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Metabolism of Phencyclidine

by

Marie Kim Phung Hoag

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Comparative Pharmacology and Toxicology

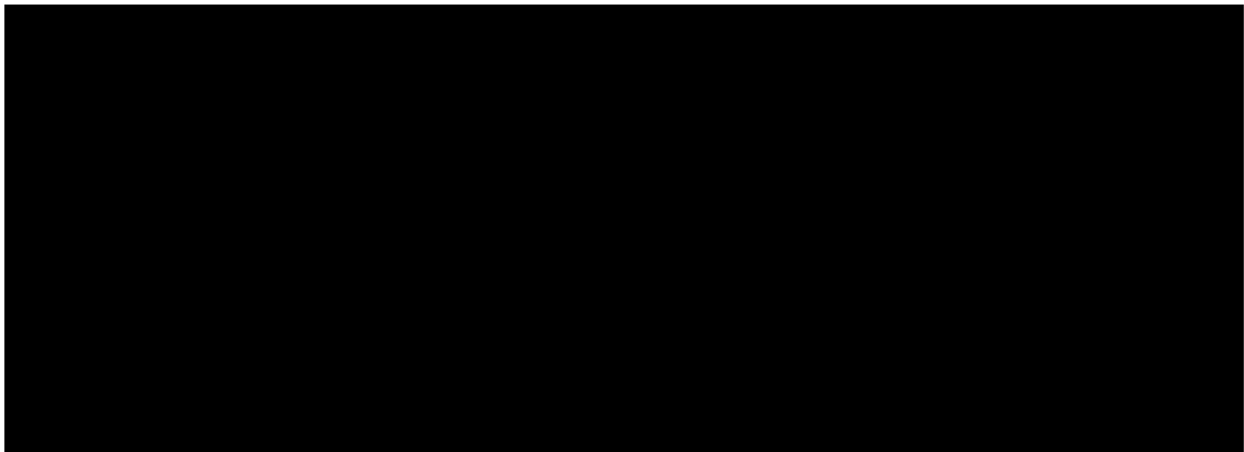
in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA

San Francisco



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To my parents, Peter and Theresa,
and
to my husband, Scott,
for their unending love and support.

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METABOLISM OF PHENCYCLIDINE

Marie Kim Phung Hoag

ABSTRACT

Phencyclidine (PCP) is an arylcyclohexylamine which was developed in the 1950's as an intravenous anesthetic agent. Because of undesirable side effects which became apparent during clinical trials, its use in humans was discontinued. PCP has since become a drug of abuse which may produce, in some users, a persistent schizophreniform psychosis. Possible explanations for the mediation of such long term effects of the drug include metabolic activation of the parent compound to reactive species which may interact covalently with biological macromolecules and thereby modify their normal function. This hypothesis is consistent with the demonstration of metabolism-dependent covalent binding of radiolabeled PCP *in vivo* and *in vitro* to macromolecules in rodent lung, liver, and kidney.

The electrophilic iminium ion of PCP has been proposed as an intermediate in the metabolism of this compound. Evidence for its formation was provided by the isolation and characterization of the corresponding alpha-aminonitrile after coincubation of PCP and sodium cyanide in a rabbit liver microsomal system. Evidence for the involvement of the PCP iminium ion in covalent binding was provided by the inhibition of binding by concentrations

of cyanide ion which did not inhibit metabolism of PCP. In the present studies, experiments were designed to characterize further the biological fate of PCP by identifying possible macromolecular targets of the reactive metabolite(s). Using rabbit liver microsomal preparations, the effect of PCP on drug metabolizing enzymes was studied.

Incubation of PCP with rabbit liver microsomes resulted in an NADPH-dependent loss of ketamine N-demethylase activity and reduction in microsomal cytochrome P-450 content. This effect was concentration-dependent, exhibited pseudo-first order kinetics, and was irreversible, characteristics of a mechanism-based process. Sodium cyanide, at concentrations which trap the iminium intermediate as the corresponding cyano adduct, antagonized the inhibition of N-demethylase activity by PCP. Furthermore, incubation of synthetic PCP iminium perchlorate ($PCP\text{-}Im^{+}ClO_4^{-}$) with liver microsomes resulted in the loss of N-demethylase activity and reduction in cytochrome P-450 content. These effects also were concentration-dependent, time-dependent (exhibiting pseudo-first order kinetics), and irreversible. Incubation of radiolabeled PCP iminium perchlorate with liver microsomes resulted in covalent binding of radioactive material to macromolecules. While some covalent binding and enzyme inhibition by $PCP\text{-}Im^{+}$ were observed in the absence of NADPH, maximal effects required NADPH in the incubation mixture suggesting that these effects were

mediated by a metabolite or group of metabolites derived from PCP-Im$^+$. PCP-Im$^+$ was metabolized by liver microsomes in the presence of NADPH and this metabolism was inhibited by SKF 525A and carbon monoxide. Using HPLC with radiochemical and diode array UV detection, a metabolite of PCP-Im$^+$ was isolated. The NADPH-dependent formation of this metabolite also was observed upon incubation of PCP with rabbit liver microsomes. Comparison of high resolution EI-MS, NMR, UV, and HPLC characteristics of the isolated metabolite with those of the synthetic standard led to the identification of its chemical structure as 1-(1-phenylcyclohexyl)-2,3-dihydro-4-pyridone.

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INTRODUCTION

Background.

In recent years abuse of phencyclidine [1-(1-phenylcyclohexyl)piperidine, PCP, 1] has been a growing problem in the United States, particularly among adolescents and young adults (Lerner and Burns, 1978; Petersen and Stillman, 1978). The drug is administered usually by smoking, nasal inhalation ("snorting"), or oral ingestion to achieve an experience which apparently is not duplicated by alcohol, marijuana or hallucinogens (Aniline and Pitts, 1982). However, agitated, hostile and aggressive behavior, depressive states, a toxic psychosis, and a large number of deaths including suicides and murders have been associated with PCP abuse (Petersen and Stillman, 1978).

A solution of PCP as the hydrochloride salt originally was marketed by Parke, Davis and Company in the late 1950's as an intravenous anesthetic under the trade name of Sernyl (Munch, 1974). PCP was found to act principally on the central nervous system either by stimulation or by depression (Chen et al., 1959). Signs and symptoms varied considerably according to species of animals and dosage. Hyperactivity was observed more prominently in rats and mice. A certain degree of central depression, as manifested by the loss of muscle coordination of the limbs, also could be detected in these animals at all dose levels. In pigeons, guinea pigs, hamsters, rabbits, cats, dogs, and

monkeys, PCP produced a quieting effect at low doses and an effect ranging from a cataleptoid response to general anesthesia at higher levels. Convulsive seizures occurred in pigeons, guinea pigs, dogs, and monkeys receiving PCP at doses higher than those which produced general anesthesia. In rabbits, cats, hamsters, frogs or fish, however, convulsion or excitement was not noticed with a wide range of doses of PCP. Chronic dosing of PCP in monkeys (Balster and Chait, 1976; Chait and Balster, 1978) and rats (Brocco et al., 1983) was reported to produce tolerance to some of its behavioral effects.

Hepatic mixed-function oxidase activity is enhanced in mice and rats by chronic PCP administration (Radzialowski and Opperman, 1974; Ho et al., 1981) and in vitro metabolism of PCP itself is induced (Kammerer et al., 1984) Chronic treatment of rats resulted in lower PCP concentrations in brain and plasma, higher plasma concentrations of metabolites, and higher ratios of metabolites to free PCP concentrations in plasma compared to levels achieved with acute treatement (Misra et al., 1980). Rats treated with multiple doses of PCP exhibited significantly shorter hexobarbital sleeping times than animals treated with saline (Misra et al., 1980).

When given to humans in doses of 0.25 mg/kg intravenously, PCP produced anesthesia sufficient for minor

and major surgery without notable respiratory or cardiovascular depression (Greifenstein et al., 1958). The apparent terminal half-life of PCP is relatively long and varies fairly widely among individuals. In a study of 16 subjects to whom a 1 mg dose of radiolabeled PCP was administered, the mean half-life was 17.6 hours (Cook et al., 1983). The half-life was greater than 50 hours in 2 subjects and only 7 hours in another (Cook et al., 1983). Because 10% to 20% of the surgical patients developed postanesthetic delirium associated with unmanageable behavior often lasting more than 12 hours (Greifenstein et al., 1958), human use was discontinued. In 1967 the patent was changed to permit the manufacture of the drug in solution by Philips Roxane under the trade name of Sernylan. Phencyclidine hydrochloride solution continued to be used as a first choice anesthetic in primates and for other veterinary anesthetic purposes until discontinuance in 1979 when the drug was reassigned to Schedule II of the Controlled Substances Act. As a result, the two legitimate manufacturers of PCP, Parke-Davis and Philips Roxane, voluntarily withdrew their new animal drug applications, citing inability to comply with Schedule II requirements. Consequently, PCP is no longer commercially manufactured in the United States.

Illicit use of phencyclidine first surfaced in 1965 in California (Lerner and Burns, 1978). In the 1970's PCP use

spread rapidly into all U.S. urban centers and it has become a common form of drug abuse. In 1980 it was estimated that 7 million Americans had tried PCP (Dogoloff, 1980). In 1978 alone, an estimated 310 people died in PCP-related accidents and more than 14,000 persons were admitted for emergency treatment. In terms of actual extent of abuse of a drug, PCP was thought to be exceeded only by the minor tranquilizers (Librium* and Valium*).

Clinical pharmacology and toxicology of phencyclidine.

Acute PCP intoxication presents clinically in at least one of several ways depending on the dose, how recently it has been ingested, and frequency of prior use. Some signs of acute PCP intoxication are violent and bizarre behavior, hypertension, tachycardia, tachypnea as well as apnea, acute organic brain syndrome, lethargy, ataxia, vertical and horizontal nystagmus, assaultiveness or catatonic staring, and generalized anesthesia (Burns and Lerner, 1976; McCarron et al., 1981a) Complications of acute phencyclidine intoxication include grand mal seizures, autonomic dysfunction such as bronchospasm and urinary retention, aspiration pneumonia, muscle rigidity, and rhabdomyolysis (breakdown of muscle associated with excretion of myoglobin in urine) with occasional acute renal failure (McCarron et al., 1981b). PCP also may exhibit the lethal properties of a central nervous system depressant including coma and death. The diagnosis of PCP

overdosage frequently is missed because the presenting symptoms so often closely resemble those of an acute schizophrenic episode (Fauman et al., 1976; Fauman and Fauman, 1977).

Adverse reactions to phencyclidine can be divided into four different categories (Smith and Wesson, 1980). The first, acute PCP toxicity, includes reactions which are a direct result of PCP intoxication. Their onset may be minutes to hours following PCP administration. The second category, PCP toxic psychosis, consists of a prolonged toxic psychosis associated with chronic PCP abuse. The third type, PCP-precipitated psychotic episode, occurs in some individuals when a single dose of PCP may precipitate a psychotic reaction lasting a month or more which appears clinically similar to schizophrenia. The fourth category of adverse reaction is termed PCP-induced depression. Chronic PCP abuse can produce cerebral dysfunction and depressive reactions in many individuals. This may follow any of the previous stages and may last for several weeks.

Several patterns of recovery from acute phencyclidine intoxication exist (Burns and Lerner, 1976). A state of confusion lasting less than 8 hours appears to follow a typical street dose (35-75 mg). Individuals who either smoke greater amounts or take a higher dose orally or by insufflation ("snorting") may present in stupor or coma.

This initial state usually lasts less than 3 hours and is followed by the dose related state of confusion which lasts for 24 to 72 hours. Massive oral "overdoses", involving up to 1 gram of street purchased material, have resulted in periods of stupor or coma of several hours to 5 days in duration, marked by potentially lethal respiratory depression, reiterative seizure activity, and hypertension. The prolonged recovery period with a confusional state may last up to 15 days. In some cases, the phencyclidine confusional state is followed by a psychosis which may persist for several weeks despite abstinence from further PCP and is refractory to pharmacological or supportive treatment (Petersen and Stillman, 1978; Fauman et al., 1976; Burns and Lerner, 1976; Foltz et al., 1980). Development and duration of the psychosis appears to be unrelated to drug dose. One reported patient was comatose for 5 days with a spinal fluid phencyclidine concentration that exceeded the hospital's laboratory standards. Yet this patient was only briefly psychotic (Fauman et al., 1976).

Treatment of the PCP-precipitated psychotic episode consists basically of prevention of injury and reduction of stimuli. During the PCP-related psychotic break, the drug appears to be precipitating an underlying thought disorder rather than producing a direct toxic psychosis, defined as a major break with reality secondary to an intoxicant (Smith and Wesson, 1980). For the PCP toxic psychosis,

many clinicians use a major non-phenothiazine tranquilizer such as haloperidol to avoid the possibility of potentiation of PCP anticholinergic effects by a phenothiazine (Petersen and Stillman, 1978; Smith and Wesson, 1980). However, for the PCP-precipitated psychosis, once the possibility of anitcholinergic drug intoxication has been eliminated, the antipsychotic phenothiazine chlorpromazine is recommended for amelioration of the psychosis as well as for sedation (Luisada, 1978). Alternatively, the use of a non-sedating antipsychotic agent in combination with a sedative-hypnotic, such as diazepam, has been suggested (Luisada, 1978; Smith and Wesson, 1980). The response of PCP psychosis to even the most aggressive treatment is characteristically slow, distinguishing it from paranoid schizophrenia, in which equally agitated patients respond much more rapidly (Petersen and Stillman, 1978; Luisada, 1978).

In experiments carried out before the dangers of PCP were recognized, administration of PCP exacerbated the condition of diagnosed schizophrenics and the subjects appeared to suffer relapses. In fact, all pathologic behaviors were exaggerated with PCP. It was hypothesized that the effect of PCP was to expose a latent psychosis (Fauman et al., 1975). More recently, PCP has been thought to produce a mental state which is difficult to distinguish

from schizophrenia and is more likely to produce this effect in the presence of underlying psychiatric illness (Fauman et al., 1975). Phencyclidine users who become psychotic include schizophrenics, alienated, poorly functioning, or sociopathic persons, as well as those with no evidence of poor premorbid functioning (Fauman et al., 1976). The proportion who suffer severe psychiatric illness appears to be relatively small in view of the common use of PCP. Nevertheless, it is thought that the consistent finding of this illness generally lasting 4-6 weeks suggests some sort of chemical interaction (Fauman et al., 1976).

Drug metabolism and bioactivation.

One possible mechanism by which a chemically stable compound, such as PCP, can exert toxic effects is the metabolic generation of reactive intermediates which may interact with critical biological macromolecules and thereby modify their function. The neurotoxin 6-hydroxydopamine destroys catecholamine neurons (Javoy et al., 1975). Although the precise molecular mechanisms by which 6-hydroxydopamine exerts its neurotoxicity are not well established, it has been suggested that interaction of electrophilic oxidation products with nucleophilic functionalities present on neuronal constituents might be crucial (Saner and Thoenen, 1971; Adams et al., 1972). Several studies have confirmed that oxidation products of

6-hydroxydopamine are covalently bound to proteins of the cytoplasm and axonal membranes in the course of 6-hydroxydopamine toxicity (Jonsson, 1976; Rotman et al., 1976). The extent of covalent binding was found to correlate with toxicity (Jonsson, 1976).

The *in vivo* biotransformation of relatively inert chemicals to highly reactive metabolites, a phenomenon commonly referred to as "metabolic activation", is now recognized or suspected to be an obligatory initial event in several kinds of chemical-induced toxicities (Miller and Miller, 1966; Gillette et al., 1974; Boyd, 1980; Nelson, 1982; Miller and Miller, 1985). Reactive metabolites may be formed by enzymes involved in drug metabolism (Cummings and Prough, 1983). In some cases a single enzymatic reaction is involved, while in other cases several enzymatic and/or spontaneous chemical reactions are involved in the production of an "ultimate" toxic metabolite. Highly reactive, toxic metabolites of xenobiotics may interact with cells in a number of potentially detrimental ways including covalently binding to cellular constituents and stimulation of peroxidation leading to decomposition of cellular lipids (Miller and Miller 1966; Judah et al., 1970; Gillette et al., 1974; Gillette, 1974; Holtzman, 1982). Pioneering studies led to the initial formulation and development of the concept of metabolic activation as a mechanism accounting for the

biological activities of certain types of chemical carcinogens (Miller and Miller, 1966). More recently, attention has also been focused on the role of metabolic activation in the pathogenesis of more acute forms of cell injury, particularly cell necrosis (Potter et al., 1973; Jollow et al., 1973).

Drugs and other chemicals that do not normally occur in the body are metabolized by a wide variety of enzymes. Some of these enzymes are particularly effective in metabolizing xenobiotics while others catalyze normal reactions of cellular metabolism. Most drugs are metabolized to more polar compounds which can be removed rapidly from the body via the kidneys. Some drugs, termed prodrugs, are transformed to therapeutically active metabolites. One example of such a prodrug is phenacetin which is activated to acetaminophen (Flower et al., 1985). The enzymes that catalyze these processes, however, also catalyze the formation of reactive, toxic metabolites from some drugs such as acetaminophen, a hepatotoxin when administered in very high doses (Hinson, 1980; Prescott, 1983). Whether a metabolic reaction produces a detoxification product or a potentially toxic metabolite is determined in large part by structural features of the drug and its metabolites as well as dose of the drug.

Drug metabolism is primarily a hepatic event, although

it occurs to some extent in other organs such as the intestine (Borm et al., 1983), kidney (Orrenius et al., 1973), lung (Bend et al., 1973), placenta (Juchau et al., 1975), skin (Alvares et al., 1973) and brain (Paul et al., 1977; Marietta et al., 1979). The enzymes that catalyze the metabolism of nonpolar xenobiotics are most abundant in the liver (Vessey, 1982). The cytochrome P-450-dependent monoxygenases represent a very important class of drug metabolizing enzymes. Well over half of the drugs metabolized in the liver are oxidized by this family of hemoprotein enzymes (Vessey, 1982).

Cytochrome P-450 is found in many tissues including the adrenal cortex (Cooper et al., 1973), lungs (Bend et al., 1973), skin (Pohl et al., 1976) and kidney (Orrenius et al., 1973), but it is especially highly concentrated in the liver (Ichikawa and Yamano, 1967). The cytochromes are a group of hemoproteins which are responsible for certain forms of electron transport (Lehninger, 1975). Cytochrome P-450, like most cytochromes, is composed of a polypeptide subunit and an iron porphyrin prosthetic group (Haugen et al., 1975). Mammalian liver cytochrome P-450 in reality constitutes a family of isozymes with somewhat different substrate specificities and different sensitivities to induction and to inhibition (Lu and West, 1980). The mixed function oxidase activity of hepatocytes is associated primarily with the membranes of the endoplasmic reticulum

(Brodie et al., 1955; Omura and Sato, 1964a and 1964b). Upon mechanical disruption of the cells, the endoplasmic reticulum fragments and forms small vesicles called microsomes. The microsomes can be isolated from whole cell homogenates by differential centrifugation techniques (Mazel, 1971).

The primary source of electrons for the cytochrome P-450 system is NADPH which transfers electrons via a second enzyme, NADPH-cytochrome P-450 reductase. The second of the two electrons involved in the reaction also can be supplied to cytochrome P-450 via the cytochrome b_5 respiratory chain. This minor pathway contributes to approximately 10% of the overall rate of steady state reduction of P-450 (Hildebrandt and Estabrook, 1971). Microsomal preparations contain the enzymes necessary for cytochrome P-450 metabolism but must be supplied either with NADPH or an NADPH-generating system such as $NADP^+$, glucose-6-phosphate and glucose-6-phosphate dehydrogenase.

Metabolism of phencyclidine.

