Lowry Protein Determination.

[2]Based on CPNO formed. Assay system was described in experimental procedure.

TABLE IX

REQUIREMENTS OF CHLORPORMAZINE-N-OXYGENASE

Composition of Reaction Medium	CPNO Formed
	mμmoles/min
Complete[1]	25.4
Minus Chlorpromazine	0
Minus NADPH Generating System	1.0
Minus Buffer	8.0
Heat Inactivated Enzyme[2]	0

[1]Incubation mixture contained a final concentration of chlorpromazine, 10 mM; glucose-6-phosphate, 5mM; NADP, 0.2mM; nicotinamide, 20 mM; $MgCl_2$, 4mM; 2 units of glucose-6-phosphate dehydrogenase; Tris chloride buffer, pH 8.5, 40mM; and 1.5 mg of enzyme protein.

[2]Enzyme was kept in boiling water bath for 5 minutes before addition to the assay system.

Enzyme Activity Versus Protein Concentration

The assay mixture was incubated with various levels of enzyme purified through the protamine sulfate step, for thirty minutes at 37°C. The reaction is linear with protein concentration up to about 1.8 mg of protein per 5.0 ml total volume (Figure 8). Normal assays, therefore, contained enough enzyme to produce 10 to 30 mμmoles chlorpromazine-N-oxide per minute.

Time Course of Reaction

The reaction mixture was incubated with 0.3 ml of enzyme, purified through protamine sulfate step at 37°, and the reaction stopped at selected time intervals by the addition of zinc sulfate and barium hydroxide as described in the experimental procedure. Figure 9, shows the time course of the reaction. Routine assays were conducted for thirty minutes to obtain sufficient chloromazine-N-oxide for determination by thin layer chromatography.

Effect of Temperature on the Enzyme Activity

The reaction mixture was incubated with 0.3 ml of enzyme, purified through the protamine sulfate step at the temperatures indicated (Figure 10) for thirty minutes and then assayed for CPNO formed. Temperature was optimum between 30-37°C and loses about 70% of the activity at 45°C.

Effect of pH on the Enzyme Activity

The reaction mixture was incubated with 0.3 ml of enzyme, purified through the protamine sulfate step at different pH values, using acetate,

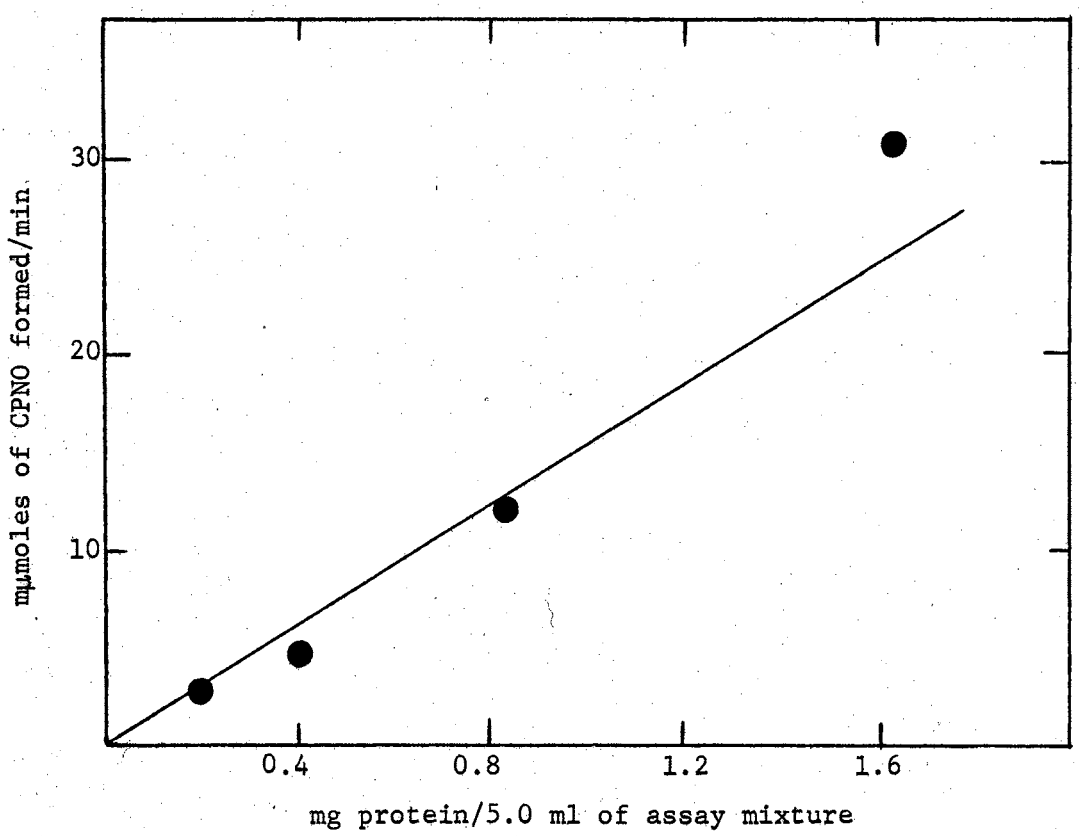


Figure 8. Enzyme Activity Versus Protein Concentration.