The metabolism of phencyclidine (PCP) *in vivo* and *in vitro* has been studied extensively (Holsztynska and Domino, 1985-1986). PCP is metabolized mainly by the liver to hydroxylated metabolites which earlier investigators identified in the urine and blood of humans, monkeys, dogs, and rats (Ober et al., 1963; Wong and Biemann, 1975; Wong

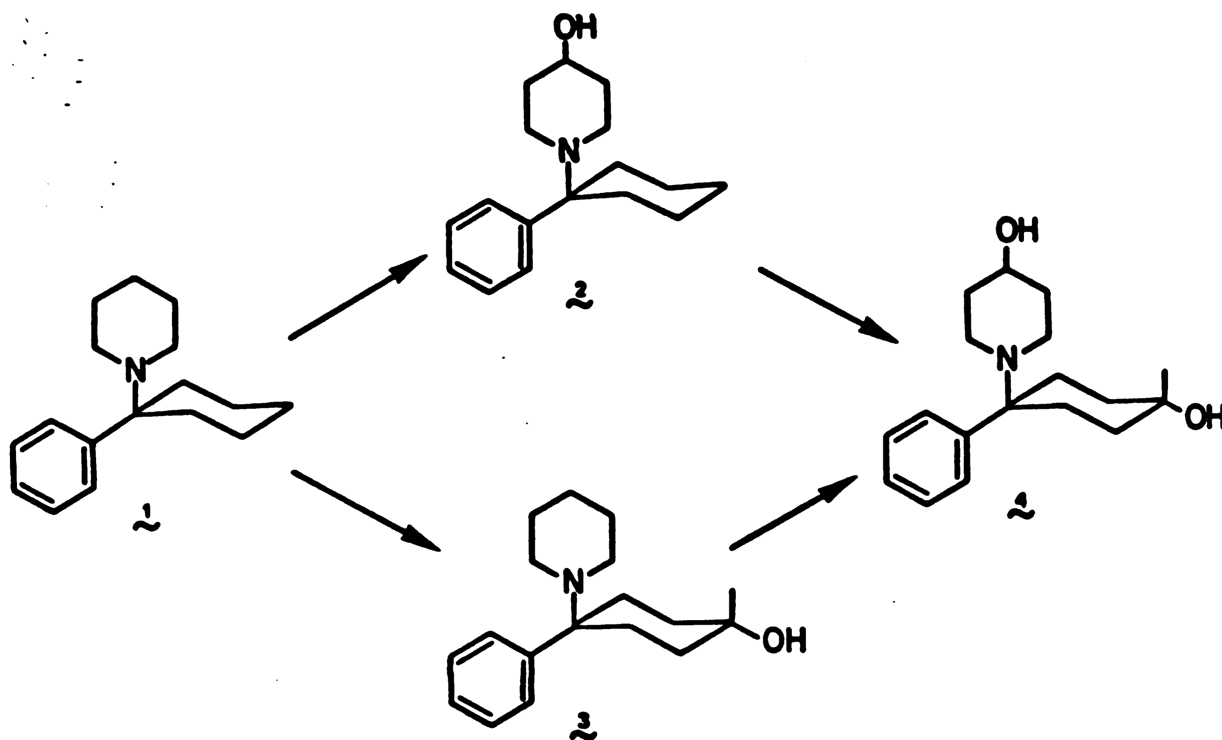


Figure 1. Pathway of metabolic formation of hydroxylated PCP metabolites.

and Biemann, 1976; Lin et al., 1975) as 1-(1-phenylcyclohexyl)-4-hydroxypiperidine (**2**) and 4-phenyl-4-piperidinocyclohexanol (**3**; see Fig. 1). Very small quantities of the 4-phenyl-4-(4-hydroxypiperidino)cyclohexanol (**4**) in which both the cyclohexyl and piperidinyl rings are hydroxylated were detected in urine of monkeys (Lin et al., 1975). These metabolites later were found to be formed in rabbit liver preparations (Kammerer et al., 1981). Further studies have shown that the cyclohexyl and piperidinyl rings may be hydroxylated at the 3 positions as well. Treatment of urine samples with beta-glucuronidase was found to yield higher concentrations of metabolites suggesting that conjugation with glucuronic acid is a pathway in the metabolism of PCP (Wong and Biemann, 1976). A subsequent study showed that in human volunteers, 72.8% of i.v. or orally administered tritium-labeled PCP was excreted in the urine withing 10 days (Cook et al., 1982). Enzymatically hydrolyzable conjugates constituted 31% of urinary radioactivity. Ninety percent of the aglycones were identified as compounds **2**, **3**, and **4** in a ratio of 2.5:6.4:1.

Two piperidine ring-opened metabolites, the amino acid (**5**; see Fig. 2) and the amino alcohol (**6**), were characterized in dog urine and rabbit liver preparations (Baker et al., 1981; Kammerer et al., 1981; Cho et al.,

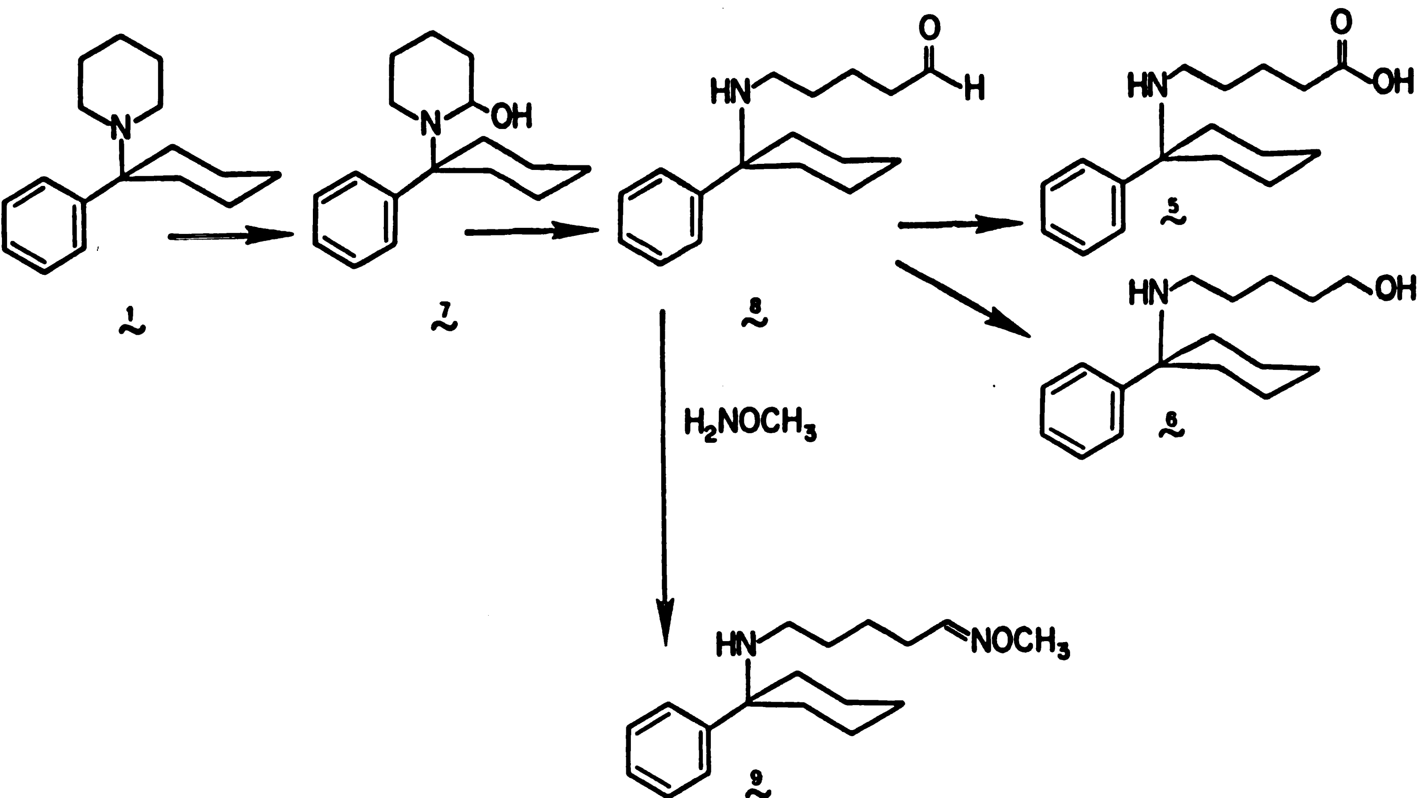
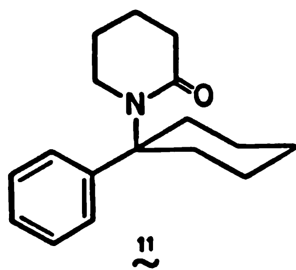


Figure 2. Pathway of metabolic formation of ring-opened metabolites of PCP.

1981). Microsomal as well as soluble enzymes were required for the formation of 5 and 6 in liver preparations. The opening of the piperidine ring was postulated to proceed via oxidation of the carbon atom alpha to the nitrogen atom to form a carbinolamine (7). Spontaneous ring cleavage formed the aldehyde (8) and subsequent oxidation or reduction led to 5 or 6, respectively. Attempts to trap the unstable aldehyde with methoxyamine-HCl led to characterization of the O-methyloxime (9, Hallstrom et al., 1983). The eneamine (10) also was identified in rabbit liver microsomal preparations and was thought to be a work-up elimination product of the carbinolamine (7). DPEA (2,4-dichloro-6-phenylphenoxyethylamine) added to liver preparations inhibited the disappearance of PCP as well as the formation of 2, 3, 4, and 6 (Kammerer et al., 1981). DPEA had been shown to bind both to microsomes and soluble cytochrome P-450 and to inhibit the interaction of many substrates with cytochrome P-450 (Soliman et al., 1974). The inhibitory effect of DPEA suggested the involvement of cytochrome P-450 dependent enzymes in these metabolic reactions. While oxidative metabolism of many piperidines and pyrrolidines produces an amino acid and a corresponding lactam (Hucker et al., 1960; Kaiser et al., 1972), formation of the lactam (11) of phencyclidine does not appear to be a significant pathway in the metabolism of this drug (Baker and Little, 1985).



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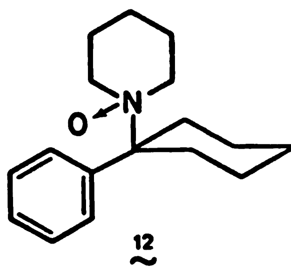

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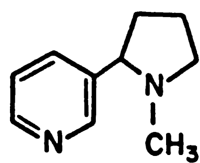

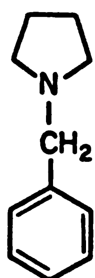

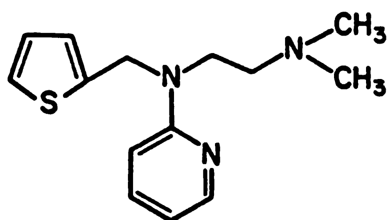

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PCP N-oxide (12) has been reported to be a minor metabolite of PCP in rat (Misra et al., 1979; Misra et al., 1980). However, no unequivocal evidence for its chemical identity has been furnished. Metabolic oxidation of the piperidine nitrogen of PCP to the corresponding N-oxide might be catalyzed by both flavoprotein and cytochrome P-450 dependent monooxygenases (Rose and Castagnoli, 1983). In thorough studies designed to investigate the possible role of 12 in PCP metabolism, Cho et al. (1983) were unable to demonstrate N-oxide formation in liver incubates from rat, rabbit or guinea pig.

Early studies provided evidence of biological oxidation of the aromatic ring of PCP (Wong and Biemann, 1976). The identification of pentadeuterophenol in urine of rats dosed with deutero-PCP (phenyl ring labeled) ring led to the suggestion that an arene oxide might be an intermediate in the oxidative hydroxylation of the phenyl ring of PCP. Reactive arene oxides, intermediates in biological oxidation of aromatic rings, are known to be capable of covalent binding to tissue RNA, DNA, and proteins (Daly et al., 1972).

Consistent with the proposed formation of chemically reactive metabolites, PCP was shown to undergo metabolism-dependent covalent binding to biomacromolecules (Law and Farquharson, 1980; Law, 1981; Ward et al., 1982b)

which apparently was mediated by one or more cytochrome P-450 isozymes (Law and Farquharson, 1980; Ward et al., 1982b). Based on the recent identification of ring-opened metabolites, the electrophilic iminium ion of PCP (PCP-Im$^+$, 13) was proposed as an intermediate in the metabolism of this drug (Ward et al., 1982a and 1982b).

Metabolism and toxicity of tertiary amines.

Generation of iminium ions may occur during the oxidative metabolism of tertiary amines. Iminium ions may be formed by dehydration of initially formed carbinolamines. The formation of carbinolamines has been thought to occur during the oxidation of a number of substituted amines (Testa and Jenner, 1978). Carbinolamines may break down to aldehydes and ketones and also may exist in equilibrium with imines or iminium ions (Beke, 1963). Alternatively, PCP iminium ion formation may proceed via a one electron oxidation of the nitrogen of PCP to produce an aminium radical cation intermediate (14; see Fig. 3; Hanzlik and Tullman, 1982; MacDonald et al., 1982) which would undergo a subsequent one electron oxidation to yield the iminium ion. Studies on the cytochrome P-450 catalyzed oxidative metabolism of (d)-nicotine (15; Murphy, 1973; Nguyen et al., 1979), 1-benzylpyrrolidine (16; Ho and Castagnoli, 1980), and methapyrilene (17; Ziegler et al., 1981) have provided strong evidence for the formation of electrophilic iminium ions through characterization of the

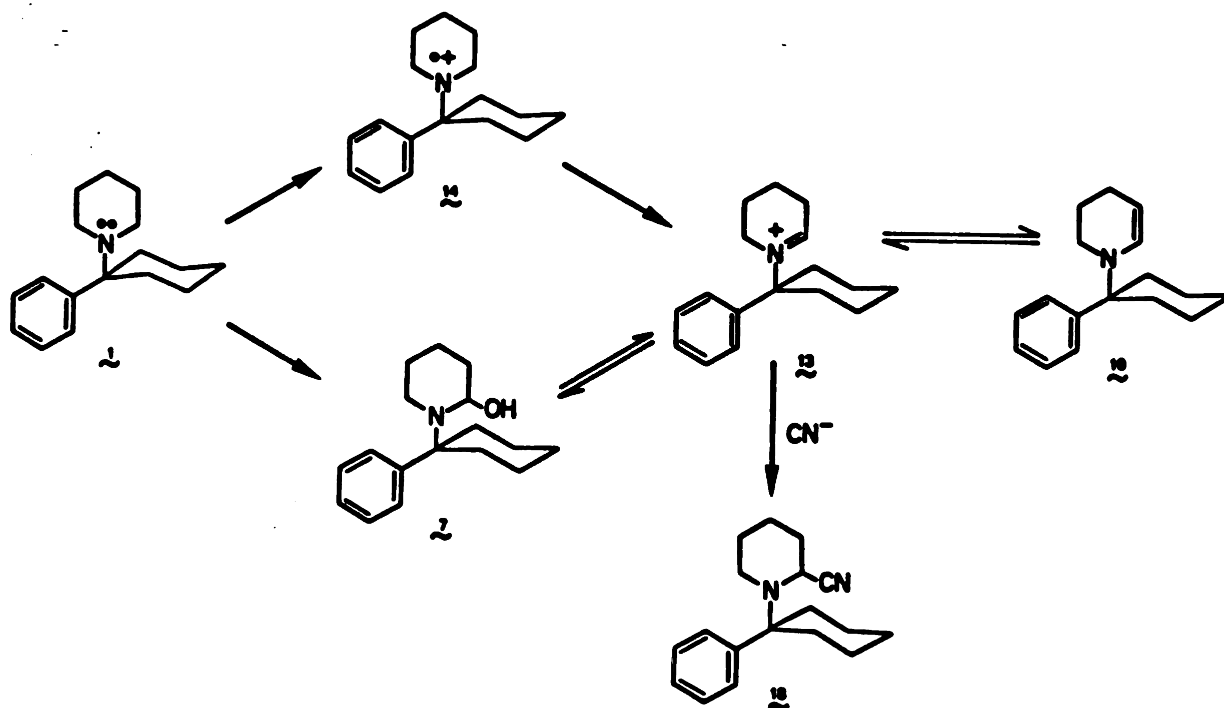
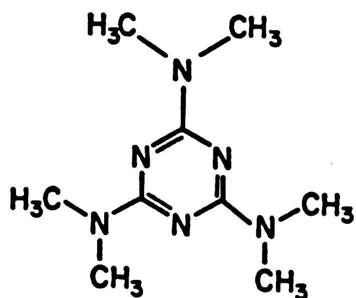


Figure 3. Pathway of metabolic formation of iminium ion metabolite of PCP.

corresponding alpha-cyano adducts upon their _in situ_ reaction with nucleophilic cyanide ion.

Evidence for PCP iminium ion formation and involvement in covalent binding was provided through the use of the nucleophilic cyanide ion. At concentrations which did not affect the oxidative metabolism of PCP itself, the $PCP-Im^+$ was trapped as the corresponding stable alpha-aminonitrile (18) and metabolism-dependent covalent binding was blocked. Based on these results it has been postulated that the metabolic pathways responsible for PCP iminium ion formation and for the formation of reactive intermediates that covalently bind to macromolecules are the same (Ward et al., 1982b).

Reactive, electrophilic iminium ions may be generated by the metabolism of a variety of N-alkylamines and N-alkylamides (Overton et al., 1985). The formation of such iminium intermediates has been proposed to explain the binding of hexamethylmelamine (19) to cellular macromolecules including DNA (Ames et al., 1983) and may be involved in the antibacterial action of taurolin (20, Gidley et al., 1981). Cytochrome P-450 has been shown to catalyze the bioactivation of several tertiary amines (Murphy, 1973; Nguyen et al., 1979; Ho and Castagnoli, 1980; Low and Castagnoli, 1980; Ziegler et al., 1981). Methapyrilene (17), a tertiary amine histamine H_1



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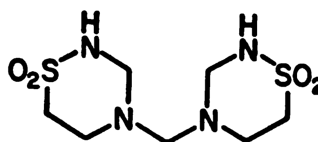

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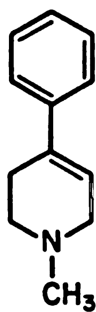

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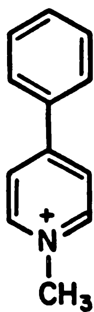

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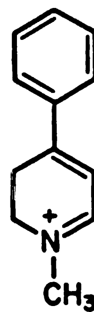

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receptor antagonist, was a constituent of many sleeping aids and antihistaminics until it was identified as a potent hepatocarcinogen in rats (Lijinski et al., 1980; Reznik-Schuller and Lijinski, 1981). Analysis of electron micrographs of rat liver revealed extensive ultrastructural changes in periportal hepatocytes with significant increases in the number of mitochondria. Administration of radiolabeled methapyrilene resulted in a selective concentration of bound activity in the mitochondria of periportal hepatocytes. Electrophilic iminium ions have been trapped with cyanide ion during the metabolism of methapyrilene by rabbit liver microsomal preparations in studies which paralleled the trapping of reactive iminium ion metabolites formed from (d)-nicotine (Murphy, 1973; Ziegler et al., 1981).

More recently, the cyclic tertiary allylamine 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (21, MPTP) has been reported to cause an irreversible parkinsonian syndrome in humans exposed to the substance either as an impurity of an illicit narcotic (Davis et al., 1979; Langston et al., 1983; Wright et al., 1984) or accidentally during synthesis of the chemical (Langston and Ballard, 1983). This compound also causes a movement disorder similar to Parkinson's disease in nonhuman primates which is accompanied by selective destruction of dopaminergic neurons in the substantia nigra and a loss of dopamine

terminals from the striatum (Burns et al., 1983; Langston et al., 1984a). Enzymes present in rat brain mitochondrial fractions were found to catalyze the oxidation of MPTP (Chiba et al., 1984). The structure of a principal mitochondrial metabolite of MPTP was shown to be the 1-methyl-4-phenylpyridinium species (22, MPP^+). This was the same product reported to have been isolated from nigrostriatal structures of monkeys suffering from an MPTP-induced parkinsonian-like syndrome (Markey et al., 1983; Langston et al., 1984a). Further studies on the metabolism of MPTP demonstrated the formation of the 1-methyl-4-phenyl-2,3-dihydropyridinium ion (23, $MPDP^+$), the species previously proposed to be an intermediate in the biotransformation of MPTP to MPP^+ (Chiba et al., 1985) and structurally related to iminium metabolites of phencyclidine and the tertiary amines discussed previously. Pargyline and deprenyl, selective inhibitors of type B monoamine oxidase (MAO-B), were found to be potent inhibitors of the mitochondrial enzymes catalyzing the oxidative metabolism of MPTP (Chiba et al., 1984). Treatment of squirrel monkeys with pargyline prevented both clinical and neuropathological evidence of the neurotoxic effects of MPTP (Langston et al., 1984b).

These findings implicate monoamine oxidase type B in the bioactivation of MPTP to one or more neurotoxic species. The requirement of biotransformation for expression of MPTP

neurotoxicity and the identification of $MPDP^+$ as a metabolic intermediate support long held concepts of our group that tertiary amines may be metabolized by brain enzymes. Biotransformation pathways may lead to formation of reactive species capable of causing biochemical lesions associated with altered CNS function. While the precise mechanism by which MPTP exerts its toxic effects still is not understood, studies on the neurotoxicity of MPTP have provided a clear example of the occurrence of metabolic activation resulting in a biochemical lesion in the brain.

Research proposal.

The structural similarities of PCP and MPTP and formation of iminium ion metabolites lend support to the suggestion that the long term neurotoxicity of PCP also might be accounted for by biochemical lesions via interaction of critical brain macromolecules with metabolically generated reactive intermediates. Since PCP is known to undergo extensive hepatic metabolism, one might theorize that PCP metabolites generated in the liver can be discharged to the general circulation from which they might enter the CNS. Once in the brain, the metabolite(s) might exert toxic effects directly or undergo further metabolism to toxic species which could then act locally. Data from several studies support the view that hepatic metabolism of certain agents can result in the formation of reactive metabolites capable of leaving their site of origin.

Reactive metabolites of bromobenzene (Lau et al., 1982), vinyl chloride (Guengerich et al., 1981), and dimethylnitrosamine (Umbenhauer and Pegg, 1981) appear to be sufficiently stable to efflux from intact hepatocytes and to bind to extracellular protein or DNA. Evidence for the relative biological stability of iminium ions was provided by perfusion studies in which addition of sodium cyanide to the liver perfusate of rats treated with PCP, or other tertiary amines, led to formation of the corresponding cyano adduct (Halldin, personal communication). In addition, evidence has been reported for hepatic formation of reactive naphthalene metabolites and their subsequent export and covalent binding to extrahepatic tissues in vivo (Buckpitt and Warren, 1983). Since the brain contains drug metabolizing enzymes including cytochrome P-450 (Paul et al., 1977) it is also possible that biotransformation of phencyclidine (or a lipophilic, transportable liver metabolite) to toxic species might occur in the brain itself.

While the reported covalent binding of PCP metabolite(s) to tissue macromolecules is consistent with such a proposal, caution must be exercised in the interpretation of these studies with respect to neurotoxicity. Reactive metabolites may be bound to macromolecules which are neither essential to the life of cells nor to their function in the body. Moreover,

metabolite-macromolecule conjugates may be replaced rapidly in the cell by repair mechanisms (Reed, 1985). Furthermore, the mechanism by which a given kind of toxicity may be manifested is usually dependent on a host of other factors in addition to the extent of covalent binding of the reactive metabolite to target macromolecules (Gillette, 1974). Even when the reactive metabolite becomes covalently bound to target macromolecules as well as to nonessential macromolecules, the relative proportion of these metabolite-macromolecule conjugates may vary markedly with the foreign compound and the tissue. Examples of compounds which covalently bind to macromolecules but are not toxic include 3-hydroxyacetanilide and 2-hydroxyacetanilide (Roberts and Jollow, 1978; Roberts and Jollow, 1979), two regioisomers of the hepatotoxin acetaminophen, and p-bromophenol (Lau et al., 1984), a metabolite of the hepatotoxin bromobenzene.

A question which arises, then, is whether the reactive metabolite(s) of PCP responsible for the observed covalent binding are also involved in mediating the chronic neurotoxicity of PCP or whether the metabolic pathway leading to covalent binding is irrelevant to toxicity. In an effort to address this question, studies were designed to evaluate the consequences of covalent binding of PCP with specific target molecules. While it is recognized that the brain is the ultimate site of toxic effects from

PCP, the observation of altered biochemical function in any organ system mediated by one or more reactive metabolites of PCP would indicate that modification of critical biological macromolecules is, in fact, possible. Although such an occurrence in the brain would be dependent on the presence of specific P-450 isozymes and their relative concentrations in the brain, the finding of altered biochemical function in any organ system would encourage further investigation of the likelihood that metabolic activation plays a role in PCP-induced psychosis. Identification of an experimental system in which metabolism-dependent covalent binding of PCP is accompanied by functional changes would be useful to examine any possible association between the two events as well as to characterize the metabolic pathways leading to formation of reactive species. Obvious target molecules for cytochrome P-450-generated reactive metabolites include the components of the cytochrome P-450 enzyme system itself. Although P-450 is present in the brain (Paul et al., 1977), the liver is known to contain an especially high concentration of cytochrome P-450 and the enzyme activity is localized in the microsomal fraction. For this reason, but still recognizing that the isozymes in liver and brain may not be identical, hepatic microsomal preparations were used to examine the effects of PCP on the activity of cytochrome P-450-dependent drug metabolizing enzymes by measuring the rates of metabolism of other substrates after exposure of

the microsomes to PCP.

Since PCP iminium ion formation appears to be critical for generation of reactive intermediates which covalently bind to macromolecules, studies to characterize further the iminium ion pathway were undertaken. Chemical synthesis of $PCP\text{-}Im^{+}$ itself made it possible to examine directly its reactive properties with respect to covalent binding to macromolecules and modification of drug metabolizing enzymes. In addition, the availability of the iminium ion intermediate made feasible studies on its further metabolism.

EXPERIMENTAL METHODS AND MATERIALS

Chemicals.

Potassium chloride, N-2-hydroxyethylpiperazine-2-ethane-sulfonic acid (HEPES), bovine serum albumin, D-glucose-6-phosphate, sodium dithionite, B-$NADP^+$, D,L-cysteine, and tris(hydroxymethyl)aminomethane hydrochloride (Sigma Chemical Company, St. Louis, MO), potassium phosphate (monobasic) and sodium cyanide (Allied Chemical, Morristown, NJ), glutathione (reduced) and glucose-6-phosphate dehydrogenase (Calbiochem-Behring Corporation, La Jolla, CA), sucrose and sodium phenobarbital (Mallinckrodt, Inc., Paris, KY), semicarbazide hydrochloride (Nutritional Biochemicals Corporation, Cleveland, OH), SKF-525A (Research Biochemicals, Inc., Wayland, MA), and L-cysteine ethyl ester (Aldrich Chemical Company, Milwaukee, WI) were obtained from the identified vendors. HPLC grade solvents were obtained from Fisher Scientific Company. All other chemicals were reagent grade.

Phencyclidine hydrochloride was obtained from Philips Roxane, Inc. (St. Joseph, MO). 1-(1-phenylcyclohexyl)-2,3,4,5-tetrahydropyridinium (PCP iminium, PCP-Im^+, 13) as its perchlorate salt was prepared by mercuric acetate oxidation of PCP (Hoag et al., in press). Structural characterization included elemental analysis, NMR, IR, and fast atom bombardment/MS. Tritium labeled 1-(1-phenylcyclohexyl)-2,3,4,5-tetrahydropyridinium

perchlorate (^{3}H-PCP-Im$^+$ ClO_4^-) was synthesized by the same reaction starting with PCP, obtained from the National Institutes on Drug Abuse, which was generally tritium labeled in the phenyl ring (10 mCi/mmol). The product was diluted with unlabeled PCP iminium perchlorate and recrystallized from ethanol. The product was pure, melted at 116-117°C, and showed a single UV absorbing peak on HPLC which coeluted with over 98% of the radioactivity (Fig. 4). The final specific activity was calculated to be 0.415 mCi/mmol. N-benzylcyclopropylamine (BCA) was synthesized as described previously (Bumgardner et al., 1972) and further purified as its hydrochloride salt. Benzphetamine hydrochloride from Upjohn Company (Kalamazoo, MI), ketamine hydrochloride from Warner-Lamber Company (Ann Arbor, MI), and norketamine hydrochloride from Parke, Davis and Company (Detroit, MI) were gifts.

Analytical methods.

Proton NMR spectra were obtained by Dr. P. Caldera-Munoz and E. A. Johnson at 240 MHz with a custom built instrument or a GE 500 MHz, both linked to a Nicolet 1180 computer; chemical shifts are reported in parts per million (ppm) relative to tetramethylsilane in $CDCl_3$ or CD_3CN. Spin multiplicity is given as (s) singlet, (d) doublet, (t) triplet, (q) quartet, or (m) multiplet. A 50x Nicolet FT-IR instrument was used to obtain infrared spectra and a Beckman DU-50 for UV spectra. Low resolution CI mass

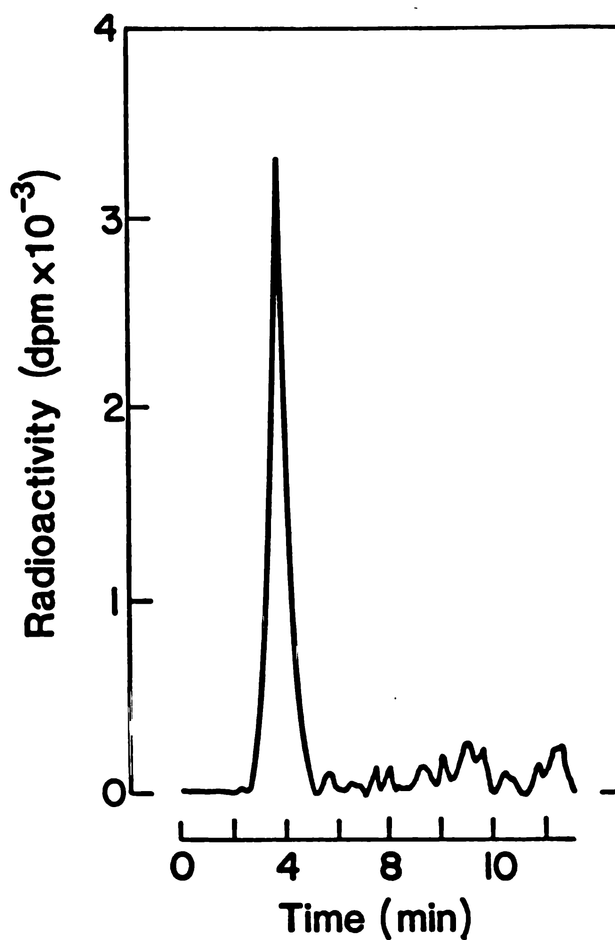


Figure 4. Radiochemical purity of ^{3}H-PCP iminium perchlorate.

Normal phase HPLC separation of $^{3}H\text{-}PCP\text{-}Im^{+}ClO_4^{-}$ (35 nmol) using a radiochemical detector.

spectra were obtained by Dr. T. Shinka using a modified AEI MS 902S at 8 kV with isobutane (ca. 1 torr) as reagent gas. The high resolution EI mass spectrum the biological isolate was obtained by Dr. T. Baillie at the University of Washington using a VG 1170E instrument; the corresponding spectrum of the synthetic sample was obtained by Dr. M. Schmidt-Peetz at the Mass Spectrometry Laboratory of the University of California, Berkeley using a Kratos MS 50S. Microanalyses were performed by the Microanalytical Laboratory, University of California, Berkeley, CA.

Synthesis of 1-(1-phenylcyclohexyl)-2,3-dihydro-4-pyridone (28).

Synthesis of this compound was carried out in collaboration with Dr. M. Schmidt-Peetz.

1-(1,4-dioxa-8-azaspiro[4,5]decane)-cyclohexane-1-carbonitrile (26)

To a cooled mixture (icebath) of 5 g (90mmol) 1,4-dioxa-8-azaspiro[4,5]decane (24), concentrated HCl was added and the pH was adjusted to 3-4. Then 8.8 g (90 mmol) cyclohexanone (25) and a solution of 5.85 g (90 mmol) KCN in 70 ml H_2O were quickly added (Fig. 5). The mixture was allowed to warm up to room temperature and stirring was continued for three hours. The emulsion was extracted with dichloromethane (3 x 80 ml), the combined organic layers

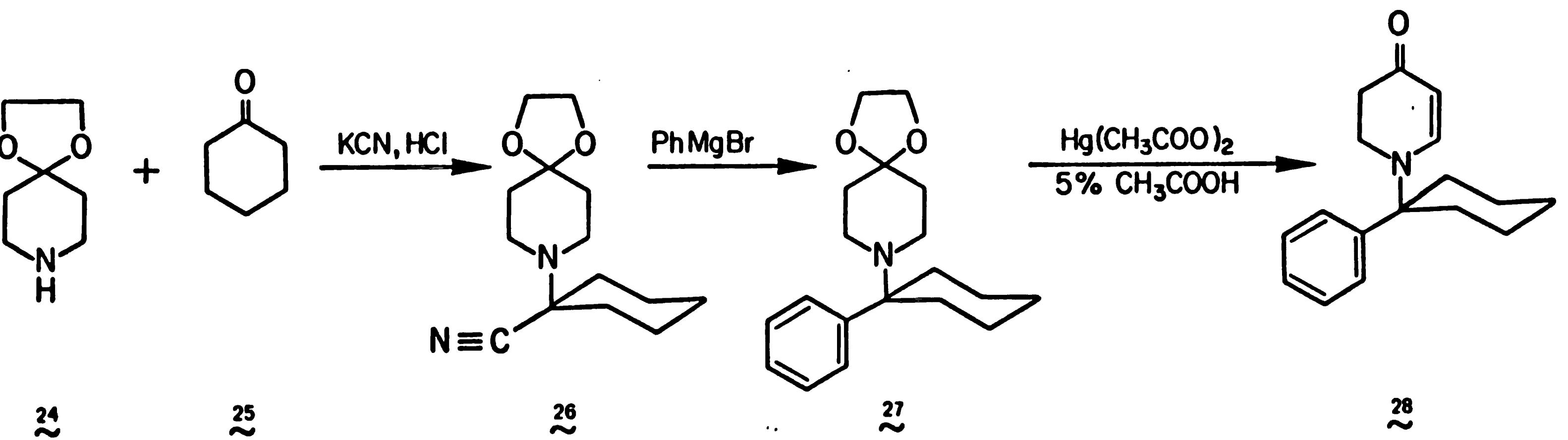


Figure 5. Synthetic pathway for 1-(1-phenylcyclohexyl)-2,3-dihydro-4-pyridone.

were dried with calcium chloride, filtered and the solvent was evaporated under reduced pressure. The oily residue was purified by flash chromatography using a silica column [30 x 200 mm; Kieselgel 60, 70-200 mesh ASTM, Fa. Merck; mobile phase: toluene/ethylacetate (4:3)]. Recrystallization from diisopropylether gave 13.3 g (60.8%) of colorless crystals: mp 108°C. 80 MHz-NMR ($CDCl_3$): δ 1.1-2.5 (m, 10H), 1.22 (t, 4H, 6 Hz), 2.7 (t, 4H, 6 Hz), 3.95 (s, 4H). IR (CCl_4sol.): 2214 cm^{-1}: mass spectra (CI-MS): 224 (MH^+ - HCN). Anal. ($C_{14}H_{22}N_2O_2$) C, H, N.

8-(1-phenylcyclohexyl)-1,4-dioxa-8-aza[4,5]decane (27)

5.5 g (22 mmol) of 26 were dissolved in a mixture of 80 ml toluene/ether (absolute) under vigorous stirring. 11 ml of a 3 M phenylmagnesium bromide solution in ether was added under nitrogen atmosphere. The mixture was refluxed for six hours, cooled in an ice bath and hydrolysed with a saturated ammonium chloride solution. The organic layer was separated and the aqueous phase was extracted twice with ether. The combined organic layers were shaken with ice cold 2 N sulfuric acid (2 x 80 ml) and the aqueous layer was quickly poured in a mixture of 2 N NaOH/ether. The ether was separated and the aqueous layer extracted twice with ether. The combined organic phases were dried over calcium chloride and then filtered. The solvent was

removed under reduced pressure. Recrystallization of the residue from diisopropylether gave 3.54 g (53%) colorless crystals: mp 118°C. 240 MHz-NMR ($CDCl_3$):δ 1.25-1.9 (m, 6H), 1.65 (t, 4H, 5.5 Hz), 2.05 (t, 4H, 5.5 Hz), 2.3-2.47 (m, 4H), 3.87 (s, 3H); mass spectrum (CI-MS): 302 (M+1). Anal. ($C_{19}H_{27}O_2$) C, H, N.

1-(1-phenylcyclohexyl)-2,3-dihydro-4-pyridone (28)

8.3 g (26 mmol) mercuric acetate were suspended in 60 ml 5% acetic acid and heated to 90°C. 2 g of 27 (6.6 mmol) were added. Stirring and heating were continued for 5 hours. The reaction mixture was cooled to 10°C and the gray mercurous acetate precipitate was separated by filtration and carefully washed with 5% acetic acid. The filtrate was saturated with hydrogen sulfide. The precipitate was treated with 5 N NaOH in the cold and extracted four times with dichloromethane. The combined organic layers were dried over calcium chloride and filtered. The solvent then was removed under reduced pressure. Recrystallization of the residue in diisopropylether/ethanol (8:2) gave 380 mg (22.3%) of colorless crystals: mp 122°C. 500 MHz-NMR (CD_3CN): δ 1.36-1.7 (m, 6H), 1.88-2.34 (m, 4H), 2.2 (t, 2H, 7 Hz), 4.85 (d, 1H, 8 Hz, s after decoupling at 7.53), 7.3 (t, 1H), 7.34-7.46 (m, 4H), 7.53 (d, 1H, 8 Hz, s after decoupling at 4.85). UV: 314 nm (CH_3CN). IR (CCl_4-

sol.): 1630, 1564 cm^{-1}. High resolution EI-MS: 255 (M, 17%), 160 (7), 159 (57), 158 (7), 129 (5), 117 (11), 115 (7), 98 (14), 92 (8), 91 (100), 81 (18), 77 (3). Anal. ($C_{17}H_{21}N O$) C, H, N.

Sources of tissue.

The livers of New Zealand White male rabbits and Sprague-Dawley male rats were used for preparation of microsomes. For studies using phenobarbital-treated animals, rabbits received 0.1% (w/v) sodium phenobarbital, pH adjusted to 7.0 with hydrochloric acid, administered in drinking water for 6 days prior to microsomal preparation. Rats were treated by administration of 72 mg/kg/day i.p. sodium phenobarbital in 0.9% (w/v) saline for 3 days. Control rats were injected with the same volume of 0.9% saline. Human liver microsomes were obtained from liver samples of donors in the Stanford University Heart Transplantation Program which were provided through the courtesy of Dr. J. Trudell, Department of Anesthesia, Stanford University Medical Center.

Preparation of liver microsomal fractions.

All buffers were bubbled with nitrogen gas just prior to use. After the animal was euthanized by carbon dioxide asphyxiation each liver was perfused *in situ* with 250 ml for rabbit or 50 ml for rat of ice cold 0.25 M sucrose, 0.05 M Tris buffer, pH 7.4. Livers were homogenized in 3

volumes (w/v) of the same buffer. The homogenate was centrifuged at 10,000g for 20 min. The pellet was discarded and the supernatant was centrifuged at 100,000g for 75 min. The resulting pellet, the microsomal fraction, was resuspended in 0.15 M KCl, 0.02 M potassium phosphate buffer, pH 7.4, and centrifuged at 100,000g again for 75 min. The pellet was resuspended in phosphate buffer and stored under nitrogen at -70°C until use. Prior to incubation, the thawed microsomal suspension was diluted to a concentration of 8-15 mg of microsomal protein/ml and homogenized in phosphate buffer. Protein concentration was determined by the method of Lowry et al. (1951) using bovine serum albumin as the standard.

Metabolic incubations.

Incubation mixtures consisted of 1-2 mg of microsomal protein per ml of 100 mM HEPES buffer, pH 7.6, which contained an NADPH-generating system (composed of 0.5 mM $NADP^+$, 8 mM glucose-6-phosphate, 1 unit/ml glucose-6-phosphate dehydrogenase, and 4 mM magnesium chloride) and 1 mM EGTA. The incubations were carried out under air at 30°C.

Effects of PCP on N-demethylase activity.