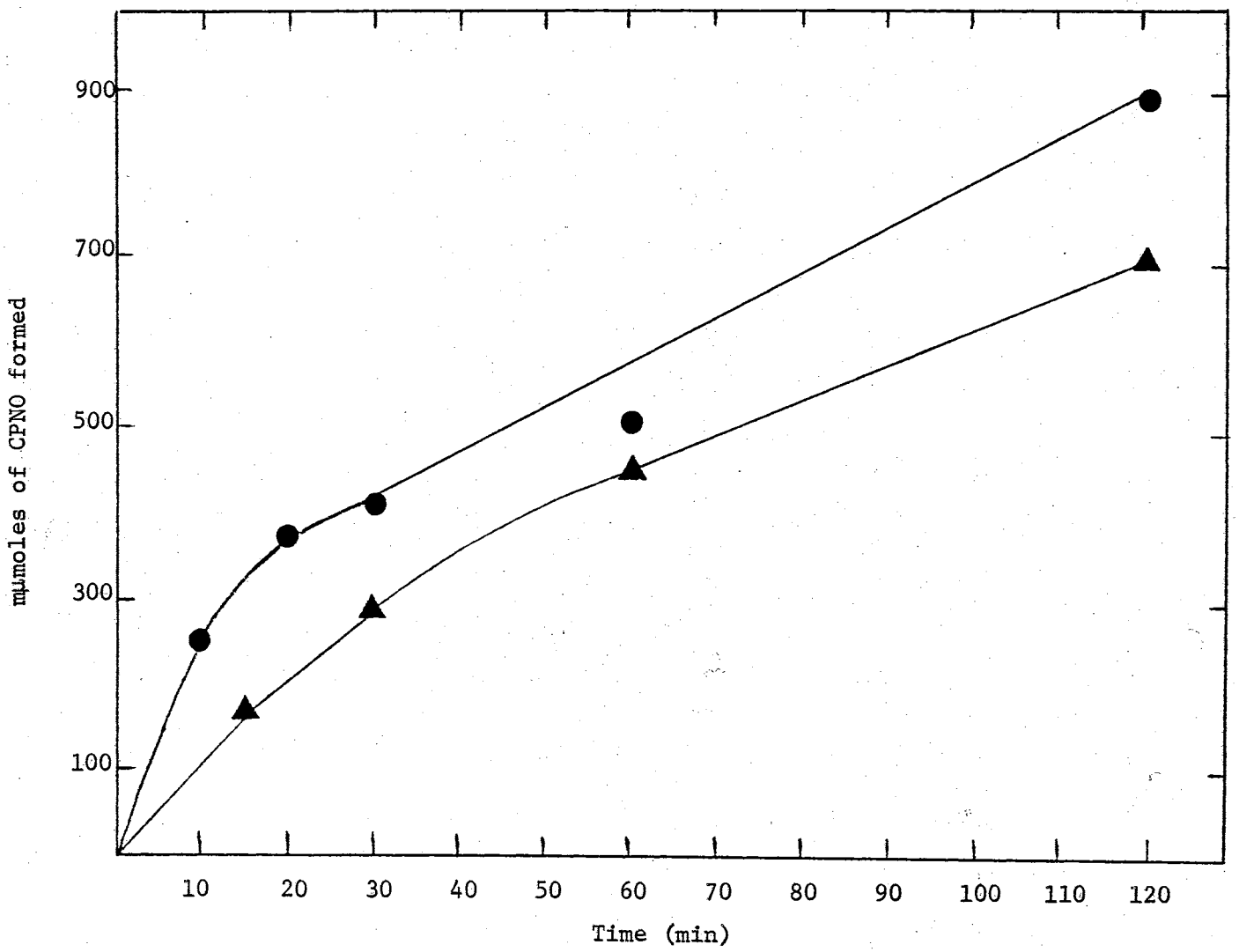
Incubation mixtures containing a final concentration: chlorpromazine, 10mM; glucose-6-phosphate, 5 mM; NADP, 0.2 mM; nicotinamide, 20 mM; $MgCl_2$, 4 mM; 2 units of glucose-6-phosphate dehydrogenase; Tris chloride buffer, pH 8.5, 40 mM; and various levels of enzyme purified through the protamine sulfate step, were incubated at 37° for thirty minutes and then assayed for the amount of CPNO formed.

Figure 9. Time Course of Reaction.

Incubation mixtures as described under Figure 8, were incubated with 1.5 mg of enzyme protein preparation in a final volume of 5.0 ml for the time intervals indicated at 37° and then the amount of CPNO formed was determined.

● = Freshly prepared protamine sulfate supernatant.

▲ = Preparation stored at -20° for approximately two weeks.


900
700
500
300
100
mμmoles of CPNO formed
10
20
30
40
50
60
70
80
90
100
110
120
Time (min)

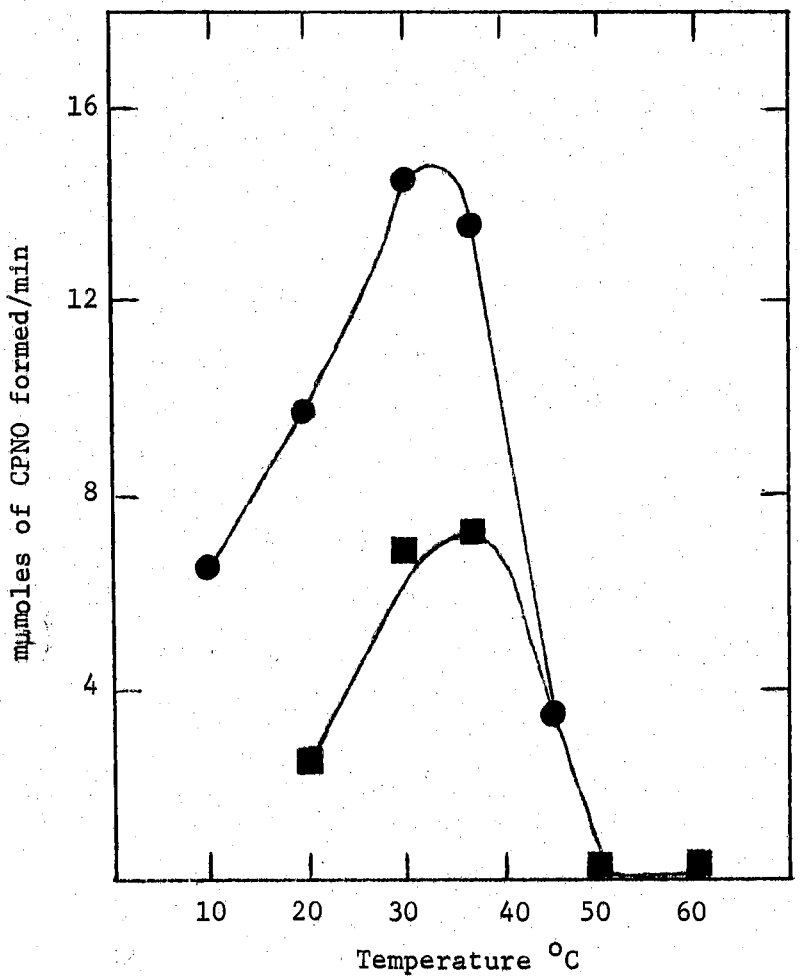


Figure 10. Effect of Temperature on the Enzyme Activity.

Assay mixtures as described under Figure 8, were incubated with one mg of enzyme protein preparation at temperatures indicated for thirty minutes and then assayed for the amount of CPNO formed.

● = Freshly prepared protamine sulfate supernatant.

■ = Preparation stored at -20° for approximately 3 weeks.

potassium phosphate, Tris chloride and sodium glycinate (Figure 11). From the pH curve, optimum pH was at 8.5.

Velocity Versus Chlorpromazine Concentration

In this experiment 0.2 ml of enzyme was incubated for thirty minutes at 37°C with increasing concentrations of chlorpromazine (Figures 12 and 13). A graphical determination of the Michaelis constant for chlorpromazine gave a Km value of 3.57×10^{-3}M.

Competitive Inhibition of Chlorpromazine on the N-Oxidation of N,N-Dimethylaniline

The reaction mixture for N,N-dimethylaniline oxygenase as described in the experimental procedure was incubated with 0.3 ml of the enzyme and with increasing concentration of N,N-dimethylaniline in the presence (2×10^{-3}M) and in the absence of chlorpromazine, at 37° for 15 minutes. Then N,N-dimethylaniline N-oxide was determined as already described. Chlorpromazine at the concentration of 2×10^{-3}M shows competitive inhibition (Figure 14) indicating fairly broad specificity of this enzyme.

Effect of Inhibitors on Chlorpromazine-N-Oxygenase

Incubation mixtures as described in the experimental procedure were incubated with 0.3 ml of the enzyme in the presence of certain inhibitors shown in Table X. In the case of PCMB, EDTA and iodoacetamide, 6 μmoles of reduced NADP were added instead of NADPH generating system along with a control. Little or no inhibition by cyanide and azide indicates that hemoprotein is not involved in this N-oxidation reaction mechanism but a slight inhibition by EDTA indicates a metal involvement.

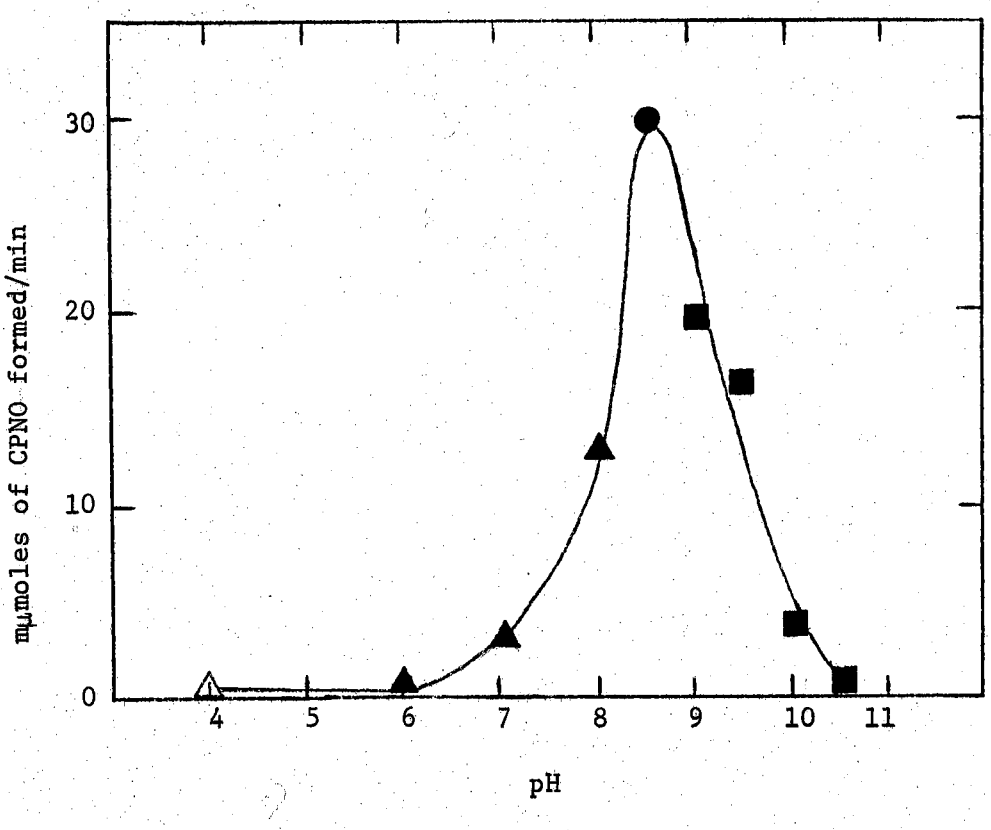


Figure 11. Effect of pH on the Enzyme Activity.

Reaction mixtures as described under Figure 8, using acetate, phosphate, Tris chloride and glycine buffers with different pH values, were incubated at 37° for thirty minutes with 1.5 mg of enzyme protein and the amount of CPNO formed was determined as described under experimental procedures.

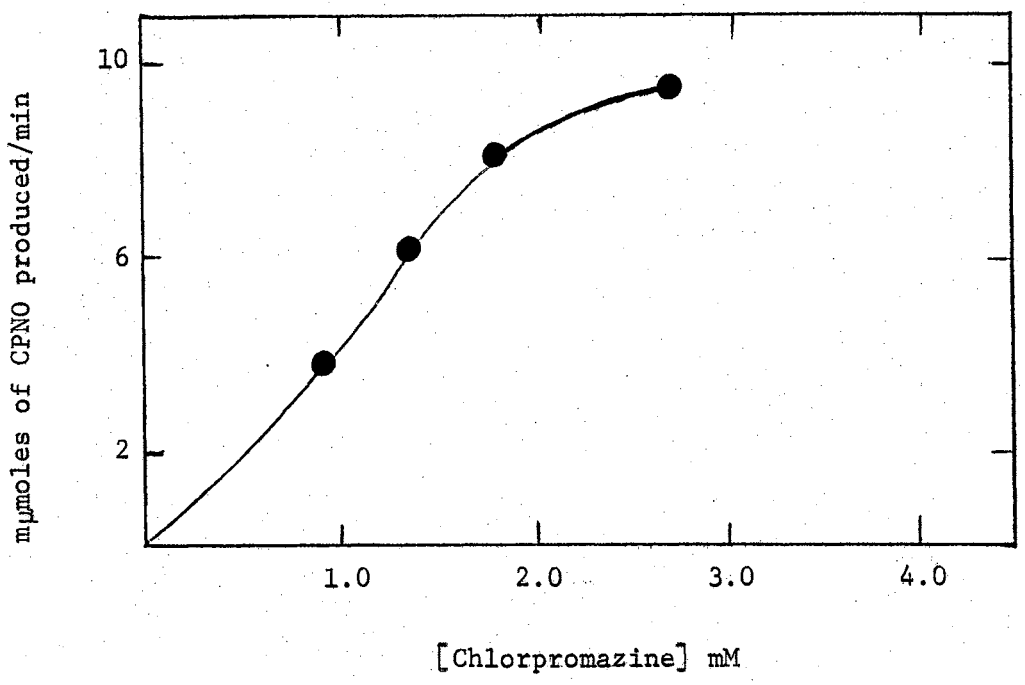
△ = Acetate, ▲ = Potassium phosphate, ● = Tris chloride, ■ = Sodium glycinate.

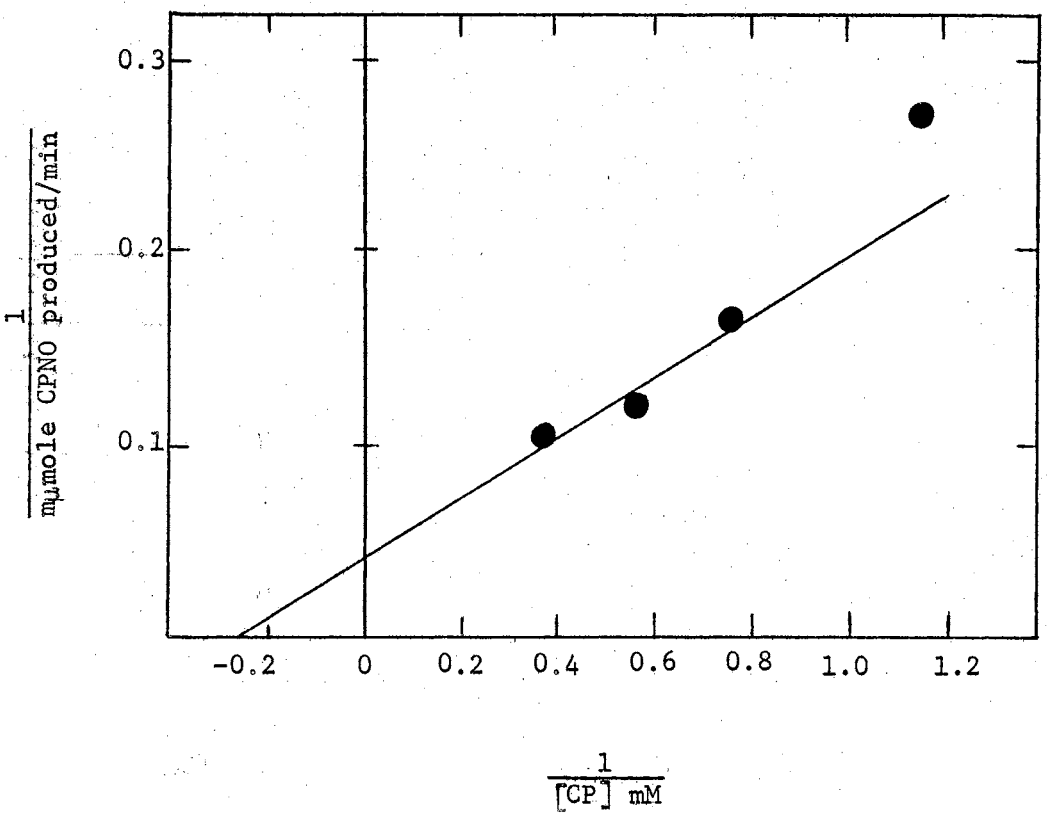
Figure 12. Velocity of Reaction Versus Chlorpromazine Concentration.