Two methods were used to examine the effects of PCP on the N-demethylase activities of liver microsomal preparations. In method A, PCP (0.01-2.00 mM) was

preincubated with microsomes in buffer with the cofactors (+/- $NADP^+$) for a period of 0-60 min. Controls were incubated for the same length of time but did not contain PCP. Ketamine (1 mM) and semicarbazide (5 mM) were then added to the incubation and at this point PCP was added to control samples to correct for any competitive effects of PCP which would occur during the 5-min assay with ketamine. The reaction was terminated after 5 min by addition of 20% (w/v) zinc sulfate. Ketamine (1 mM) was most often the substrate but in some experiments benzphetamine (1 mM) or norketamine (1 mM) was used. Formaldehyde generated by N-demethylation of ketamine or benzphetamine was trapped with the semicarbazide and measured by the Nash modification of the Hanztsch reaction (Nash, 1953). Ketamine and its primary metabolite, norketamine, were analyzed by GLC (Adams et al., 1981) in some instances for comparison with the colorimetric method. This method was also used to examine the hydroxylation of norketamine when it was used as a substrate. In experiments employing GLC analysis, the metabolic reactions were stopped with 0.1 N HCl. Norketamine incubations were carried out for 45 min rather than 5 min. In method B, PCP was similarly preincubated with microsomes for 0-60 min. Control samples were not exposed to PCP during this time but did undergo incubation as well as all subsequent procedures. Metabolic activity was stopped by placing the incubation mixture on ice. The mixture then was centrifuged at 100,000g for 15

min. The microsomal pellet was resuspended in 0.15 M KCl, 0.02 M potassium phosphate buffer, pH 7.4, and again centrifuged for 15 min at 100,000g. The resulting pellet was resuspended in the same buffer and homogenized. This microsomal suspension was used in a 5-min incubation with ketamine as substrate or a 45-min incubation using norketamine as the substrate under the conditions described above.

Effects of PCP iminium perchlorate on N-demethylase activity.

To examine the possible effects of the iminium intermediate of PCP on N-demethylase activity, PCP-Im^+ as its perchlorate salt was incubated with microsomes (1.5-3.0 mg protein/ml) in 0.1 M HEPES buffer, pH 7.6, in the presence or absence of an NADPH-generating system for 0-60 min. The reaction mixture was cooled to 2-4°C, the microsomes sedimented by centrifugation, the microsomal pellet washed with buffer and resedimented before final suspension in fresh buffer. The resulting suspension of washed microsomes was incubated in 0.1 M HEPES buffer, pH 7.6, with ketamine (1 mM) in the presence of semicarbazide (5 mM) and an NADPH-generating system for 5 min. Formaldehyde generated by N-demethylation was measured using the Nash modification of the Hantzsch reaction (Nash, 1953).

Determination of cytochrome P-450 concentrations.

Cytochrome P-450 concentrations were determined using an Aminco DW-2 spectrophotometer by measuring absorbance differences between dithionite-reduced, carbon monoxide-treated samples and an unreduced, carbon monoxide-treated reference sample (Estabrook et al., 1972).

Covalent binding of tritium labeled PCP iminium perchlorate.

To determine the extent of covalent binding to microsomal macromolecules, ^{3}H-PCP iminium perchlorate (0.5 mM, 0.415 µCi) was incubated with microsomes in a total volume of 2.0 ml as described above. The reaction was stopped by the addition of 3 ml of ice cold 3 M TCA. The precipitate was pelleted by centrifugation and washed 3 times with 3 ml volumes of 0.6 M TCA. The precipitate was then washed repeatedly with 3 ml volumes of methanol/water (80:20, v/v) until no further radioactivity could be removed (10 to 15 times). After dissolving the precipitate overnight in 1 ml of 1 N NaOH, 0.5 ml of the resulting solution was neutralized with 0.125 ml of 4 N HCl. Five ml of liquid scintillation fluid was added and radioactivity was counted using a Packard Prias, PLD Tri-Carb liquid scintillation counter. The remainder of the dissolved protein precipitate was used for determination of protein concentration (Lowry et al., 1951).

HPLC analysis.

Metabolism of PCP-Im$^+$ was studied by HPLC analyses of the PCP-Im$^+$ concentration remaining after incubation with microsomes. The reaction was terminated by addition to the incubation mixture of an equal volume of ice cold 0.1 M sodium bicarbonate, pH 8.6 and PCP added as internal standard. Basic and neutral components were extracted into ethylacetate. The concentration of PCP-Im$^+$ in the ethylacetate fraction was determined using a Beckman Model 330 liquid chromatographic system equipped with a 254 nm UV detector and an Altex Ultrasphere-ODS reverse-phase column (5µm particle diameter; 4.6 mm x 25 cm) and an Upchurch Uptight precolumn packed with Alltech C18 pellicular guard column refill. When metabolism of tritium-labeled PCP-Im$^+$ was being studied, a Flow-One Model IC radioactive flow detector for HPLC (Radiomatic Instruments) was used. The mobile phase consisted of acetonitrile/0.1 M acetic acid, pH adjusted to 5.6 with triethylamine (85:15, v/v). The flow rate was 2.0 ml/min. The ratios of PCP-Im$^+$ to PCP peak heights were used to determine PCP-Im$^+$ concentrations. Since the possibility existed that PCP might undergo chemical reduction back to the parent compound, a sample from which PCP was omitted was prepared for HPLC analysis of PCP-Im$^+$. The resulting chromatogram showed no peak with a retention time corresponding to that of PCP, indicating that under the metabolic conditions used, PCP-Im$^+$ was not converted to

PCP and that PCP was a suitable internal standard.

In some cases, HPLC analysis was performed using a Beckman model 114M liquid chromatographic system equipped with the normal phase column and precolumn described above and a Hewlett Packard model 1040A diode-array detector. When metabolism of radiolabeled compounds was being studied, a Flow-One Beta Model IC radioactive flow detector (Radiomatic Instruments) was employed to confirm the purity of the labeled substrate and to detect radiolabeled metabolites.

Purification of PCP iminium ion metabolite.

For purification of the $PCP\text{-}Im^+$ metabolite, PCP iminium perchlorate (0.5 mM) was incubated with microsomes prepared from livers of phenobarbital-treated rabbits (1.5 mg protein/ml) in 0.1 M HEPES buffer, pH 7.6, containing the NADPH-regenerating system and 1.0 mM EGTA. After a 30 min incubation the metabolic reaction was stopped by addition to the incubation mixture of an equal volume of ice cold 0.1 M sodium bicarbonate. Basic and neutral components were then extracted into 1.5 volumes of methylene chloride. The $PCP\text{-}Im^+$ metabolite was separated from other components of the incubation mixture by normal phase HPLC using an Alltech Econosil silica column (5µm particle diameter; 4.6 mm x 25 cm) and an Upchurch precolumn packed with Alltech silica pellicular guard

column refill. The mobile phase was 0.5% propylamine in acetonitrile at a flow rate of 1.5 ml/min. Over repeated injections, the fraction containing the metabolite peak was collected. One day prior to analysis, the mobile phase was evaporated off at room temperature under vacuum. The metabolite was then redissolved in methylene chloride and repurified using the same HPLC system and again collecting the metabolite fraction. The mobile phase was again evaporated off under vacuum. For analysis by NMR the sample was further dried overnight in a dessicator under vacuum to remove any remaining water.

Preparation of brain homogenate and mitochondrial fractions.

Following the method of Roth (1976), male New Zealand White rabbits (2-3 kg) were euthanized by inhalation of carbon dioxide and whole brains were removed and homogenized in 0.32 M sucrose/0.1 M potassium phosphate buffer at pH 7.4 to make a 10% homogenate. In some experiments the whole homogenate was used. To prepare mitochondrial fractions, the homogenate was centrifuged for 10 min at 700g. The supernatant fraction was then centrifuged at 10,000g for 20 min and the resulting mitochondrial pellet was resuspended in the sucrose buffer. Following a second centrifugation at 10,000g for 20 min, the recovered pellet was suspended in 0.1 M phosphate buffer, pH 7.4, and stored at $-20^{o}C$. Protein

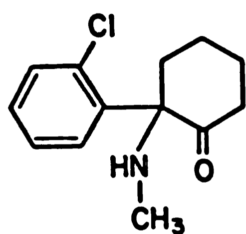
concentrations were determined by the method of Lowry et al. (1951).

BIOACTIVATION OF PHENCYCLIDINE

INTRODUCTION

The observation of metabolism-dependent covalent binding *in vivo* and *in vitro* of PCP to macromolecules (Law and Farquharson, 1980; Law, 1981; Ward et al., 1982b) is consistent with the proposal that the prolonged toxic effects of the drug are mediated by biochemical lesions caused by the interaction of chemically reactive metabolites of PCP with critical biological macromolecules. To investigate this hypothesis in greater depth, studies were designed to evaluate the consequences of covalent binding of PCP to specific target molecules. One of the most obvious targets for cytochrome P-450 generated reactive metabolites is the P-450 system itself. To maintain continuity with previous *in vitro* studies (Law and Farquharson, 1980; Ward et al., 1982a and 1982b), rabbit liver microsomal preparations were used to examine the effects of PCP on the activity of drug metabolizing enzymes. Following exposure of microsomes to PCP, the rates of ketamine N-demethylation (29) were determined. In some cases, other substrates for cytochrome P-450 dependent enzymes were used.

Ketamine (29) is an anesthetic agent which is metabolized extensively by hepatic drug-metabolizing enzymes (White et al., 1982). A major pathway involves cytochrome P-450 mediated N-demethylation of ketamine



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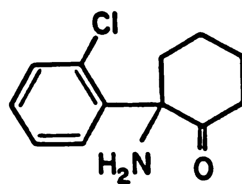

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(Adams et al., 1981) to form norketamine (30) and formaldehyde which can be quantitated by a colorimetric assay (Nash, 1953). Thus, N-demethylase activity can be determined by measuring the rate of formaldehyde formation. The hepatic microsomal N-demethylase was used to investigate whether, under conditions in which covalent binding had been observed, enzyme activity would be modified and whether factors which were known to influence covalent binding also would affect enzyme activity.

RESULTS

Use of ketamine to measure N-demethylase activity.

In order for production of formaldehyde from ketamine to be an accurate estimation of N-demethylase activity, it was necessary for the ketamine concentration to be sufficient to saturate the enzyme. Maintenenance of saturating substrate concentrations would permit the interpretation of changes in the rate of formaldehyde generation as representing changes in enzyme activity rather than differences in substrate availability. To establish the appropriate ketamine concentrations for the experiments to follow, metabolic incubations were carried out using rabbit liver microsomes (1.0 mg protein/ml) and five different concentrations of ketamine, ranging from 0.4-2.0 mM. With the exceptions that PCP was not added and varying concentrations of ketamine were used, the experiment was

carried out as described in the Methods section under the heading "Effects of PCP on N-demethylase activity." The rate of formaldehyde production from the five different initial ketamine concentrations ranged from 19.2-20.7 nmol/mg protein/min. The mean rate was 20.2 with a sample standard deviation of 0.60 nmol/mg protein/min. It appeared, therefore, that the N-demethylase was saturated at all of the concentrations (0.4-2.0 mM) of ketamine that were studied. In a subsequent experiment, concentrations of 0.01-0.40 mM ketamine were incubated with microsomes. From analysis of the rates of ketamine metabolism, the V_{max} was determined to be 53.8 nmol/mg protein/min and the K_m, 0.255 mM (Fig. 6). The concentration of 1.0 mM ketamine appeared sufficient to saturate the enzyme and was used in future experiments in which N-demethylase activity was studied.

Effect of PCP on ketamine N-demethylase activity.

The procedures used in experiments designed to determine the effect of PCP on microsomal N-demethylase activity are described in the Methods section. Briefly, incubation method A involved a 0-60 min period of preincubation of microsomes (1-2 mg protein/ml) and PCP (0.01-2.00 mM) with the necessary cofactors for cytochrome P-450 dependent enzyme activity. Sample controls were preincubated without PCP. Following preincubation, ketamine was added for a 5-min incubation. At this time PCP was added to sample

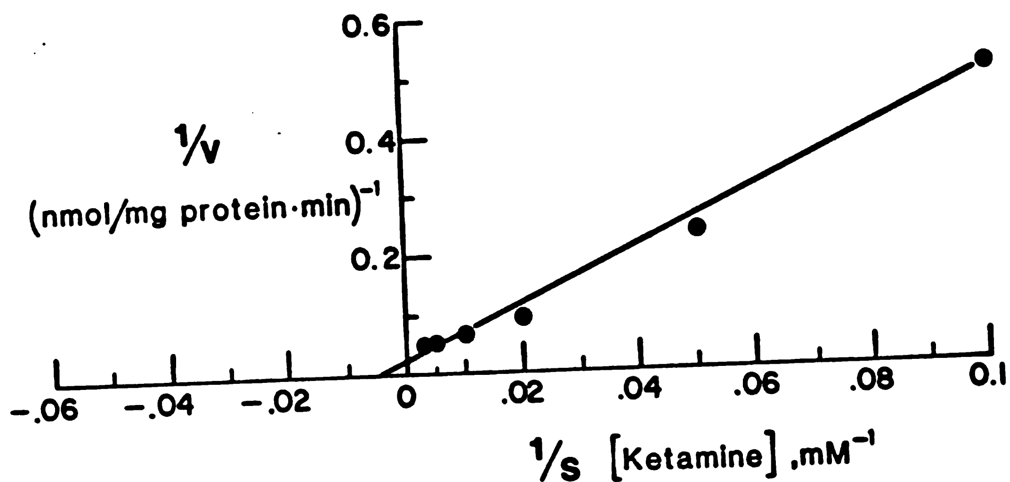


Figure 6. Kinetics of ketamine N-demethylation by rabbit liver microsomes.

Ketamine (0.01-0.40 mM) was incubated with microsomes (1.0 mg protein/ml) for 5 min. The rate of formaldehyde production was determined using the Nash modification of the Hantzsch reaction.

controls to correct for any competitive effects which might occur during the 5-min assay with ketamine. At the end of the ketamine incubation, the metabolic reaction was terminated and the amount of formaldehyde formed during the 5-min interval was determined by a colorimetric assay.

In addition to samples preincubated with PCP (sample 1) and preincubated without PCP but later supplemented with the drug (sample 2), a control sample, to which PCP was never added (sample 3), was included. The rate of formaldehyde generation from sample 3, the experimental control, was designated as 100% enzyme activity. The enzyme activities of sample 1 and sample 2 were expressed as a percentage of the activity of sample 3. The net effect of PCP exposure was expressed as the difference in the N-demethylase activities of samples 1 and 2. The rationale for this analysis was as follows. The presence of PCP during the preincubation period might result in non-competitive effects while the presence of the drug during the 5-min incubation with ketamine might result in a competitive inhibition of N-demethylase activity. In sample 2, PCP was present only during the incubation with ketamine when the possible competitive effects of PCP might result. By subtracting the effects observed in sample 2 from those observed in sample 1, an estimate of non-competitive effects occurring during the period of exposure to PCP was be made.

However, in experiments conducted according to this method (A), the correction for the reversible (competitive) component of PCP inhibition of N-demethylase during the 5-min assay with ketamine as substrate would probably include a certain degree of irreversible inhibition as well. While this should not interfere with measurements of the rate of change in enzyme activity with time, comparisons of the effects of PCP with those of other compounds might be complicated by differences in their competitive and irreversible inhibitory effects. Experiments to establish the NADPH dependence of N-demethylase inactivation by PCP also were complicated by the fact that the presence of NADPH was necessary during the enzyme assay. Therefore, in experiments to compare effects of different substrates, or to establish the NADPH dependence of effects, an alternative method (B) was developed to remove PCP, or other compounds being studied, prior to assessment of the remaining enzyme activity. In this way, the reversible, competitive component of inhibition was separated from the irreversible component. The procedures used for method B, described previously in the Methods section, involved a preincubation period of 0-60 min similar to that in method A. PCP, or the compound to be studied, then was removed from microsomes by washing with phosphate buffer and the remaining enzyme activity of the washed microsomes was subsequently determined.

Experimental controls were preincubated without any substrate but with cofactors and were carried through the identical washing procedures.

In an experiment designed to assess the effectiveness of the washing procedure in removing PCP from microsomes, ^{3}H-PCP (1.0 mM, 0.5 mCi/mmol) was incubated for 30 min at 30°C with rabbit liver microsomes (1.0 mg protein/ml) in either the presence or absence of the NADPH-generating system. Radioactivity in the supernatants from microsomal washes was measured by liquid scintillation counting. Recovery of radioactivity from washes of samples with and without NADPH was 82% and 90%, respectively.

Upon a 30-min incubation with liver microsomes isolated from untreated rabbits and using method B, 1.0 mM PCP caused a small (14%) but significant ($p<0.05$) decrease in ketamine N-demethylase activity (Fig. 7A) compared to a control which was preincubated in the absence of substrate. N-benzylcyclopropylamine (BCA), which has been reported to inhibit liver microsomal enzymes in a metabolism-dependent process (Hanzlik and Tullman, 1982; MacDonald et al., 1982) was used as a positive control. Preincubation with 1.0 mM BCA for 30 min produced marked (69%) inhibition. PCP inhibition of ketamine N-demethylation also was observed in experiments using liver microsomes from untreated rats (30% inhibiton after a 30-min exposure to 1.0 mM PCP) as well as

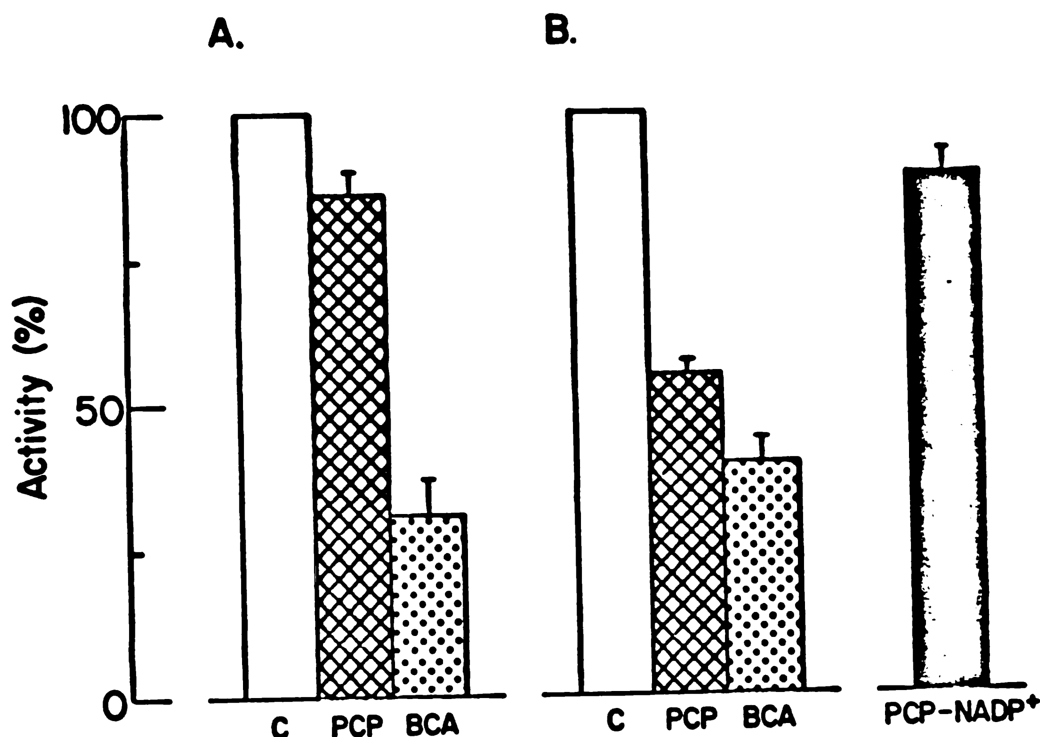


Figure 7. Effect of PCP on ketamine N-demethylase activity.

Liver microsomes from untreated rabbits (A) or phenobarbital-treated rabbits (B) plus NADPH were exposed to PCP (1 mM), BCA (1 mM), or as a control (C), no drug for 30 min in the incubation system described as method B. Percentage activity ± SEM (N=8) is based on control rates of N-demethylation which were 9.1 nmol/mg protein/min in uninduced and 33.6 nmol/mg protein/min in phenobarbital-induced liver microsomes.

human liver microsomes (16% inhibition).