Incubation mixture as described under Figure 8, with various concentration levels of chlorpromazine were incubated with 1.0 mg of enzyme protein for thirty minutes at 37° and then assayed for the amount of CPNO formed.

Figure 13. Lineweaver-Burk Plot of Reaction; Reciprocal Velocity Versus Chlorpromazine Concentration.

Same conditions as Figure 12.


10
6
2
mμmoles of CPNO produced/min
1.0
2.0
3.0
4.0
[Chlorpromazine] mM


0.3
0.2
0.1
1/mμmole CPNO produced/min
-0.2
0
0.2
0.4
0.6
0.8
1.0
1.2
1/[CP] mM

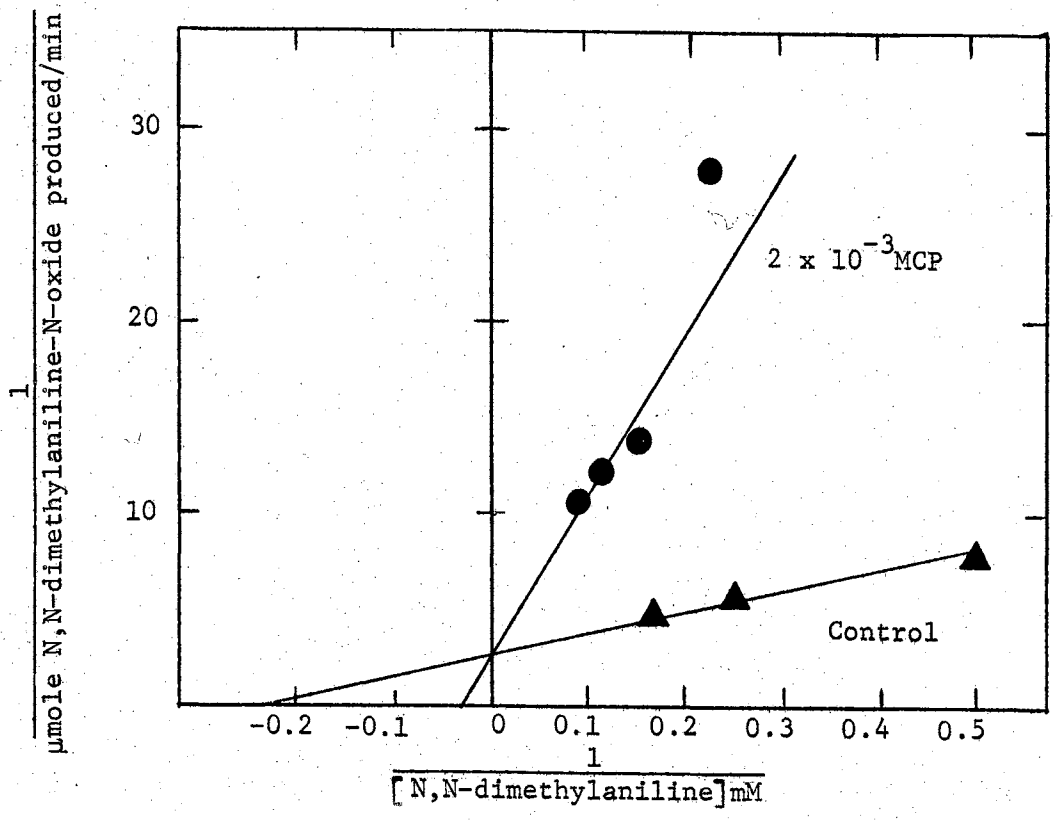


Figure 14. Lineweaver-Burk Plots suggesting the Competitive Inhibition of Chlorpromazine on the N-oxidation of N,N-Dimethylaniline.

▲ = -CP; ● = +2 x 10^{-3}M CP

SKF-525A does not show any significant inhibition of N-oxidation. Inhibition by PCMB and iodoacetamide indicates the sulfhydryl nature of this enzyme.

Effect of Inhibitors on Methanol Oxidation

Reaction mixture for methanol oxidase as described in the experimental procedure was incubated with 0.3 ml of enzyme in the presence of certain inhibitors shown in Table XI. At the end of the reaction, formaldehyde was determined as described in the methods. A comparison of the effects of inhibitors on chlorpromazine and methanol oxidation by the enzyme is shown in Table XI. Methanol oxidation is inhibited up to 50-70 percent by cyanide and azide but no significant inhibition on chlorpromazine-N-oxidation, indicating that methanol oxidase constitutes a separate enzyme system from chlorpromazine-N-oxygenase.

Carbon Monoxide Difference Spectra

Carbon monoxide difference spectra of pig liver microsomes were measured as described in experimental procedure (Figure 15). Table XII shows the absorbance increment in a CO difference spectrum at 450 mμ and 420 mμ in microsomes and protamine sulfate supernatant. As the enzyme is purified, instead of an increase in the ratio of cytochrome to protein, P-450 is decreased followed by an increase in P-420. This indicates that P-450 is partly solubilized during purification procedure and further supports the concept that P-450 is not involved in N-oxygenation reaction.

TABLE X

EFFECT OF INHIBITORS ON CHLORPROMAZINE-N-OXYGENASE ACTIVITY

Addition	Conc.	CPNO Formed	Inhibition
	(M)	mμmoles/min	%
None	-----	26.6	-----
Azide	10^{-3}	29.3	0
PCMB[1]	10^{-3}	3.2	84.4
KCN	10^{-3}	26.2	1.5
SKF 525 A	10^{-3}	25.4	4.5
EDTA[1]	10^{-3}	18.2	9.9
Iodoacetamide[1]	10^{-3}	14.8	26.8
None[1]	-----	20.2	-----

[1]TPNH (6μmoles) was added instead of TPNH generating system.

TABLE XI

EFFECT OF INHIBITORS ON CHLORPROMAZINE AND METHANOL OXIDATION

		CP-N-Oxidation		Methanol Oxidation	
Addition	Conc.	CPNO Formed	Inhibition	HCHO Formed	Inhibition
	(M)	mμmoles/min	%	mμmoles/min	%
None	---	26.6	---	4.65	---
KCN	10^{-3}M	26.2	2	2.1	54.8
Azide	10^{-3}M	29.3	0	1.2	74.2

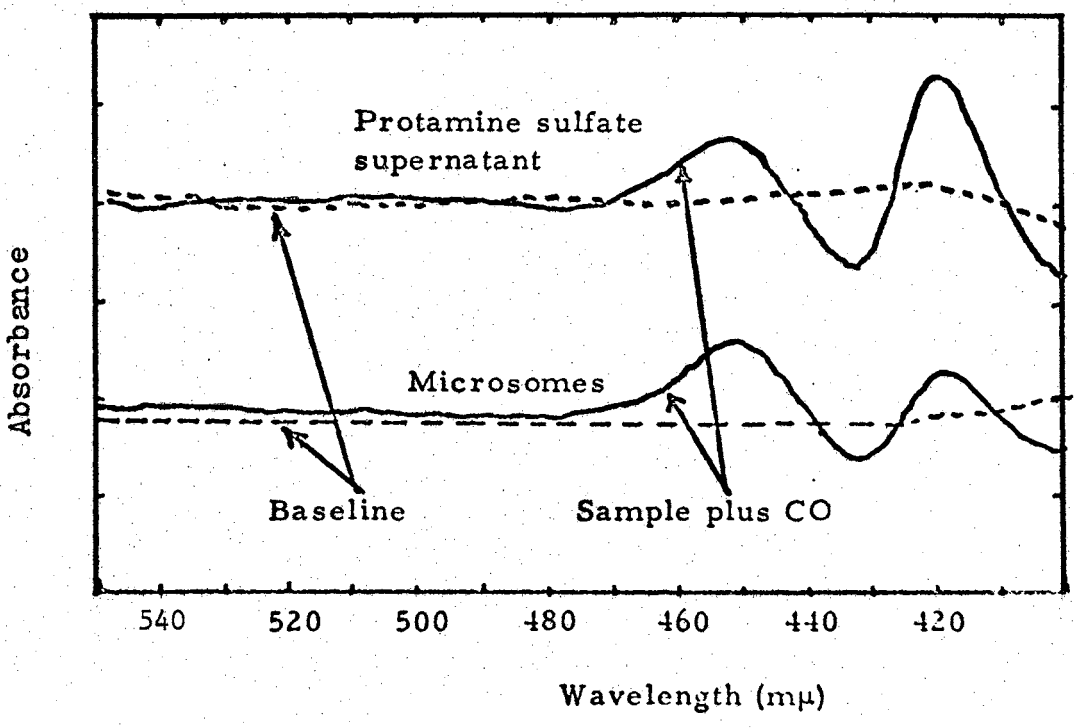


Figure 15. Carbon Monoxide Difference Spectra.

Carbon monoxide difference spectra were measured as described with a Cary 14 recording spectrophotometer. Microsomal and protamine sulfate supernatant preparations contained 1.5 mg of protein/ml of 0.1 M phosphate buffer, pH 7.0.

TABLE XII

P-450 AND P-420 IN MICROSOMES AND PROTAMINE SULFATE SUPERNATANT

Fraction	CP-N-Oxygenase Specific Activity	Absorbance increment in CO difference spectrum	
		450 mμ	420 mμ
	mμmoles/mg protein/min	ΔA/mg protein	ΔA/mg protein
Microsomes	1.76	0.009	.005
Protamine sulfate supernatant	32.8	0.007	.012

CHAPTER V

DISCUSSION

A large amount of research has been conducted on the detection and estimation of chlorpromazine metabolites but a satisfactory comprehensive systematic procedure which can be used routinely in enzymatic studies on chlorpromazine metabolism has still not been described. Phenolic metabolites of chlorpromazine yield a purple color whereas nonphenolic metabolites produce a pink color when treated with sulfuric acid spray reagent. For enzymatic studies, the specific metabolite must be separated from all other metabolites and chlorpromazine and determined quantitatively. Table I shows a separation of seven nonpolar chlorpromazine metabolites and chlorpromazine, in three solvent systems, by thin layer chromatography. CP-N-oxide, sulfoxide, and demethylated metabolites can be separated from a mixture of all 8 reference compounds. This technique can be utilized for determination of sulfoxidation, N-oxidation and demethylation of chlorpromazine by enzyme preparations.

After spraying the paper chromatogram, Posner et al. (5) scanned the strips with the Spinco model RB Analytrol whereas Kurland et al. (139) determined the metabolite by extracting into 50% H_2SO_4 and reading the acid extract at 525 mμ in a Bausch and Lomb "Spectronic 20". Complete extraction of the color was obtained for CPNO with sulfuric acid from the thin layer separation whereas with paper this was not the case. Table II shows absorption maximum for certain chlorpromazine metabolites

in the ultraviolet region. Chlorpromazine and chlorpromazine-N-oxide have λmax at 256 mμ and almost similar spectra (Figure 4). For this reason chlorpromazine-N-oxide cannot be determined by a spectrophotometric method unless separated from chlorpromazine. The sulfuric acid extracts of CP and CP-N-oxide show two peaks in the ultraviolet region at 267 and 277 mμ . Thus, the acid extract of chlorpromazine-N-oxide can be determined at 269 mμ , 277 mμ or its pink color at 525 mμ . In this work, CP-N-oxide was determined at 525 mμ.

Jollow et al. (61) determined N-oxidation of chlorpromazine based on the s NADPH oxidation by 1000 fold purified microsomal mixed function oxidase from pork liver. The preparation was found to be free from demethylase, monoamine oxidase, NADPH-cytochrome C reductase and NADH-cytochrome C reductase. However, CP-N-oxygenase purified about 900 fold from pork liver in this laboratory, catalyzes conversion of chlorpromazine to CPNO, a small amount of N,S-dioxide and a major unidentified metabolite (orange when sprayed with sulfuric acid reagent, max 273.5 mμ) in the presence of NADPH. Besides, there is the possibility of sulfoxidation of chlorpromazine which was shown to require NADPH (7). In liver homogenate and microsomes, NADPH oxidase and demethylase are present, which will also utilize NADPH. Thus, oxidation of NADPH is not only due to N-oxidation alone. Therefore, enzyme activity was based on the determination of CP-N-oxide by thin layer chromatography. Although the determination of CPNO, as an assay technique for CP-N-oxygenase, requires more time, it was the method of choice as the product of the reaction was determined.

Fishman et al. (6) reported that man excretes more mono-and didemethylated sulfoxide metabolites than N-oxide whereas dog excretes more

sulfoxide and monodemethylated sulfoxide than N-oxide in urine. Further, they reported that pharmacological and behavioral tests on nor_1 CP and CPNO in animals indicate that these compounds approach CP in activity, whereas CPSO is markedly less active. In the light of above facts, it is of interest to find that liver microsomes of mammalian species shown in Table V, produce more N-oxidation than demethylation. Beckett and Hewick (10) also showed occurance of N-oxidation (24%), demethylation (12%), hydroxylation (2%) and sulfoxidation (10%), when 2.5 μm of ^{35}S chlorpromazine hydrochloride were incubated with male rat liver microsomes (equivalent to 5.8 mg of protein) in the presence of NADPH.