When liver microsomes from phenobarbital-treated rabbits were used, PCP preincubation in the presence of NADPH decreased ketamine N-demethylase activity to 55% of control ($p < 0.001$). In the absence of NADPH, enzyme activity decreased to 89% of control (Fig. 7B). Pretreatment with phenobarbital had no apparent effect on the extent of ketamine N-demethylase inhibition due to BCA. Because phenobarbital pretreatment of the rabbits and rats enhanced the inhibitory effect of PCP on N-demethylase activity, liver microsomes from phenobarbital-pretreated animals were used in subsequent experiments. Experiments conducted by method A, as described above, gave similar results. GLC analysis for ketamine and its N-demethylated product confirmed the results obtained for experiments (method B) analyzed by the Nash colorimetric procedure. An experiment in which benzphetamine was substituted for ketamine as the substrate was carried out and gave a similar result to that obtained with ketamine. Incubation according to method A of 1.0 mM PCP plus NADPH at $30^{\circ}C$ for 30 min decreased metabolism of benzphetamine to 56% of control. To examine the effect of PCP on enzymes catalyzing reactions other than N-demethylation, norketamine (30, the demethylated analog of ketamine) was substituted for ketamine as substrate. The metabolism of norketamine primarily involves hydroxylation at the 4, 5, or 6 position of the

cyclohexanone ring (Adams et al., 1981). Preincubation of microsomes with 1.0 mM PCP using method B resulted in an inhibition of norketamine metabolism to 56% of control activity. When NADPH was omitted from the preincubation with PCP, no decrease in norketamine metabolism was observed.

Characteristics of PCP effect.

Inhibition of rabbit liver microsomal ketamine N-demethylase activity by PCP was concentration-dependent (Fig. 8). Half-maximal inhibition after a 30-min preincubation period was observed at 80 μM PCP and maximal inhibition (ca. 50%) at 0.5 mM PCP. Enhanced loss of ketamine N-demethylase activity was observed when the length of preincubation time with PCP was increased over the period of zero to 60 min. The enzyme inhibition exhibited pseudo-first order kinetics (Fig. 9) and half-maximal inhibition by 1.0 mM PCP occurred at 14.4 min. However, exposure of the enzyme to PCP in the presence of NADPH for up to 60 min failed to completely inhibit its activity even at 1.0 mM, under which conditions the remaining activity was 35% of control.

To determine whether this enzyme (ketamine N-demethylase) inactivation by PCP was saturable, enzymes which already had been exposed to PCP under conditions of metabolism were incubated once again with the drug. PCP

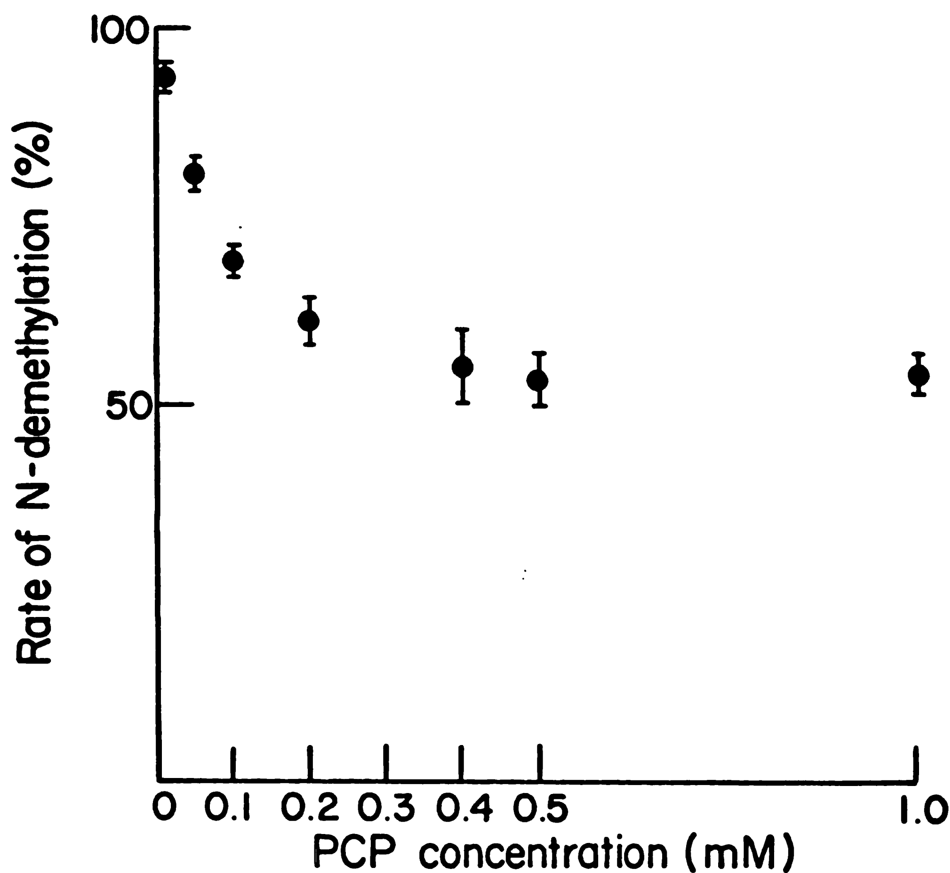


Figure 8. Concentration dependence of PCP effect on ketamine N-demethylase activity.

Liver microsomes from phenobarbital-treated rabbits plus NADPH were exposed in the incubation system described as method A to PCP for 30 min prior to addition of ketamine (1 mM) for 5 min and measurement of N-demethylase activity. N-demethylase activity is expressed as a percentage of samples to which no PCP was added. (N=6)

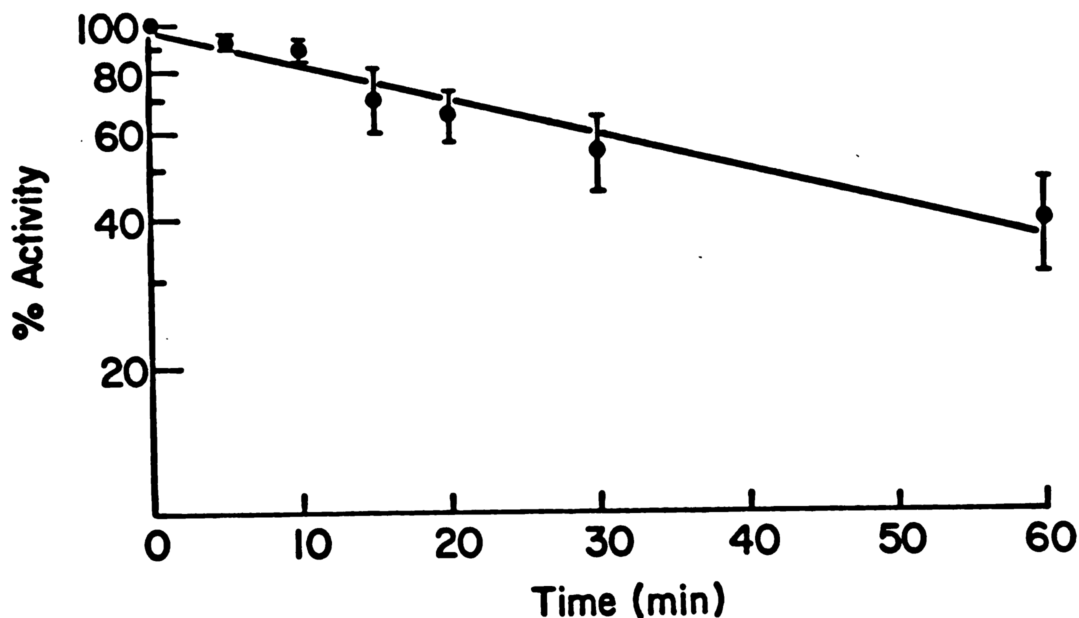


Figure 9. Kinetics of PCP effect on ketamine N-demethylase activity.

Liver microsomes from phenobarbital-treated rabbits plus NADPH were exposed in the incubation system described as method A to PCP (1 mM) for various times prior to addition of ketamine (1 mM) for 5 min and measurement of N-demethylase activity. (N=6)

(1.0 mM) plus NADPH was preincubated with microsomes for 30 min using method B. Under these conditions, maximal inhibitory effects of PCP on N-demethylase activity were observed as in previous experiments. Prior to addition of ketamine to the incubation mixture to assess the remaining N-demethylase activity, the microsomes were again incubated for 15 min with 1.0 mM PCP and NADPH. The activity of microsomal enzymes which had been re-exposed to PCP in the second preincubation was the same as the activity of enzymes which had not been re-exposed. Thus, once maximal inactivation was achieved, no further inactivation could be obtained by subsequent exposure to PCP.

The possibility that a "preferred" substrate, ketamine, would protect against the metabolism-dependent inactivation of N-demethylase by PCP was investigated. Again using the procedure outlined in method B, ketamine (at a concentration of 1.0 mM which saturates the enzyme) and PCP were preincubated together in equimolar amounts in the presence of NADPH for 30 min. Following resedimentation and washing, no loss of ketamine N-demethylase was observed.

Effect of PCP on cytochrome P-450 concentration.

Incubations were conducted by Method A as described in the Methods section. Control samples were incubated in the absence of substrate with or without NADPH. After a period

of preincubation with PCP in the presence or the absence of NADPH, the measurements were made. Cytochrome P-450 determinations were made for each set of samples (for example, PCP with NADPH and without NADPH). At the end of the incubation, carbon monoxide was bubbled through the microsomal suspensions and then three readings were made. The baseline reading was obtained using the PCP without the NADPH mixture in both the reference and the sample cuvettes. Dithionite was then added to the sample cuvette and mixed well and the second reading was made. From measurement of the absorbance difference, the concentration of cytochrome P-450 in the PCP without NADPH sample was determined. Finally, the contents of the sample cuvette were replaced by the PCP with NADPH mixture which had been exposed to carbon monoxide and reduced with dithionite. From this absorbance reading, the cytochrome P-450 concentration in the PCP with NADPH mixture was determined using a millimolar extinction coefficient of 100.

Cytochrome P-450 concentrations after incubation of microsomes without substrate were the same for samples containing NADPH and those without NADPH. Measurements of cytochrome P-450 concentrations indicated a metabolism-dependent loss of the chromophore of cytochrome P-450 after exposure to PCP. Incubation of 0.2 mM PCP with microsomes plus NADPH for 30 min at 30°C produced a 20-30% decrease in cytochrome P-450 concentration as

Table 1

Effect of nucleophiles on ketamine N-demethylase activity

	Nucleophile concentration (mM)	N	Control activity (%)	Activity after 0.2 mM PCP (%)
None		20	100	52±6
GSH	1.0	10	95±2	56±1
D,L-cysteine	1.0	2	92	58
Cysteine ethyl ester	0.4	4	94	47*
Cysteine ethyl ester	1.2	2	85	43*
Cysteine ethyl ester	2.0	2	67	34*
NaCN	0.5	10	87±7	68±20
NaCN	0.2	8	97±5	86±10
NaCN	0.1	8	97±3	85±5
NaCN	0.02	2	96	75
NaCN	0.004	2	100	59
NaCN	0.001	2	100	53

Microsomes from livers of phenobarbital-treated rabbits were incubated with or without PCP [0.2 mM or 0.4 mM(*)] in the presence of NADPH plus nucleophiles as described by method B. Ketamine then was added and N-demethylase activity determined. N-demethylase activity is expressed as a percentage (±SEM) of control rates when no nucleophile was present.

Table 2

Effect of cyanide ions on loss of microsomal cytochrome P-450

Incubation	nmol P-450/mg protein -NADPH (control)	nmol P-450/mg protein +NADPH	% of control	% of no substrate
no substrate	3.77	3.77	100	100
PCP (0.2 mM)	3.81	3.17	83	84
CN- (0.2 mM)	3.61	3.25	90	86
PCP + CN-	3.73	3.45	93	92

Liver microsomes from phenobarbital-treated rabbits were incubated for 30 min prior to determination of cytochrome P-450 concentration. (N=4)

compared to controls. No decrease was observed when NADPH was absent from the incubation.

Effects of nucleophiles.

Sodium cyanide added to the preincubation mixture antagonized the inactivation of ketamine metabolism by PCP (Table 1). The protective effect of sodium cyanide was concentration-dependent, ranging from no observable effect at 4 μM NaCN to maximal protection at 100 μM NaCN. Since concentrations of sodium cyanide greater than 500 μM inhibited metabolism of phencyclidine (Ward et al., 1982b), possible protective effects at higher concentrations could not be determined. Glutathione (GSH), at concentrations up to 10 times that of PCP, which did not inhibit ketamine metabolism, failed to protect against the loss of N-demethylase activity caused by PCP. Neither cysteine, cysteine ethyl ester, nor N-acetylcysteine demonstrated any protective effects (Table 1). Sodium cyanide coincubated with PCP (0.2 mM) in an equimolar concentration for 30 min with NADPH prevented the loss of microsomal cytochrome P-450 content (Table 2).

DISCUSSION

The results of these experiments provide evidence that PCP, which is known to be metabolized to reactive intermediates capable of covalently binding to tissue

proteins, can cause changes in biochemical functions and this effect of PCP is dependent on its metabolism.

The washing procedure used to remove PCP from liver microsomes prior to measurement of enzyme activity was unable to remove 10-20% of the PCP. If the initial concentration was 1.0 mM, the concentration of PCP still present in the microsomes would be 0.1-0.2 mM. The presence of this concentration of PCP in a 30-min incubation would lead to a 30-40% inhibition of ketamine N-demethylase activity. The contribution of this residual PCP to the inhibition of N-demethylase during the 5-min incubation with ketamine was not determined. Lipophilic molecules, such as PCP, which may partition into the microsomes can be removed more effectively by the addition of bovine serum albumin to the phosphate buffer wash (Correia et al., submitted). This technique should be used to examine the reversibility of the PCP effect on microsomal ketamine N-demethylase.

When NADPH was absent during the microsomal incubation with PCP (1.0 mM), 88.8% of ketamine N-demethylase activity was recovered after washing with buffer. When NADPH was present, only 55.1% of the enzyme activity was recovered. (It should be noted that the incubation period for determination of N-demethylase activity was only 5 min rather than 30 min.) The observation that the loss of

N-demethylase activity after exposure of microsomes to PCP was recovered to a greater extent when NADPH was not present during the PCP incubation suggests that partitioning of PCP into the microsomes alone is not sufficient to explain the persistent inhibitory effect. Nevertheless, the use of bovine serum albumin to remove PCP from microsomes would have been preferable for examining the reversibility of the PCP effect on microsomal ketamine N-demethylase activity. In further discussion of the results of experiments in which microsomes were washed with buffer prior to assessment of enzyme activity, the term "irreversible inhibition" will refer to the failure to recover enzyme activity after washing the microsomes.

Exposure of the hepatic microsomal enzyme system to PCP produced an inhibition of cytochrome P-450-dependent N-demethylase activity. Loss of activity was dependent on PCP concentration and occurred at concentrations which are physiologically relevant. At 1 hr after i.p. administration of 1.8 mg/kg PCP to rats, the concentration of drug in liver is reported as 25 µM (Law, 1981) which is within the range of concentrations at which inhibition of N-demethylase activity _in vitro_ was observed. Chronic administration of PCP to rats has been shown to cause significant increases in the rate of metabolism of hexobarbital, aminopyrine, and zoxazolamine by rat liver 10,000g supernatant fraction (Radzialowski and Opperman,

1974). In mice, significant increases in barbiturate and aniline hydroxylase activities, N-demethylase activity, and NADPH-cytochrome c reducatase levels were reported after chronic PCP administration (Ho et al., 1981). When rats were chronically pretreated with PCP, the *in vitro* metabolism of PCP in liver incubates was increased by approximately 50% during either a 30-min or a 60-min incubation (Kammerer et al., 1984). However, the effect of PCP administration *in vivo* on the rate of ketamine N-demethylation remains to be studied.

As indicated by the requirement for NADPH, the metabolism dependence exhibited by covalent binding of PCP to tissue proteins (Law and Farquharson, 1980; Ward et al., 1982b) also was apparent in the inhibitory effect on N-demethylase activity. The inhibition exhibits several characteristics of mechanism-based enzyme inactivation including NADPH dependence, pseudo-first order kinetics, and irreversibility. Similar characteristics have been reported for the inhibition of cytochrome P-450 by cyclopropylamines (Hanzlik and Tullman, 1982; MacDonald et al., 1982). The inhibitory effect of PCP on N-demethylation of benzphetamine and hydroxylation of norketamine is an indication that other P-450 catalyzed reactions are affected as well.

Inability to inhibit completely enzyme activity is

consistent with evidence for the multiplicity of species of mammalian cytochrome P-450 (Lu and West, 1980) and suggests that certain isozymes of P-450 are susceptible to the inhibitory effect while others are not. Phenobarbital pretreatment of rabbits induces formation of form 2 (also called LM_2 and $P-450_1$) to the extent that it becomes the major species of cytochrome P-450 in liver microsomes of the animals. Enhancement of the PCP effect by phenobarbital pretreatment of animals implies that form 2 may be a species of P-450 susceptible to PCP inhibition. In fact, Osawa and Coon (1986) have reported recently that cytochrome P-450 isozyme 2 is inactivated selectively by PCP in a mechanism-based process. In addition to these characteristics, coincubation of ketamine, which is assumed to be a preferred substrate for N-demethylase, with PCP protected the enzyme(s) from the inhibitory effect of PCP. Although the specificity of ketamine as a substrate for isozyme 2 is not known, this result is consistent with the idea that enzymes which catalyze the demethylation of ketamine also can metabolize PCP to species which then can inactivate the enzyme itself.

Iminium species are known to react readily with cyanide ion to generate the corresponding alpha-cyanoamines which are relatively stable compounds (Murphy, 1973; Nguyen et al., 1979; Ho and Castagnoli, 1980; Ziegler et al., 1981). $PCP-Im^+$, formed in the oxidative metabolism of PCP, also

reacts with cyanide ion to generate the corresponding alpha-aminonitrile which appears to be stable (Ward et al., 1982a and 1982b). Sodium cyanide, added to microsomal incubation mixtures, blocked the metabolism-dependent covalent binding of PCP to microsomal proteins (Ward et al., 1982b). In an attempt to protect N-demethylase against the inhibitory effect of PCP, sodium cyanide was coincubated with the drug. The protective effect of NaCN, at concentrations which inhibited neither the metabolism of PCP nor of ketamine, against inactivation of the enzyme provides evidence that the metabolic pathways responsible for iminium ion formation and the formation of reactive intermediates which can bind covalently to macromolecules as well as inactivate cytochrome P-450 dependent enzymes are similar and possibly identical.

Glutathione was ineffective in antagonizing the effect of PCP on ketamine N-demethylation despite its inhibitory effect on the metabolism dependent covalent binding of radiolabeled PCP to microsomal proteins (Ward et al., 1982b). A similar observation has been reported in the case of BCA inactivation in which glutathione decreased covalent binding of radiolabeled BCA to liver microsomal proteins but had no effect on its inhibition of cytochrome P-450 dependent aminopyrine N-demethylase (Hanzlik and Tullman, 1982). It has been suggested that the reactive species generated by the enzyme can diffuse from the active

site and subsequently react with glutathione or other protein groups in reactions which do not lead to enzyme inactivation (Hanzlik and Tullman, 1982). Other nucleophiles such as cysteine and cysteine ethyl ester also failed to inhibit the effect of PCP. It is possible that the small, nucleophilic cyanide ions have better access to the active site. They might interact with reactive species proximal to the site of formation and thereby block their interaction with functional groups which could otherwise lead to enzyme inactivation.

The protective effect of sodium cyanide, while suggesting that the iminium ion of PCP is involved in the irreversible inhibition of N-demethylase activity, indicates that phencyclidine is not a true suicide enzyme inactivator. In examining a potential suicide inhibitor, loss of enzyme activity must be shown to be a time-dependent, first-order process. Time dependence provides good, but not definitive, evidence that covalent modification has taken place. Demonstration that the loss of enzyme activity, at constant inactivator concentrations, is first-order provides evidence that inactivation occurs before the inactivator is released from the enzyme, a fundamental property of suicide inhibition. Usually this distinguishes the inactivation process from cases in which the enzyme converts a substrate to a reactive species which, on release from the active site, reacts with the

enzyme from solution (Abeles, 1978; Walsh et al., 1978). Formation of such an intermediate may be detected by adding nucleophiles which trap the intermediate prior to its reaction with the enzyme. Such enzyme-generated inactivators generally are electrophiles and, therefore, may be trapped by appropriate nucleophiles. The experimental consequence is that addition of the nucleophile reduces the rate of inactivation. If true suicide inactivation occurs, the added nucleophile should not alter the inactivation rate. In the case of phencyclidine, the first-order loss of N-demethylase activity suggests this is a suicide inactivation process. However, the protective effect of sodium cyanide on N-demethylase activity indicates that phencyclidine is not itself a true suicide substrate. The possibility still exists that a metabolite of PCP, such as the iminium ion or an iminium ion metabolite, is a true suicide substrate for the N-demethylase. Experiments to examine the potential of PCP-Im^+ to inhibit cytochrome P-450-dependent ketamine N-demethylase activity will be discussed in the next chapter.