In 1956 Mason (150) proposed the terms oxygen transferase and mixed function oxidase to denote enzymes catalyzing the over-all reactions shown in Eqs. (B) and (A) respectively.

$$[S] + \tfrac{1}{2} O_2 \longrightarrow SO \qquad (A)$$

$$[S] + O_2 \longrightarrow SO_2 \qquad (B)$$

At the same time, Hayaishi (150) proposed the term oxygenase in view of the fact that the reactions catalyzed by this new group of enzymes are very similar to oxygenation reactions known to occur by ordinary chemical or photochemical processes. The second type of enzyme was, therefore, referred to as a mixed function oxygenase or mixed function oxidase because these enzymes are bifunctional, carrying out oxidase activity on one site and oxygenase activity on the other. Recently the term hydroxylase has been preferred to mixed function oxidase or mixed function oxygenase, presumably because the former is more concise and convenient. Thus, the term oxygenase, in a broad sense, may be assigned to a group of enzymes catalyzing the activation of oxygen and

the subsequent incorporation of either one or two atoms of oxygen per mole of various substrates. The terms hydroxylase, mixed function oxygenase or mixed function oxidase reaction is characterized by a pair of requirements: (i) oxygen is necessary, as a specific oxidizing agent, and (ii) an electron donor is needed as a reductant. One of the oxygen atoms is incorporated into the substrate molecule, while the other atom is presumed to be simultaneously reduced to water (150).

Chlorpromazine-N-oxygenase requires reduced NADP, which is oxidized by O_2 in the process. According to nomenclature of International Union of Biochemistry, this enzyme belongs to hydroxylase group (1·99·1). As the reaction is not yet fully understood, no systematic names were recommended for this type of reaction (151). However, naming this enzyme as chlorpromazine hydroxylase will be misleading because this enzyme does not catalyze hydroxylation of chlorpromazine. Further enzymes were observed catalyzing hydroxylation of chlorpromazine in rabbit liver microsomes (138). Thus, it is more appropriate to name this enzyme as chlorpromazine-N-oxygenase. Chlorpormazine sulfoxidation is another process similar to N-oxidation, involving reduced NADP and oxygen (7). In the opinion of the author the name "Mixed Function Oxygenases" will be more appropriate than the name "Hydroxylases" for this group of enzymes. This group may be further divided as hydroxylases, N-oxygenases and S-oxygenases. Oxidation of N-atom of tertiary amine group in compounds such as promazine, chlorpromazine, N,N-dimethylaniline, trimethylamine and erythromycins, and oxidation of heterocyclic N-atom in compounds such as nicotinamide and chlorcyclizine by liver microsomes have been observed. In the light of this work, naming of drug metabolizing enzymes is worthy of reconsideration.

Hayaishi et al. (152) showed with experiments using ^{18}O, that one atom of molecular oxygen was incorporated into catechol by the action of salicylate hydroxylase. Katageri et al. (153) postulated the following scheme for the overall reaction of salicylate hydroxylase:

$$\text{E-FAD} + \text{salicylate} \longrightarrow \text{E-FAD-salicylate}$$

$$\text{E-FAD-salicylate} + NADH_2 \longrightarrow \text{E-}FADH_2 - \text{salicylate} + NAD$$

$$\text{E-}FADH_2\text{-salicylate} + O_2 \longrightarrow \text{E-FAD} + \text{catechol} + CO_2 + H_2O$$

Here, E denotes the protein moiety of the enzyme. Imai and Sato (154) reported that the interaction of P-450 with drug substrates is an obligatory step in the mechanism of drug hydroxylations. They proposed the following mechanism for aniline hydroxylation:

$$[\text{P-450}'''] + \text{aniline} \longrightarrow [\text{P-450}'''] - \text{aniline}$$

$$[\text{P-450}'''] - \text{aniline} \longrightarrow [\text{P-450}''] - \text{aniline}$$

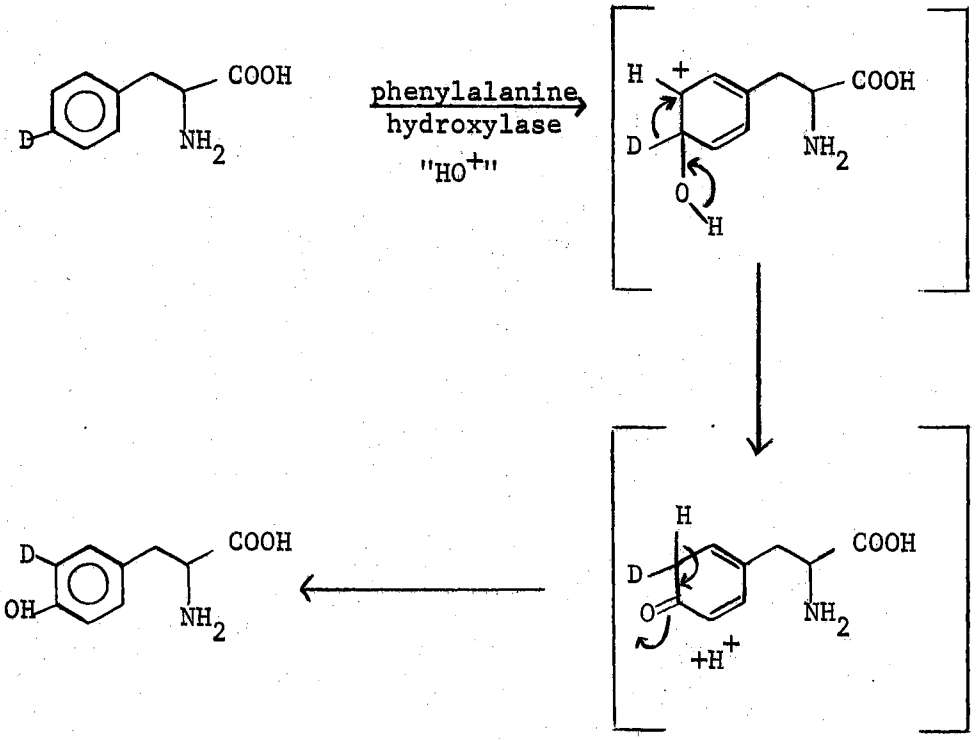
↑ A specific microsomal electron transfer pathway.

$$[\text{P-450}''] - \text{aniline} + O_2 \longrightarrow [\text{P-450}''.O_2] - \text{aniline}$$

$$[\text{P-450}''.O_2] - \text{aniline} \longrightarrow [\text{P-450}'''] + \text{p-aminophenol.}$$

where $[\text{P-450}'''] - \text{aniline}$ and $[\text{P-450}''.O_2]$ indicate that aniline and oxygen are bound to a nonheme site and the heme iron, respectively, of P-450.

Guroff and Daly (155) prepared labelled 4-deuterophenylalanine and showed that hydroxylation, catalyzed by phenylalanine hydroxylase from liver or bacteria, yielded 3-deuterotyrosine. The hydroxylating species is not known and is referred to as HO^+. Mechanism for hydroxylation was proposed thus:



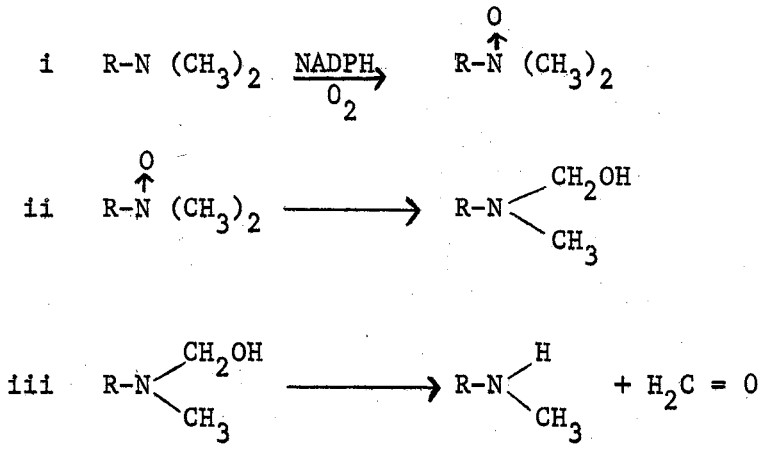
The requirement of the oxidative microsomal enzyme systems for NADPH and oxygen is not completely understood. Brodie et al. (11) have postulated that a hydroxyl donor is formed during the oxidation of NADPH by a microsomal NADPH oxidase and transferred to the drug substrates by non specific enzymes. Gillette and Kamm (7) proposed the following mechanism for sulfoxidation of 4,4'-diaminodiphenylsulfide:

$$NADPH + O^2 \xrightarrow[\text{NADPH oxidase}]{\text{microsomal}} [HO^+] + TPN$$

$$\begin{matrix} R \\ R \end{matrix}\!\!>\!S + [OH^+] \rightarrow \left[\begin{matrix} R \\ R \end{matrix}\!\!>\!SOH\right]^+ \rightarrow \begin{matrix} R \\ R \end{matrix}\!\!>\!SO + H^+$$

The mechanism of oxidative dealkylation has been the subject of a number of studies. Horning and his associates (156,157) postulate (that N-oxides may be intermediate products. They have shown that liver homogenate supplemented with DPN, AMP, and nicotinamide catalyze the

rearrangement of N,N-dimethyltyrosine oxide and N,N-dimethyl tryptophan oxide to yield formaldehyde and the secondary amines. This system also catalyzes the oxidation of N,N-dimethyltryptamine to the corresponding N-oxide. From these observations the authors have proposed that the dealkylation of alkylamines may proceed along the following pathway:



In support of the above pathway Pettit and Ziegler (146,158) demonstrated that rat and pig liver microsomes catalyze the rapid demethylation of N,N-dimethylaniline-N-oxide to methylaniline and formaldehyde in the absence of both NADPH and oxygen. The rate of N,N-dimethylaniline-N-oxide demethylation by liver microsomes was sufficiently high for the N-oxide to be considered as an intermediate. They also demonstrated formation of N,N-dimethylaniline-N-oxide at a sufficiently rapid rate for it to be an intermediate in the oxidative demethylation of N,N-dimethylaniline catalyzed by rat or pig liver microsomes. From Table VII, chlorpromazine was not converted to the N-oxide and was not demethylated to an appreciable extent in the absence of NADPH. Thus, NADPH seems to be essential for N-oxide formation from chlorpromazine and for oxidative demethylation of chlorpromazine or chlorpromazine-N-oxide by mouse liver microsomes. It was also observed that the demethylation of CP was greater than that of CPNO in the presence of NADPH. This would indicate

that CPNO is not intermediate in oxidative demethylation of chlorpromazine. In support of this observation, Mao and Tardrew (159) reported that N-oxide can not be intermediate in oxidative demethylation of erythromycins by rabbit tissues *in vitro*. This is further confirmed by the observation of Kuntzman *et al.* (160) that chlorcyclizine-N-oxide does not appear to be intermediate in the formation of norchlorcyclizine. To clarify the status of the mechanism of demethylation, Ziegler and coworkers (161) reported that pig liver microsomes could catalyze the dealkylation of only those N-oxides in which the nitrogen atom was directly attached to an aromatic ring. It is of interest to note the observation of Coccia and Westerfeld (137) that the ferrous ion catalyzes the rearrangement of the chlorpromazine-N-oxide to nor_1 CP and to CPSO as well as the reduction of the N-oxide to CP. However, this was not observed in the presence of the ferric ion.

Microsomal preparations isolated by the usual techniques contain a host of enzymes that hinder the interpretation of studies on the mechanism of the oxidative systems. The solubilization and purification of the microsomal enzyme system should prove invaluable in studying their mechanism of action. Various drug metabolizing enzymes have been solubilized using heated snake venom, pancreatic lipase, deoxycholate, freeze-thaw ultrasonication, freeze-thaw glycerol extraction and sonication followed by cholate (13). Ziegler *et al.* (60) solubilized alkylarylamine oxygenase using sonication and addition of Triton X-100 detergent. With slight modification of Ziegler's procedure and with an addition of protamine sulfate step, chlorpromazine-N-oxygenase has been solubilized and purified about 900 fold from pig liver homogenate (Table

VIII). This purified preparation was used for studying the properties of the enzyme. The requirement for flavin adenine dinucleotide by a liver microsomal oxygenase catalyzing the oxidation of N,N-dimethylaniline was observed by Ziegler et al. (162). From Table IX, reduced NADP appears to be essential for maximum enzyme activity.

From Figure 11, optimum pH appears to be at 8.5. In this connection it is of interest to note the observation of Hochstein and Dalton (163). In the range of pH 8.2-9, nicotine oxidase activity decreased, when pyrophosphate or gycine buffers were employed instead of Tris buffer. They observed an increase in activity reaching maximum at pH 8.6 and then the rate remained essentially constant up to pH 9.0, the last pH at which Tris buffers were assayed.

Jollow et al. (61) reported a Km value of 2.6×10^{-5} M for chlorpromazine, on the basis of oxidation of reduced NADP by a purified microsomal mixed function oxidase from pork liver. As discussed earlier, the amount of NADPH oxidation can not yield the exact quantity of CPNO formed. From Figure 13, we obtained a Km value of 3.57×10^{-3} M on the basis of CPNO formed. This is comparable with Km value of 1.42×10^{-3} M for N,N-dimethylaniline, which is also a lipid soluble N-methyl compound oxidized by pork liver microsomes (158). However, at pH 8.5, apparently 3.5% of the substrate is soluble and the remaining chlorpromazine was precipitated or separates into a two phase system. When the supernatant after precipitation of CP at pH 8.5, was used, the N-oxide formed was only 1/36th of the amount, which formed with the same volume of unprecipitated CP solution.