The inactivation of microsomal N-demethylase activity by a metabolism-dependent process which was not reversible by washing the microsomes with phosphate buffer suggests that a reactive metabolite of PCP interacts with one or more components of the hepatic microsomal drug metabolizing

system, thereby causing inactivation. Protein components which may be involved in such an interaction include components of the cytochrome P-450 system such as cytochromes P-450, cytochrome P-450 reductase, and cytochrome b_5. The metabolism-dependent loss of the chromophore of microsomal cytochromes P-450 revealed by the spectral data, along with the reports of metabolism-dependent covalent binding of PCP to microsomal proteins, prompts the suggestion that functional groups on protein components of the cytochrome P-450 system may represent target sites for a reactive metabolite of PCP.

The loss of only 16% (Table 2) of microsomal cytochrome P-450 content after a 30-min exposure to 0.2 mM PCP while ketamine N-demethylase activity decreased by 48% (Table 1) suggests that the isozymes which contribute to ketamine N-demethylation represent a fraction of the total P-450 population and of those, PCP is inactivating only a portion. When these isozymes are inactivated by PCP, significantly reducing N-demethylase activity, the unaffected P-450 isozymes remain and hence, the total P-450 concentration does not change dramatically.

Results of these experiments provide evidence that the previously observed metabolism-dependent covalent binding of PCP to tissue proteins, indeed, is accompanied by functional changes. Namely, hepatic microsomal drug

metabolizing enzyme activity is inhibited by PCP, also in a metabolism-dependent process. The loss of enzyme activity is accompanied by a decrease in microsomal cytochrome P-450 concentration suggesting that components of the P-450 system might represent targets of irreversible inactivation for the reactive metabolites. It appears, then, that reactive species generated by oxidative metabolism of PCP are capable of binding to macromolecules including cytochrome P-450. Interaction of the reactive metabolite(s) with P-450 appears to result in a destruction of the P-450 chromophore and loss of cytochrome P-450 enzyme activity. The protective effect of cyanide ion against enzyme inhibition and loss of P-450 as well as covalent binding is evidence that PCP-Im^+ is an obligatory intermediate in the bioactivation pathway of PCP.

BIOACTIVATION OF PHENCYCLIDINE IMINIUM ION

INTRODUCTION

Incubation of phencyclidine with rabbit liver microsomes results in metabolism-dependent covalent binding to protein (Law and Farquharson, 1980; Ward et al., 1982b) which is accompanied by a loss of liver microsomal cytochrome P-450-dependent N-demethylase activity and destruction of cytochrome P-450. Antagonism of these effects by cyanide ion, which is known to trap the iminium ion metabolite of PCP to form the stable alpha-aminonitrile, implicates PCP-Im^+ in the mediation of PCP-induced effects. The experiments discussed in this chapter represent an effort to gain a better understanding of the actual role of this intermediate in the bioactivation pathway of PCP. A question which immediately comes to mind is whether PCP-Im^+ can itself produce the same effects observed with PCP and, if so, whether it is the ultimate reactive metabolite of PCP responsible for the observed irreversible effects of the drug. Chemical synthesis of the phencyclidine iminium ion and its tritium labeled analog (Hoag et al., in press) made it possible to examine directly its reactive properties. The rabbit liver microsomal system was used again, in experiments which paralleled those carried out with PCP, to study the possible effects of PCP iminium perchlorate with respect to covalent binding and microsomal N-demethylase activity.

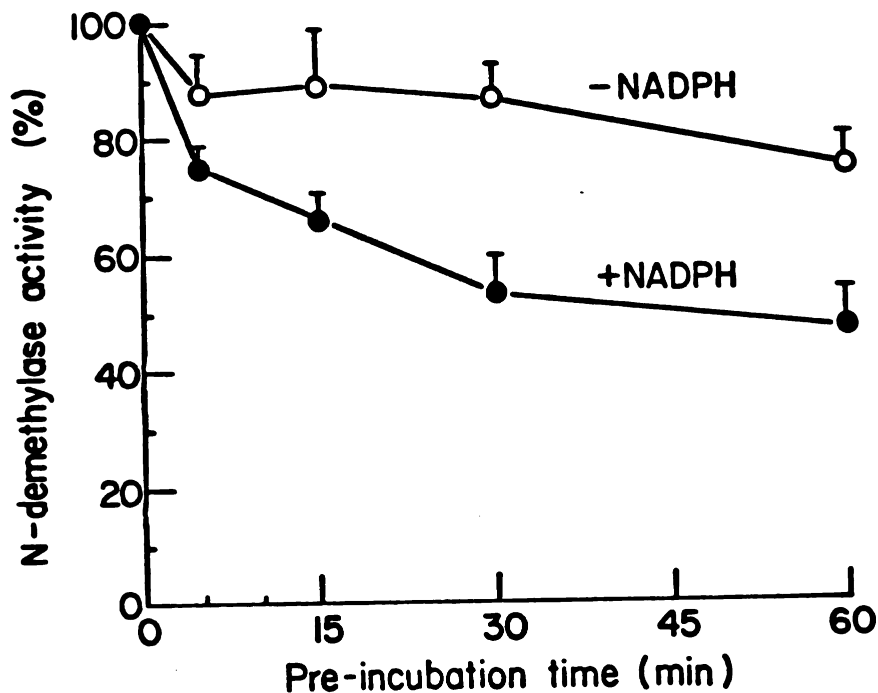


Figure 10. Effect of PCP-$Im^+ClO_4^-$ on ketamine N-demethylase activity.

Liver microsomes from phenobarbital-treated rabbits were preincubated according to method B with PCP-Im^+ (0.5 mM) either in the presence or absence of NADPH for 0-60 min. Following the washing procedure, microsomal N-demethylase activity was measured. Rates of N-demethylation are expressed as percentages of the activity of microsomes preincubated for 0 min with PCP-Im^+. For microsomes plus NADPH, the activity was 17.35±3.80 nmol/mg protein/min after 0 min preincubation with PCP-Im^+ (the incubation was cooled immediately upon addition of PCP-Im^+). For microsomes minus NADPH, the activity was 21.80±3.24 nmol/mg protein/min. (N=4)

RESULTS

Effects of phencyclidine iminium ion on ketamine N-demethylase activity.

Phencyclidine iminium perchlorate was synthesized as described earlier in the Methods section. Liver microsomes prepared from phenobarbital treated rabbits were preincubated with $PCP\text{-}Im^{+}$ (0.5 mM) for 0 to 60 min. The $PCP\text{-}Im^{+}$ was then removed from the microsomes as described for method B and the washed microsomes were used to assess remaining ketamine N-demethylase activity. Control samples (preincubated for 0 min) were cooled to 2-4°C immediately upon addition of $PCP\text{-}Im^{+}$. Preincubation of microsomes with $PCP\text{-}Im^{+}$ for 60 min in the presence of NADPH resulted in a maximum decrease in ketamine N-demethylase activity of 56% from the value obtained after 0 min preincubation with $PCP\text{-}Im^{+}$. In the absence of NADPH, $PCP\text{-}Im^{+}$ decreased enzyme activity by 28% from the initial value (Fig. 10). Both types of inhibitory effect (with and without NADPH) were dependent on preincubation time and $PCP\text{-}Im^{+}$ concentration. Neither type of inhibition was reversed by removal of the $PCP\text{-}Im^{+}$ by sedimentation and washing of the microsomal preparation. Because complete enzyme inhibition could not be achieved, the level of maximum inhibition observed at 60 min was set equal to 100% N-demethylase activity. Fifty percent of that inhibition occurred at a $PCP\text{-}Im^{+}$ concentration of approximately

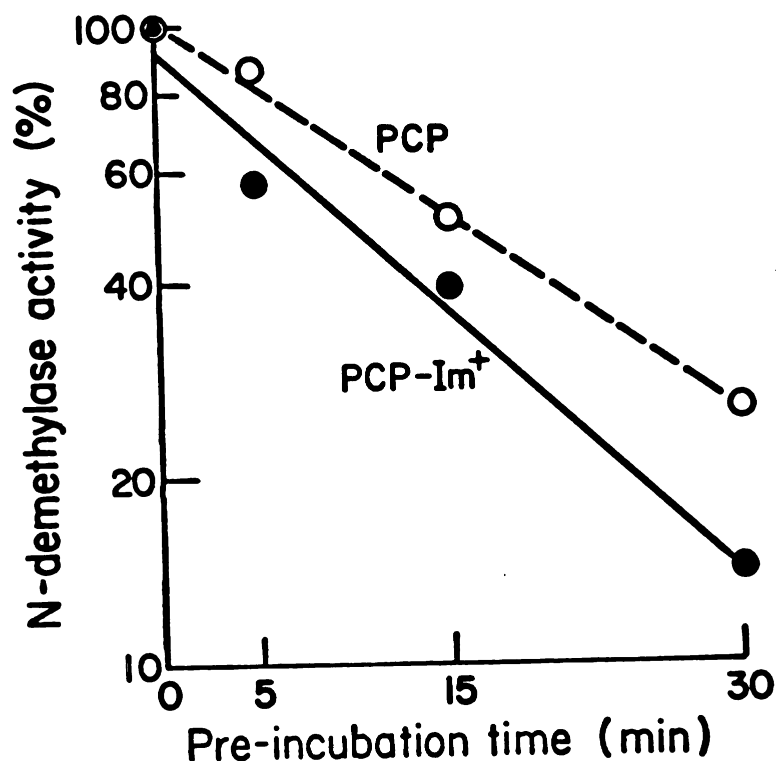


Figure 11. Kinetics of PCP-$Im^+ClO_4^-$ effect on ketamine N-demethylase.

Liver microsomes from phenobarbital-treated rabbits were preincubated for 0-30 min in the presence of NADPH and PCP-Im^+ (0.5 mM) or PCP (1.0 mM) and remaining microsomal N-demethylase activity was measured. N-demethylase activity is expressed as a percentage of that which could be inhibited at 60 min by PCP-Im^+ (0.5 mM) and PCP (1.0 mM), 56% and 60%, respectively. (N=4)

50 µM. NADPH-dependent enzyme inhibition exhibited pseudo-first order kinetics (Fig. 11), typical of a mechanism-based enzyme inactivation process (Walsh et al., 1978).

Effect of phencyclidine iminium ion on cytochrome P-450.

Incubation of PCP-Im^+ with liver microsomes led to a loss of microsomal cytochrome P-450 content which was dependent on NADPH, PCP-Im^+ concentration, and incubation time (Figs. 12A and 12B). Up to 30% loss was observed with 0.5 mM PCP-Im^+ after 30 min at 30°C. No loss of cytochrome P-450 content was observed in the absence of NADPH.

Covalent binding of radiolabeled phencyclidine iminium ion.

Incubations of tritium labeled PCP iminium perchlorate with liver microsomes in the absence of NADPH resulted in time-dependent covalent binding of radioactive material to microsomal proteins (Fig. 13). With NADPH present, covalent binding was increased by 250-300% after a 60-min incubation. The NADPH-dependent binding after 60 min represented 5.5 nmol/mg protein of PCP-Im^+ equivalents. Covalent binding of PCP (0.1 mM) to microsomal protein after a 30-min incubation was 3.85 nmol/mg (Ward et al., 1982b). Because the experimental conditions were different for PCP and PCP-Im^+, comparison of the extent of covalent binding was not possible.

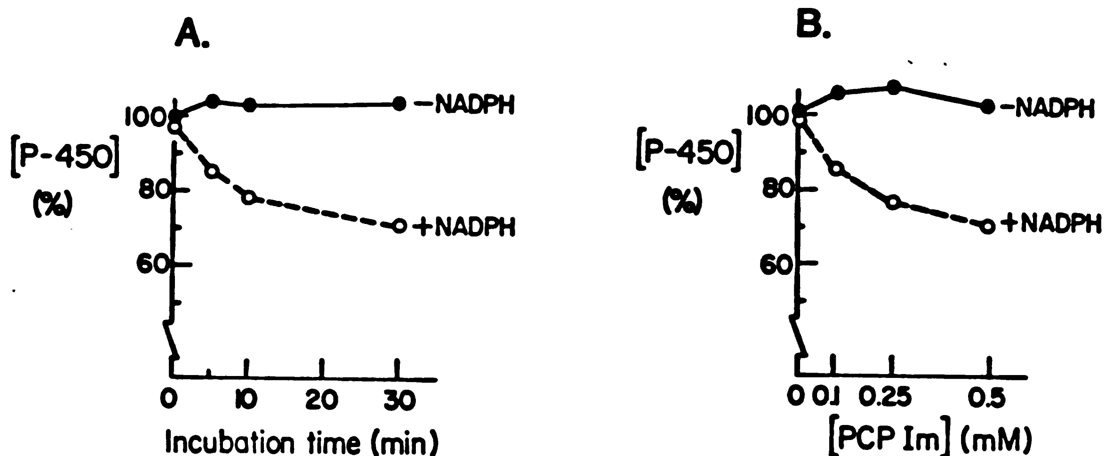


Figure 12. Effect of $PCP\text{-}Im^{+}ClO_4^{-}$ on microsomal cytochrome P-450 concentration.

A. Time dependence: Liver microsomes from phenobarbital-treated rabbits were incubated in either the presence or absence of an NADPH-generating system with $PCP\text{-}Im^{+}$ for 0-30 min. (N=4) P-450 concentrations are expressed as a percentage of the basal value which was 3.12±0.46 nmol/mg protein.

B. Concentration dependence: Microsomes were incubated with $PCP\text{-}Im^{+}$ (0.5 mM) in either the presence or absence of NADPH for 30 min. (N=4)

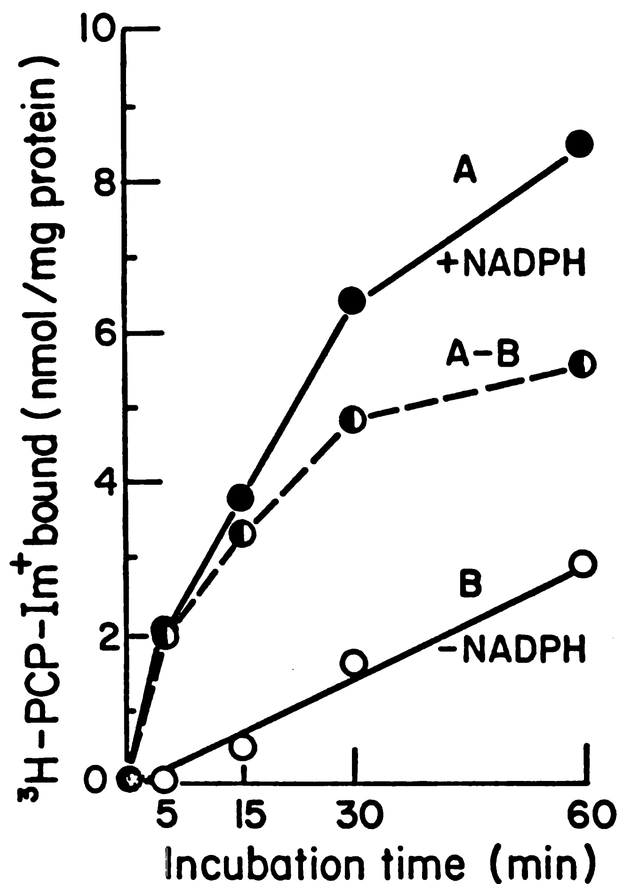


Figure 13. Covalent binding of radiolabeled PCP-Im$^+$ClO$_4^-$ to microsomal protein.

Liver microsomes from phenobarbital-treated rabbits were incubated with ^{3}H-PCP-Im$^+$ (0.5 mM; 0.415 µCi). Microsomal proteins were precipitated with TCA and unbound radioactivity was removed by repeated methanol/water extractions as described in the text. (A): +NADPH, (B) -NADPH, (A-B): NADPH-dependent covalent binding. (N=4)

DISCUSSION

Exposure of the hepatic microsomal enzyme systems to PCP-Im$^+$ *in vitro* resulted in a significant loss of cytochrome(s) P-450-dependent N-demethylase activity. The observation of decreased N-demethylase activity when NADPH was absent from the preincubation mixture suggested that PCP-Im$^+$, to a limited extent, might directly inhibit enzyme activity. This result is consistent with earlier observations that the inhibition of hepatic microsomal cytochrome(s) P-450 by PCP was dependent on its alpha-carbon oxidation to PCP-Im$^+$. In the presence of an NADPH-generating system, however, the loss of the demethylase activity was greatly enhanced. This NADPH-dependent effect exhibited characteristics of a mechanism-based enzyme inactivation process including concentration dependence, irreversibility, and pseudo-first order kinetics, characteristics similar to those observed for the inactivation of cytochrome(s) P-450 by PCP itself. The use of nucleophilic cyanide ion to probe the possibility that PCP-Im$^+$ is a true suicide substrate for the N-demethylase is not feasible because of the known chemical interaction of cyanide ion with the iminium to generate the cyano adduct (Ward et al., 1982a and 1982b). The loss of N-demethylase activity following incubation of microsomes with PCP-Im$^+$, as with PCP (Fig. 11), is consistent with the suggestion that the iminium species is

a proximal intermediate to the ultimate reactive species in the bioactivation process. This effect plus the metabolism dependent loss of the chromophore of microsomal cytochrome(s) P-450 caused by PCP-Im^+ and PCP indicate that functional groups on such proteins may represent target sites for a common reactive metabolite of both molecules. The inability of PCP and PCP-Im^+ to completely inhibit N-demethylase activity may be due to such factors as inaccessibility to all P-450 molecules in the microsomal membrane and specificity for certain of the multiple species of mammalian cytochrome(s) P-450 (Lu and West, 1980). The latter possibility would suggest that the bioactivation involves specific forms of this enzyme. As discussed in the last chapter, these speculations are consistent with a report by Osawa and Coon (1986) which states that cytochrome P-450 isozyme 2 is inactivated selectively by PCP in a mechanism-based process. More recently, Osawa and Coon (1987) have reported that PCP-Im^+ is less specific in its inactivation of P-450 isozymes and inhibits forms 2 and 3b. One possible interpretation of these results is that PCP might be a specific substrate for isozyme 2 whereas PCP-Im^+ might be a substrate for other isozymes as well, including forms 2 and 3b. Thus, metabolism of PCP by isozyme 2 would yield PCP-Im^+ which might be metabolized further by the same enzyme to reactive species which could then inactivate the enzyme. Using PCP-Im^+ as the initial substrate, on the

other hand, would allow it access to active sites of many P-450 isozymes including those for which is its a substrate and which it can potentially inactivate.

Irreversible loss of microsomal N-demethylase activity with PCP-Im^+ in the absence of NADPH is consistent with the potential reactivity of this electrophilic iminium ion. This reactivity is demonstrated further by the observed association of tritiated PCP-Im^+ with microsomal protein in the absence of NADPH. Association of PCP-Im^+ with microsomal protein also increased markedly in the presence of an NADPH-generating system. Because solvents more lipophilic than methanol were not used to wash the microsomal protein pellet, the possibility remains that the observed association of ^{3}H-PCP-Im^+ with microsomal protein represents non-specific rather than covalent binding. These experiments, therefore, should be repeated using more nonpolar solvents to reduce the likelihood of observing non-specific binding of ^{3}H-PCP-Im^+ to microsomal protein. However, the observation of increased binding upon addition of NADPH to the incubation mixture indicates that under conditions which allow metabolism, binding occurs more readily. This suggests that while the non-NADPH-dependent component of binding might be non-specific, the NADPH-dependent component could, in fact, be specific. For the sake of further discussion, the supposition shall be made that the NADPH-dependent

association of ^{3}H-PCP-Im^{+} with microsomal protein represents covalent binding.

Presumably cytochrome(s) P-450 represent only one target site for the metabolism-dependent covalent binding of PCP-Im^{+} to microsomal protein. Assuming one molecule of reactive species generated from PCP-Im^{+} reacts with one molecule of cytochrome(s) P-450, binding to this protein would represent only 13% of the total radioactivity covalently bound to microsomal protein under the present experimental conditions. Other targets for binding may represent molecules not critical for the functioning of the N-demethylase.

The radiochemical purity of the tritiated PCP-Im^{+} must be considered when interpreting the results of the covalent binding experiments. Analysis by HPLC using radiochemical detection showed the PCP-Im^{+} eluting as a single UV absorbing peak which coeluted with 98% of the radioactivity (Fig. 4). Most (70%) of the radioactive impurity coeluted with PCP, the starting material in the synthesis of PCP-Im^{+}. The question which arises is whether covalently bound (as defined above) material represents the compound of interest or an impurity. After a 60 min incubation period with PCP-Im^{+}, the NADPH-dependent component of binding accounted for approximately 1.6% of the starting radioactivity. If the observed binding was due exclusively

to the major impurity, PCP, virtually all of the PCP-derived species would have to be covalently bound. This situation is not likely since it would require that all PCP be metabolized and that each metabolized molecule of substrate undergo covalent binding. Since the metabolism of one molecule often involves more than one pathway, it is unlikely that all pathways lead to formation of reactive species. In fact, when PCP (0.1 mM) was incubated with liver microsomes, only 8% of the PCP metabolized was found to be covalently bound (Ward et al., 1982b). Therefore, the quantity of PCP present as a contaminant in the sample of PCP-Im$^+$ was not sufficient to account for all of the NADPH-dependent covalent binding of radioactivity observed. Although it is possible that one, or more, unidentified impurities might be involved in the irreversible association with microsomal macromolecules, the situation is analogous to that of PCP. Participation of such impurities, to a significant extent, in this interaction would require the unlikely event that each molecule of the impurity be metabolized to the ultimate reactive species. Hence, the major portion of covalent binding can be attributed to PCP-Im$^+$.

The results of these experiments indicate that the iminium ion metabolite of phencyclidine does produce the same effects that were previously observed with PCP. While binding of PCP-Im$^+$ to microsomal protein and enzyme

inhibition occurred to some extent when NADPH was absent from the incubations, the requirement of NADPH to produce maximal effects suggests that further metabolism was necessary and that PCP-Im^+ is not the sole ultimate reactive metabolite of PCP. However, ability of PCP-Im^+ to produce the same effects mediated by PCP is consistent with the proposal that the iminium ion is an obligatory intermediate in the bioactivation pathway of PCP.

METABOLISM OF PHENCYCLIDINE IMINIUM ION

INTRODUCTION

Results discussed in the preceding sections have established that incubation of phencyclidine with liver microsomes resulted in covalent binding to protein, inhibition of N-demethylase activity and a decrease in cytochrome P-450 content. These effects were dependent on metabolism of PCP and were antagonized by the presence of sodium cyanide at concentrations which did not affect PCP metabolism (Ward et al., 1982b; Hoag et al., 1984). Cyanide ion is known to interact with phencyclidine iminium ion (Ward et al., 1982a and 1982b) as well as other iminium species (Murphy, 1973; Ho and Castagnoli, 1980; Ziegler et al., 1981) to generate the corresponding stable alpha-aminonitriles. The protective effect of sodium cyanide was consistent with the proposed involvement of $PCP\text{-}Im^+$ in the mediation of the effects of PCP. The results obtained following $PCP\text{-}Im^+$ incubation with liver microsomes were similar to those obtained following incubations with PCP. Once again, covalent binding, inhibition of cytochrome P-450-dependent ketamine N-demethylase activity, and loss of cytochrome P-450 were observed. While binding of $PCP\text{-}Im^+$ to microsomal protein and N-demethylase inhibition occurred to some extent when NADPH was absent from the incubation mixture, maximal effects required the presence of NADPH. It appears, therefore, that $PCP\text{-}Im^+$ is an obligatory intermediate in

the bioactivation pathway and that it is metabolized further to one or more reactive species.

This finding prompted efforts to characterize the metabolism of PCP-Im$^+$ in order to gain further insight into the bioactivation pathway of PCP. The availability of chemically synthesized PCP iminium perchlorate was advantageous since using this intermediate as the substrate in a metabolic incubation might increase the likelihood of detecting metabolites which could be pertinent to the bioactivation pathway. As in previous experiments, PCP-Im$^+$ was incubated with liver microsomes prepared from phenobarbital-treated rabbits and the post-incubation mixtures were analyzed for the presence of the iminium species and its metabolites.

In addition, because the observed long term effect of phencyclidine appears to be primarily neurotoxic, the possible metabolism of PCP by brain tissue was studied. While it is known that PCP is extensively metabolized in the liver, not much information about its metabolism by brain is available. The metabolic activity of brain tissue could potentially play a role in the mechanism of PCP neurotoxicity in a number of ways. In one case, metabolic activation of PCP might occur entirely in the brain. Alternatively, or additionally, PCP-Im$^+$ (or other metabolites) formed in the liver could enter the general

circulation and thereby gain access to the brain. Once in the CNS, the intermediate(s) might be metabolized further to yield the ultimate reactive species. Although PCP-Im^+ (13) is charged and therefore may be unable to enter the brain, the corresponding uncharged eneamine conjugate base (10) should be quite lipophilic and have ready access to the CNS. Since the pKa of 13 is likely to be near 7, the partitioning between 13 and 10 would favor 10 relative to the corresponding equilibrium between the protonated PCP (approximate pKa 9) and its conjugate base. Consequently, preliminary studies were undertaken in collaboration with M. Shigenaga and Dr. M. Schmidt-Peetz to examine the stability of PCP and PCP-Im^+ in brain homogenates. These studies represent an effort to identify possible interactions of these compounds with brain.

RESULTS

Metabolism of PCP and PCP iminium ion by rabbit brain.

To examine their stability in brain tissue, PCP and PCP-Im^+ were incubated with brain fractions. The concentration of PCP or PCP-Im^+ remaining after incubation was determined by HPLC analysis. A C18 reverse phase column was used with a mobile phase consisting of acetonitrile/100 mM sodium acetate (85:15, v/v) containing 0.1% (v/v) triethylamine, final pH 5.6. The flow rate was 2.0 ml/min. The incubation reaction was stopped with the

addition of an equal volume of acetonitrile. The mixture was centrifuged to sediment the precipitate and 100 µl of the supernatant were analyzed by HPLC. PCP and $PCP\text{-}Im^{+}$ had retention times of 6.3 and 5.2 min, respectively, and appeared as broad peaks. Concentrations were estimated by peak heights. In some experiments, $PCP\text{-}Im^{+}$ concentration was assessed by a second method. The pH of the mobile phase was adjusted with triethylamine rather than NaOH. The incubation reaction was terminated by addition of an equal volume of ice cold 0.1 M sodium bicarbonate, pH 8.6. For each ml, 25 µl of 10 mM PCP-HCl was added as internal standard. Neutral and basic components then were extracted into 1.0 ml of ethyl acetate and 15 µl of the ethyl acetate phase were analyzed by HPLC.

After incubation of PCP (0.1 mM) with rabbit brain mitochondrial fractions (2 mg protein/ml) for 0 to 90 min no change in PCP concentration was observed (Fig. 14). When radiolabeled PCP was incubated with rat brain microsomal fractions, analysis of the post-incubation mixture by HPLC with UV and radiochemical detection revealed the NADPH-dependent formation of a new peak of radioactivity (Fig. 15). After a one hour incubation, this new peak accounted for 1.0% of the starting radioactivity and its retention time was the same as that of chemically synthesized 1-(1-phenylcyclohexyl)-4-hydroxypiperidine (2). However, no

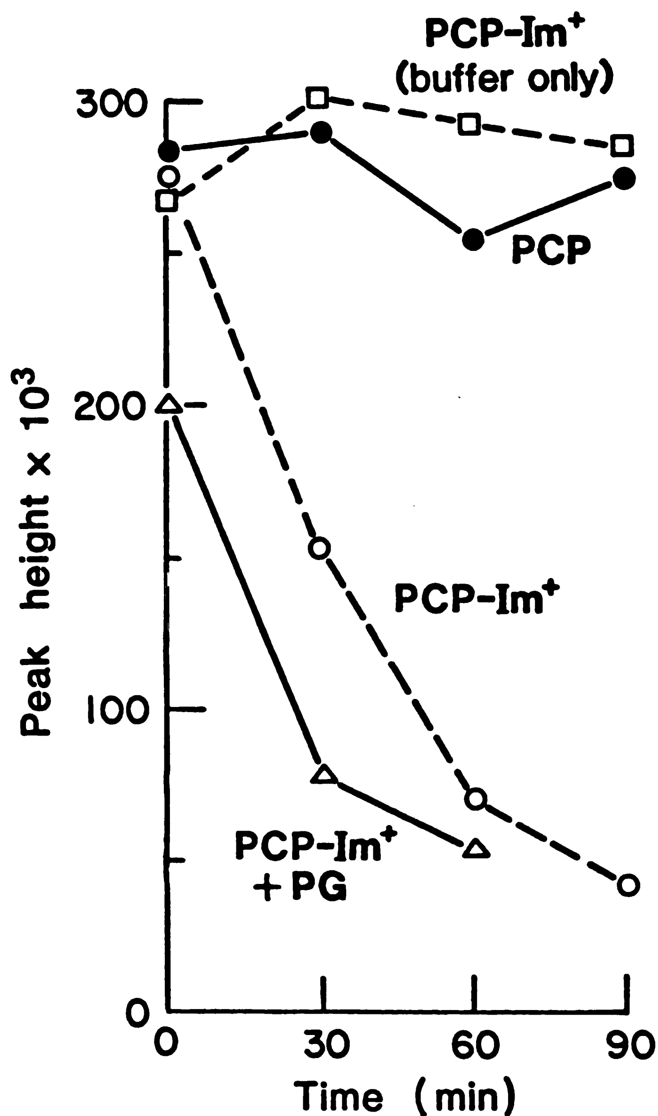


Figure 14. Stability of PCP and PCP-$Im^{+}ClO_{4}^{-}$ in rabbit brain mitochondrial fraction.

PCP (0.1 mM) or PCP-Im^{+} (0.1 mM) was incubated wtih rabbit brain mitochrondrial fraction (2 mg protein/ml) or buffer alone for 0-90 min either with or without pargyline (PG; 0.1 mM). (N=4)

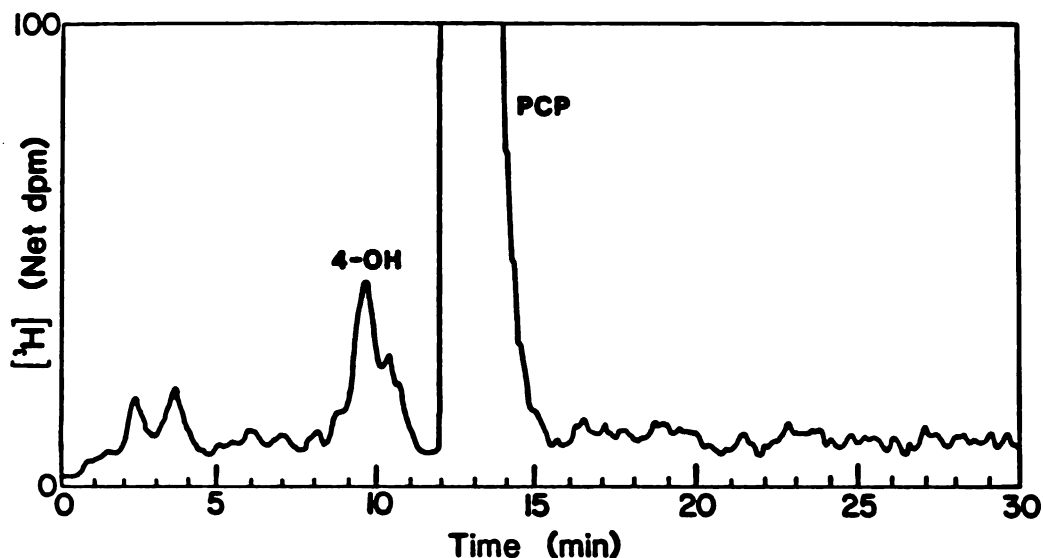


Figure 15. Radiochemical detection of HPLC separation of ^{3}H-PCP and metabolites after incubation with rat brain microsomal fraction.

^{3}H-PCP was incubated with NADPH-supplemented rat brain microsomes for 60 min. Formation of the radioactive peak which coeluted with synthetic 1-(1-phenylcyclohexyl)-4-hydroxypiperidine (4-OH) was NADPH-dependent.

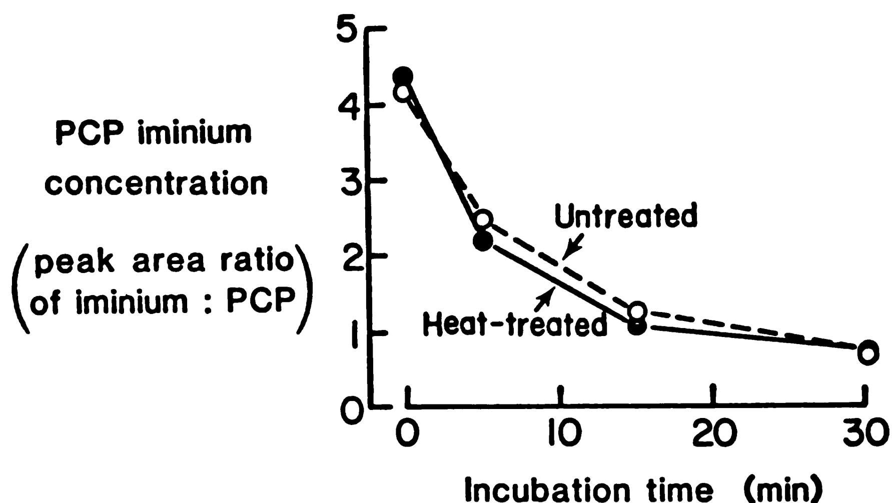


Figure 16. Stability of PCP-$Im^+ClO_4^-$ in rabbit whole brain homogenate.

PCP-Im^+ (1.0 mM) was incubated with rabbit whole brain homogenate (22-26 mg protein/ml; untreated or heat-denatured) for 0-60 min. Remaining PCP-Im^+ concentration was determined by HPLC. (N=4)

radioactivity or UV absorbance was found which had the same retention time as PCP-Im$^+$.

Incubation of PCP-Im$^+$ (0.1 mM or 1.0 mM) with rabbit whole brain homogenate (22-26 mg protein/ml) for 0-60 min resulted in a time-dependent decrease in PCP-Im$^+$ concentration. After 60 min, a concentration decrease of approximately 80% from the initial value was observed. When proteins in the whole brain homogenate were denatured by heat treatment for 10-15 min in an 80°C water bath, PCP-Im$^+$ concentration decreased at a rate similar to that observed when the homogenate was not heat denatured (Fig. 16). In neither instance was the formation of new metabolites detected by HPLC. Incubation of PCP-Im$^+$ at 37°C in buffer alone for up to 90 min resulted in no change in PCP-Im$^+$ concentration (Fig. 14).

When PCP-Im$^+$ (0.1 mM) was incubated with rabbit brain mitochondrial fraction (2 mg protein/ml) for 0-90 min at 37°C, a time-dependent decrease of PCP-Im$^+$ concentration was observed (Fig. 14). After 30 min, a decrease of approximately 45% was observed. Preincubation of the mitochondrial fraction with pargyline (0.1 mM), a concentration sufficient to inhibit 100% of monoamine oxidase (both types A and B) activity (Roth, 1979), did not inhibit this decrease. The rate of PCP-Im$^+$ decrease per mg protein was approximately 5 times faster in brain

mitochondrial fraction than in whole homogenate.

Preliminary studies of metabolism by rat brain fractions gave similar results. A 60 min incubation of PCP-Im$^+$ (100 μM) with rat brain whole homogenate (3.5 mg protein/ml) or 900g supernatant (mitochondrial fraction, 1.2 mg protein/ml) led to time-dependent decreases in PCP-Im$^+$ concentration of 59.6% and 31.9%, respectively, which were not affected by pargyline. To examine the possible involvement of aldehyde oxidase, whole brain homogenate was preincubated for 30 min with potassium cyanide to inhibit the enzyme activity. After removal of unbound cyanide ion by dialysis, the homogenate was incubated with PCP-Im$^+$. The rate of PCP-Im$^+$ disappearance was the same for homogenate preincubated either with or without potassium cyanide. Heat treatment of the brain homogenate did not affect the rate of disappearance. No decrease in PCP-Im$^+$ concentration was observed upon its incubation with the 100,000g supernatant (soluble) fraction for up to 75 min.

<u>Metabolism of PCP iminium ion by rabbit liver microsomes</u>.

In the absence of liver microsomes, PCP-Im$^+$ (0.5 mM) was stable in buffered media at 30°C for over 1 hour (Fig. 17). With microsomes present, but no NADPH, PCP-Im$^+$ concentrations decreased at the rate of 85 nmol/mg protein/30 min. Addition of an NADPH-generating

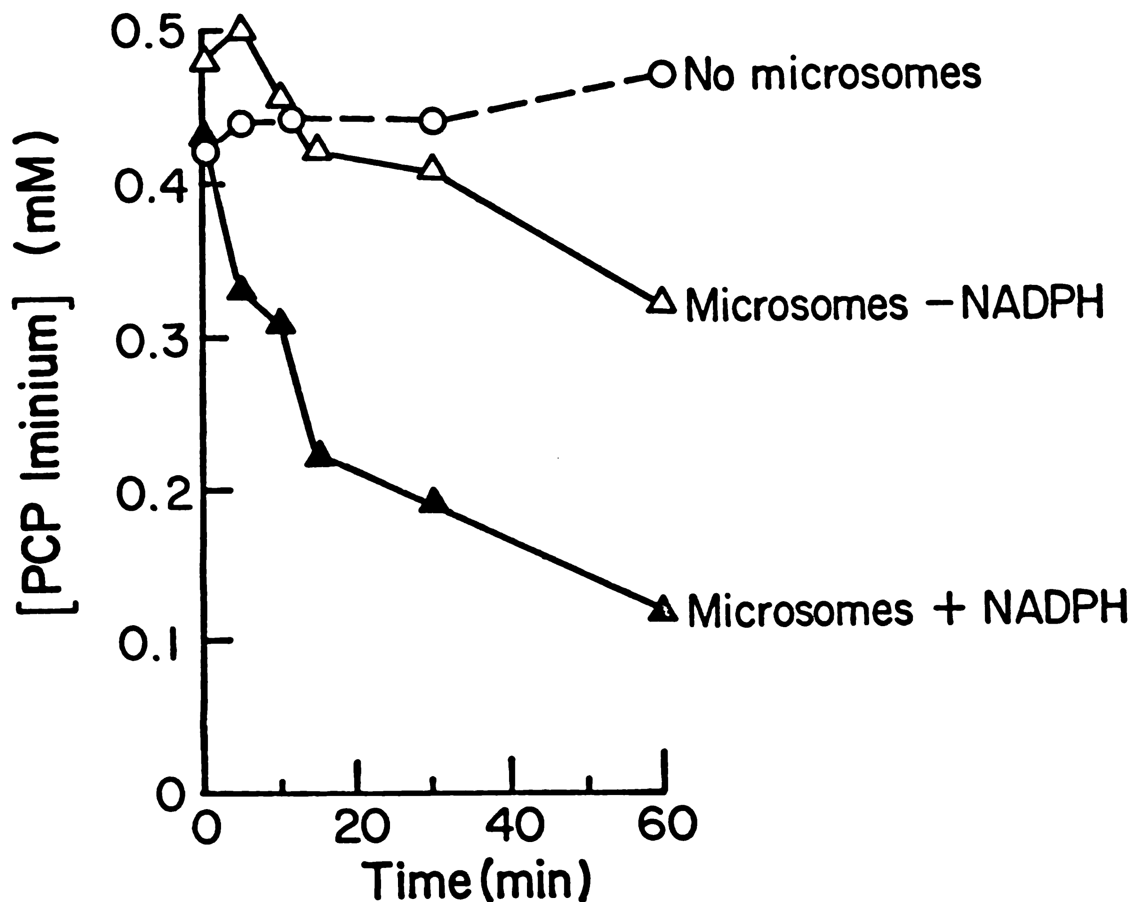


Figure 17. Stability of PCP-$Im^+ClO_4^-$ in rabbit liver microsomal fraction.