Chlorpromazine at the concentration of 2×10^{-3} M shows competitive inhibition on the N-oxidation of N,N-dimethylaniline by the protamine

sulfate supernatant (Figure 14). Ziegler et al. (61) reported that the purified oxidase from pork liver can also catalyze the oxidation of other tranquilizers, antihistamines, hallucinogens, narcotics, alkaloids containing a tropine base, ephedrine and derivatives of ephedrine. Although it shows broad specificity, it will not catalyze the oxidation of N-alkyl derivatives of purines or barbiturates. In recent years, evidence has accumulated for the view that the microsomes contain a number of enzyme systems which catalyze the same reaction. For example, there seems to be more than one N-dealkylase. This is deduced from the following facts: β-diethylaminoethyldiphenylpropyl acetate (SKF 525A) (164) and iproniazid (165) inhibit the metabolism of monomethyl-4-aminoantipyrine (MMAP) but do not block the dealkylation of N-methylaniline. 3,4-benzpyrene (53) administered to rats stimulate the dealkylation of 3-methyl-MAB about five fold, but does not alter the rate of dealkylation of MMAP by liver microsomes. Results from similar experiments have suggested that the liver microsomes also contain at least two O-dealkylases (24) and a number of aromatic hydroxylases (166,167). A fifty to 70% inhibition of methanol oxidation and no significant inhibition of chlorpromazine-N-oxidation (Table XI) indicates that methanol oxidase constitutes a separate enzyme from CP-N-oxygenase.

From Table X, inhibition of chlorpromazine-N-oxidation by PCMB and iodoacetamide indicate the sulhydryl nature of this enzyme. No significant inhibition of N-oxidation was observed by SFK-525A. This is in agreement with the findings of Coccia and Westerfeld (137). SKF-525A, a potent inhibitor of microsomal drug metabolism, in concentrations as low as 10^{-4} M blocks the in vitro oxidation of many drugs including barbiturates, codeine and aminopyrine. In addition, SFK-525A inhibits the

formation of morphine glucuronide and the hydrolysis of procaine. The microsomal enzyme (13) that hydrolyzes the amide-linkage of monoethylglycinexylidide, a metabolite of lidocaine, has been solubilized. The soluble enzyme is even more strongly inhibited by SFK-525A than is the microsomal preparation. Although SKF-525A inhibits the dealkylation of N,N-dimethylaniline-N-oxide, it does not inhibit the N-oxidation of N, N-dimethylaniline (168). Coccia and Westerfeld (137) reported that SKF-525A does not inhibit CP-N-oxidation and N-oxide reduction but inhibits demethylation, sulfoxidation and hydroxylation of chlorpromazine by rat and human liver microsomal enzyme systems. Gillette and Kamm (7) reported that SKF-525A does not inhibit sulfoxidation of chlorpromazine by guinea pig liver microsomes.

Little or no inhibition by cyanide and azide (Table X) indicates that hemoprotein is not involved in the CP-N-oxidation reaction mechanism but a slight inhibition by EDTA suggests a metal involvement. The presence in mammalian liver microsomes of a carbon monoxide-binding pigment has been reported by Klingenberg and Garfinkel (147). The CO compound of the reduced pigment has an intense absorption band at 450 mμ and thus can be readily detected in dithionite-treated microsomes by difference spectrophotometry. Omura and Sato (169) reported evidence for the hemoprotein nature of the microsomal CO-binding pigment, provisionally called "P-450" and have shown that it can be converted into a solubilized form, which was called "P-420", by treatment of microsomes anaerobically with snake venom or deoxycholate. Recent studies have established that P-450 is involved as the oxygen activating enzyme in a number of NADPH-requiring monooxygenase reactions such as drug hydroxylations by liver microsomes and steroid hydroxylations by adrenal cortex

microsomes and mitochondria (170). Imai and Sato (154) collected evidence indicating that the drug-induced spectral changes are closely related to the mechanism of drug hydroxylation by liver microsomes and that P-450 is actually involved in these changes and functions in the substrate activation. Participation of cytochrome P-450 was also reported in omega oxidation of fatty acids (171). As the CP-N-oxygenase is purified, instead of an increase in the ratio of cytochrome to protein, P-450 is decreased followed by an increase in P-420 (Table XII). This indicates that during purification procedure P-450 is partly solubilized and further supports the suggestion that P-450 is not involved in N-oxidation reaction. In confirmation of the above, after the 10th day the preparation did not show the presence of P-450, though it possessed about 70% of the N-oxygenase activity. Ziegler and Pettit (168) reported that carbon monoxide does not inhibit N-oxidation of N,N-dimethylaniline but inhibits the subsequent dealkylation of the N-oxide to the secondary amine and aldehyde. Ziegler et al. (172) further reported that P-450 is an essential pigment in the dealkylation reaction but is not involved in the initial N-oxidation of N,N-dimethylaniline. Estabrook et al. (173) reported the absorption spectral properties of two forms of cytochrome P-450 in rabbit liver microsomes inducible preferentially either by phenobarbital or 3-methylcholanthrene.

In conclusion, cytochrome P-450 does not appear to be involved in the N-oxidation reaction mechanism, whereas involvement of P-450 has been established in hydroxylation reactions by other workers. Further purification by gel filtration and development of more sensitive methods using radioactive chlorpromazine for determination of CPNO will be more useful in elucidation of mechanism of N-oxidation reactions.

SUMMARY

Chlorpromazine (CP) was converted to chlorpromazine-N-oxide by mammalian liver microsomal preparations. The assay system developed for chlorpromazine-N-oxygenase was based on a thin-layer chromatographic separation of CP metabolites. The enzyme system catalyzing the NADPH-dependent oxidation of the side chain N-atom of CP was found to be mainly in the microsomes. CP-N-oxidation and demethylation by liver microsomes of mouse, rat, dog, pig and bovine were measured. Production of N-oxide by different species was in the following order: dog = mouse > pig > rat > bovine. CP-N-oxygenase was solubilized using sonication followed by addition of Triton X-100. This enzyme was further purified by ammonium sulfate fractionation and protamine sulfate. The purified enzyme showed about 900 fold purification from the liver homogenate or 18 fold from liver microsomes. The enzyme has a Km value of 3.57×10^{-3} M for chlorpromazine and showed a pH optimum at 8.5. The temperature was optimum between 30-37°C and loses about 70% activity at 45°C. SKF-525A does not show any significant inhibition of N-oxidation. Inhibition by PCMB and iodoacetamide indicates the sulfhydrayl nature of the enzyme. A comparison of the effects of cyanide and azide on inhibition of methanol oxidation and CP-N-oxidation, indicates that CP-N-oxygenase constitutes a separate enzyme system from methanol oxidase. Chlorpromazine at a concentration of 2×10^{-3} M shows competitive inhibition on

the N-oxidation of N,N-dimethylaniline by protamine sulfate supernatant. Little or no inhibition on CP-N-oxidation by cyanide and azide indicate that hemoprotein is not involved in this N-oxidation reaction mechanism but a slight inhibition by EDTA suggests a metal involvement. It is further confirmed by CO difference spectra that P-450 is not involved in CP-N-oxidation reaction mechanism.

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