PCP-Im^+ (0.5 mM) was incubated with liver microsomes from phenobarbital-treated rabbits for 0-60 min. Remaining PCP-Im^+ concentration was determined by HPLC. (N=4)

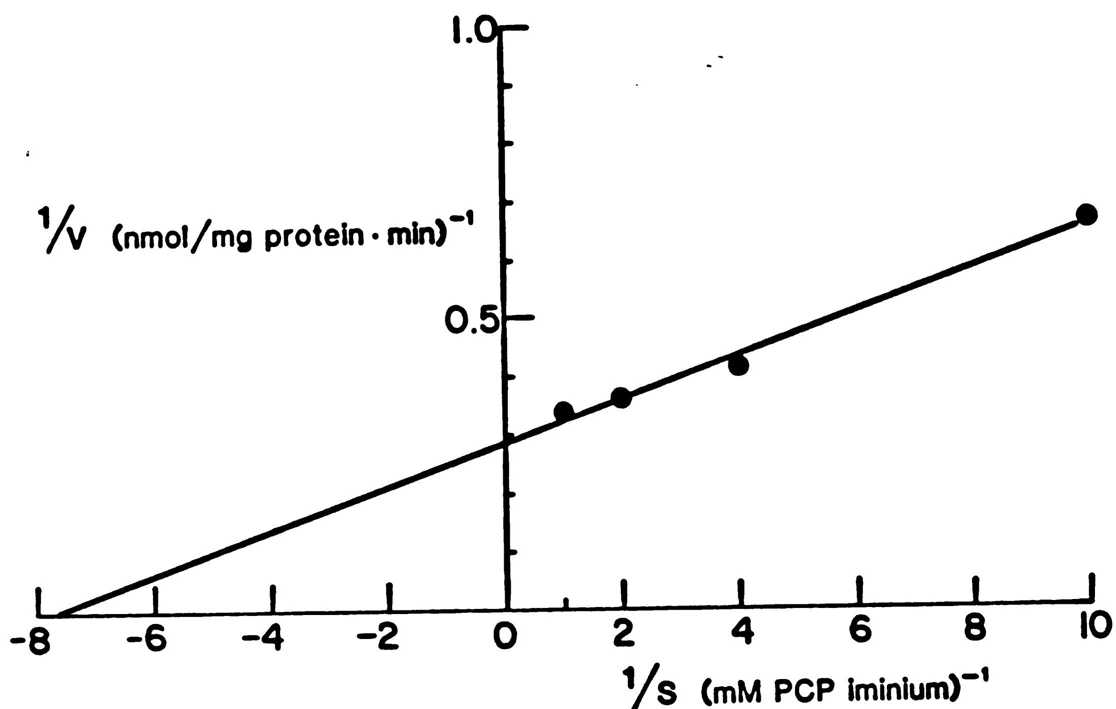


Figure 18. Kinetics of PCP-$Im^{+}ClO_4^{-}$ metabolism by rabbit liver microsomes.

PCP-Im^{+} (0.1-1.0 mM) was incubated with liver microsomes from phenobarbital-treated rabbits (1.65 mg protein/ml) for 30 min. Remaining PCP-Im^{+} concentration was determined by HPLC. (N=4)

system increased the rate of disappearance of PCP-Im^+ to 228 nmol/mg protein/30 min. From a study of the kinetics of PCP-Im^+ metabolism by liver microsomes (1.65 mg protein/ml) during a 30 min incubation, an apparent K_m of 0.13 mM and an apparent V_{max} of 3.45 nmol/mg protein/min were calculated (Fig. 18). Preincubation of the microsomes with SKF 525A (0.2 mM) inhibited NADPH-dependent metabolism of PCP-Im^+ by greater than 80%. When the incubation mixture was purged with carbon monoxide/oxygen (50:50, v/v), PCP-Im^+ metabolism also was inhibited by more than 50% (Fig. 19). To examine the possible role of microsomal flavin-containing monooxygenase in metabolism of PCP and PCP-Im^+, liver microsomes were coincubated with N-octylamine (3.0 mM), which is an inhibitor of cytochrome P-450 (Jeffcoate et al., 1969) and a positive effector for microsomal flavin-containing monooxygenase activity (Ziegler, 1980) inhibited metabolism of PCP (1.0 mM) by approximately 30%. In contrast, N-octylamine (3.0 mM) coincubation with PCP-Im^+ (0.5 mM) enhanced the disappearance of the iminium species by 20%. Coincubation of octylamine with 1.0 mM PCP-Im^+ resulted in a 90% greater disappearance as compared to PCP-Im^+ with an NADPH-generating system only. Further study revealed that the decrease in PCP-Im^+ concentration in the presence of microsomes and octylamine occurred even when NADPH was not present.

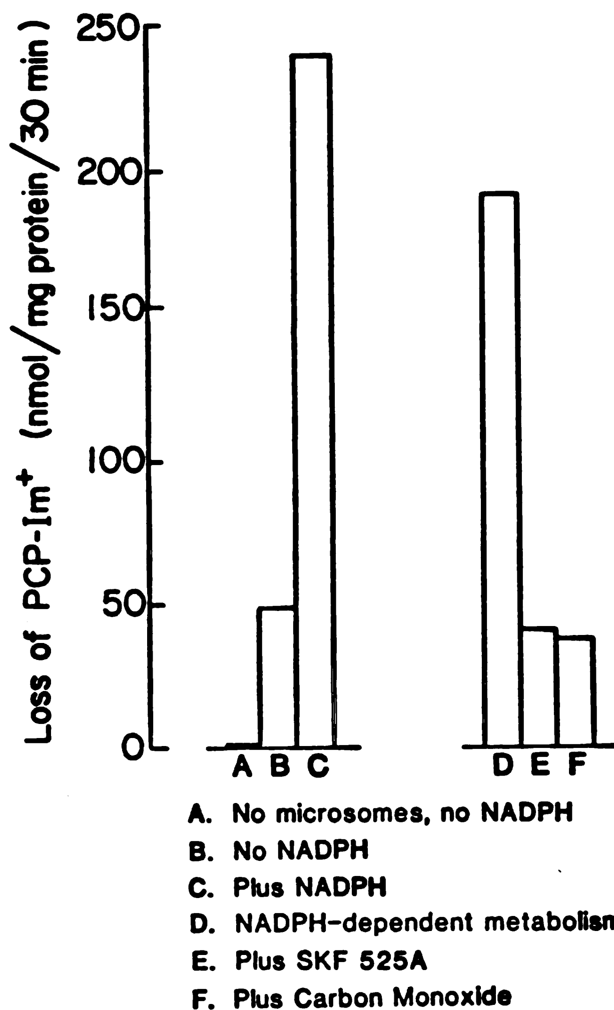


Figure 19. Microsomal metabolism of $PCP\text{-}Im^{+}ClO_4^{-}$.

Rates of disappearance of $PCP\text{-}Im^{+}$ (0.5 mM initial concentration) when incubated for 30 min (A) in the absence of both microsomes and NADPH, (B) in the presence of microsomes but absence of NADPH, (C) in the presence of both microsomes and NADPH, (E) following a 5-min preincubation of microsomes with SKF 525A (0.2 mM) and NADPH, and (F) in the presence of air/CO (50:50, v/v) and NADPH are shown. The net NADPH-dependent metabolism (C-B) is shown as bar D. (N=4)

Characterization of PCP iminium ion metabolite.

Analysis of incubations of tritium labeled PCP-Im^+ with liver microsomes by reverse phase HPLC using a C18 column revealed the NADPH-dependent and time-dependent appearance of 2 new peaks of radioactivity which eluted before PCP-Im^+ (Fig. 20). Attempts to isolate the material in these peaks were unsuccessful, however, due to poor resolution.

In an effort to achieve better separation, a normal phase HPLC system was examined. Extracts from incubations of radiolabeled PCP-Im^+ with liver microsomes were separated by normal phase silica column HPLC. Monitoring by radioactivity flow and UV detection revealed the NADPH-dependent and time-dependent formation of a UV absorbing peak of radioactivity with a retention time longer than that of PCP-Im^+ which presumably represented one or more metabolites of the iminium species. The metabolite(s) behaved chromatographically as a more polar molecule (Fig. 21). After a 30 min incubation, the NADPH-dependent decrease in PCP-Im^+ concentration was approximately 58% (Table 3). The metabolite(s) accounted for 23% of the starting radioactivity. Thus approximately 40% of the metabolized PCP-Im^+ could be accounted for as this product. The identity of the remaining 60% of the metabolites has not yet been established. Diode array analysis of this metabolite displayed a chromophore (λ_{max}

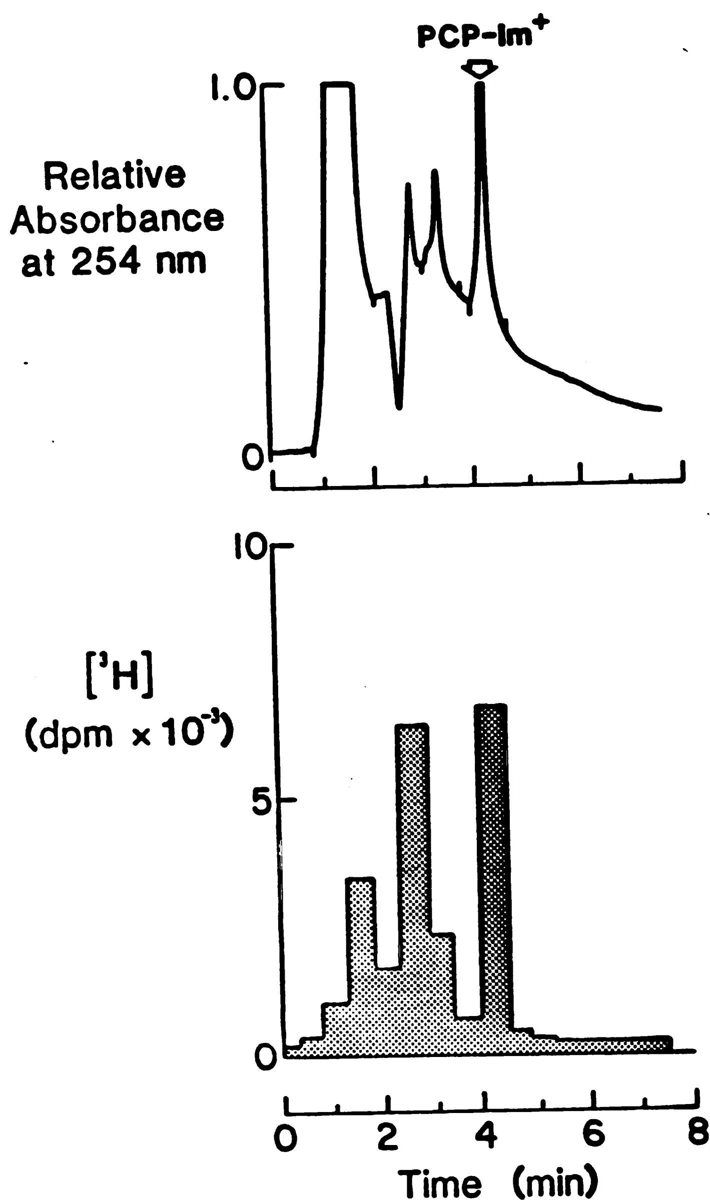


Figure 20. Reverse phase HPLC separation of PCP-Im^+ and its metabolites.

After a 30-min incubation of ^{3}H-PCP-Im^+ (0.5 mM; 0.415 mCi/mmol) with NADPH-supplemented rabbit liver microsomes, proteins were precipitated by addition of acetonitrile. The supernatant was subjected to reverse phase HPLC separation. UV absorption was monitored at 254 nm. Fractions were collected every 0.5 min for determination of radioactivity by liquid scintillation counting. Formation of the 2 peaks of radioactivity eluting prior to PCP-Im^+ was NADPH-dependent. Their identity was not determined.

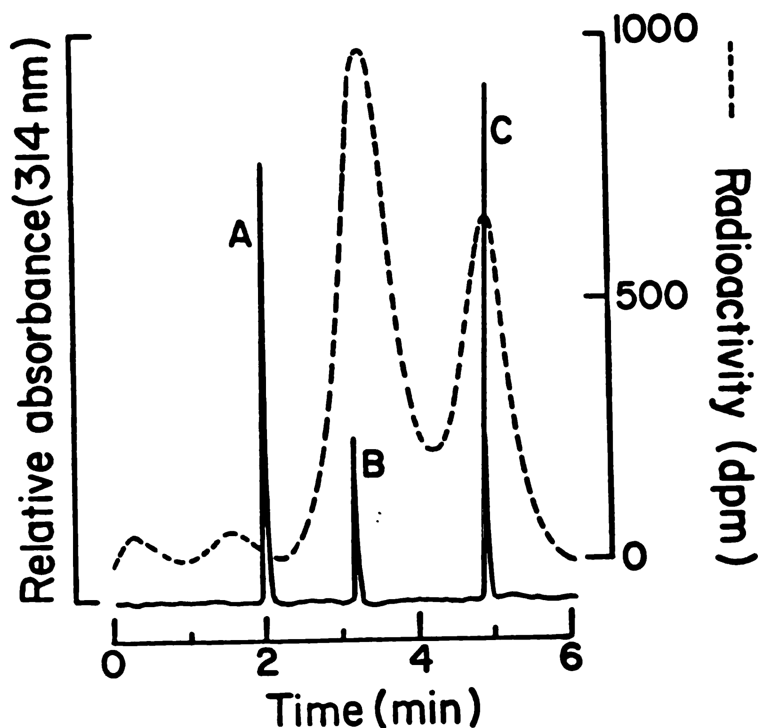


Figure 21. Normal phase HPLC separation of PCP-Im$^+$ and its metabolites.

Methylene chloride extracts from NADPH-supplemented liver microsomal incubation mixtures with ^{3}H-PCP-Im$^+$ (0.5 mM) were separated by normal phase HPLC. UV absorption was monitored at 314 nm and the radioactivity with an online flow counter. A: solvent front; B: PCP-Im$^+$; C: metabolite(s).

Table 3

Metabolic profile of radiolabeled PCP iminium perchlorate

Incubation	Radioactivity (dpm) PCP-Im$^+$	Metabolite(s)
0 min + NADPH	35397	0
30 min + NADPH	11251	6272
30 min - NADPH	26988	0

NADPH-dependent decrease of PCP-Im$^+$: (26988-11251)/26988 = 58.3%

PCP-Im$^+$ conversion to metabolite: 6272/26988 = 23.2%

% of metabolized PCP-Im$^+$ found as metabolite: 6272/(26988-11251) = 39.9%

^{3}H-PCP-Im$^+$ (0.5 mM) was incubated with liver microsomes from phenobarbital-treated rabbits. PCP-Im$^+$ was separated from metabolites(s) by normal phase HPLC. Radioactivity under the peaks was measured by radiochemical detection.

314 nm, Fig. 22) which is not present in the spectra of previously characterized PCP metabolites. This metabolite also was formed in microsomal incubations of PCP, although at a slower rate. After a 20 min incubation, metabolite formation from PCP was approximately 25% less than from PCP-Im^{+}. The relative rates of formation of the metabolite from the two substrates (Fig. 23) suggest that the conversion of PCP to this metabolite proceeds via PCP-Im^{+}.

A preparative scale microsomal incubation was carried out in order to isolate adequate quantities of this material for further characterization. Repeated injections and collections led to the isolation of a sample which, by HPLC, appeared to be homogeneous (Fig. 24). Direct insertion probe chemical ionization mass spectral analysis of this isolate showed a protonated molecular ion of mass 256 consistent with the introduction of one oxygen atom into the phencyclidine iminium ion substrate together with one additional degree of unsaturation. A strong fragment ion (57%) at m/z 159 is best accomodated by the phenylcyclohexenyl radical cation which suggests that the phenylcyclohexyl moiety of PCP remains unmodified in the metabolite. A second revealing fragment ion was observed at m/z 98. Analysis by high resolution EI-MS confirmed these results (Fig. 25A). The empirical formula C_5H_8NO is consistent with this fragmentation which is best

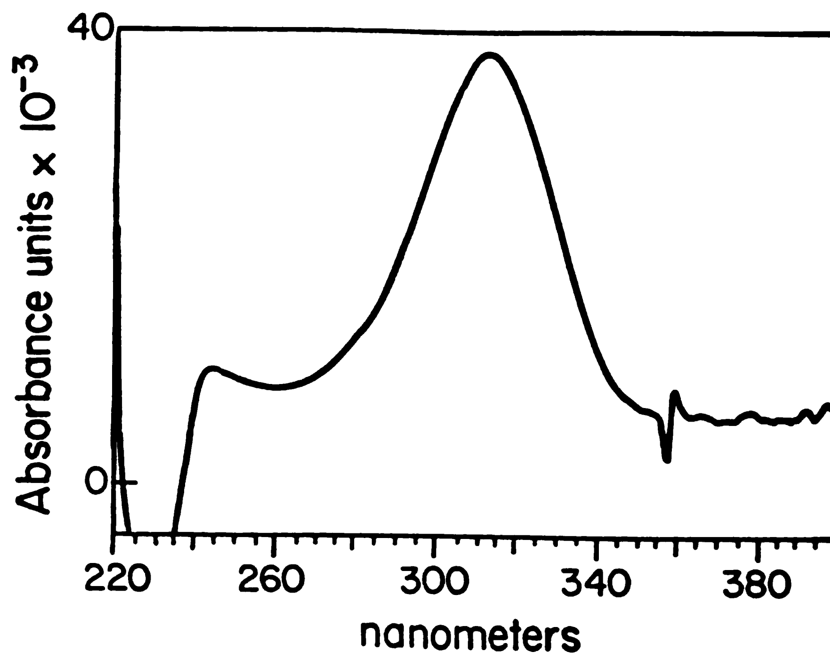


Figure 22. UV spectrum of $PCP-Im^{+}$ metabolite(s).

The spectrum of the material eluting as peak C (Fig. 21) was obtained with an online diode array detector.

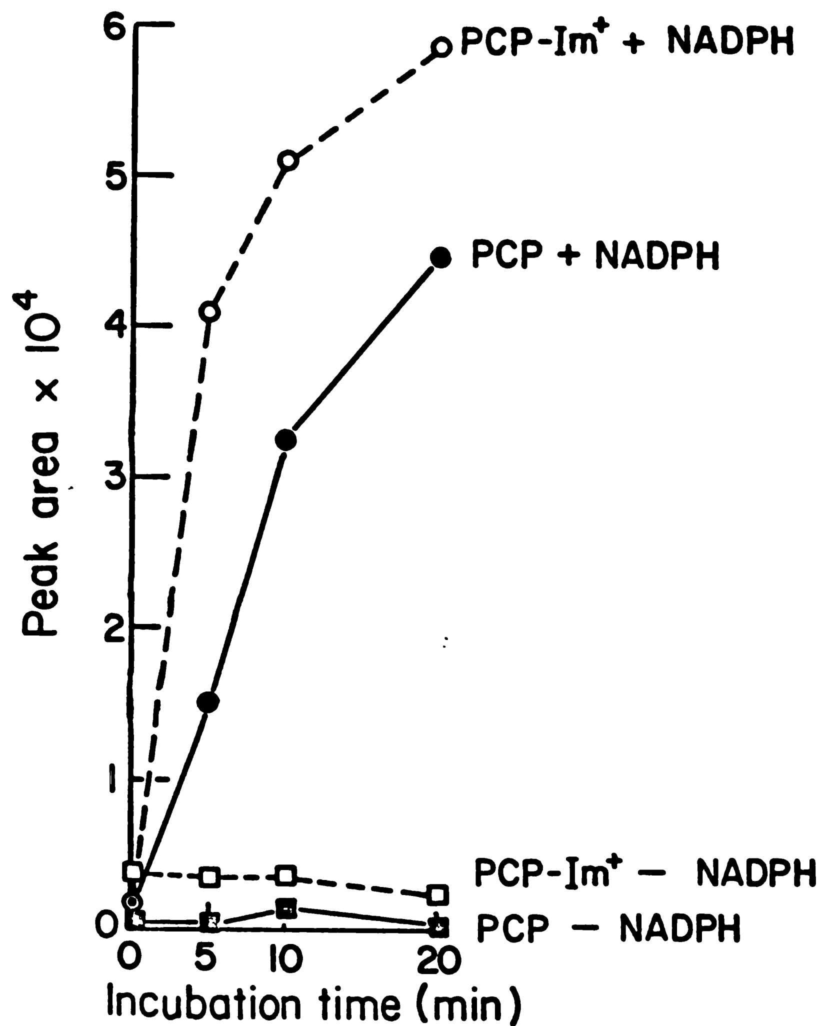


Figure 23. Formation of metabolite(s) from PCP and PCP-Im^+.

PCP (0.5 mM) or PCP-Im^+ (0.5 mM) was incubated with or without NADPH and liver microsomes from phenobarbital-treated rabbits for 0-20 min. Metabolite formation was measured by an HPLC assay monitoring 314 nm absorbance. (N=4)

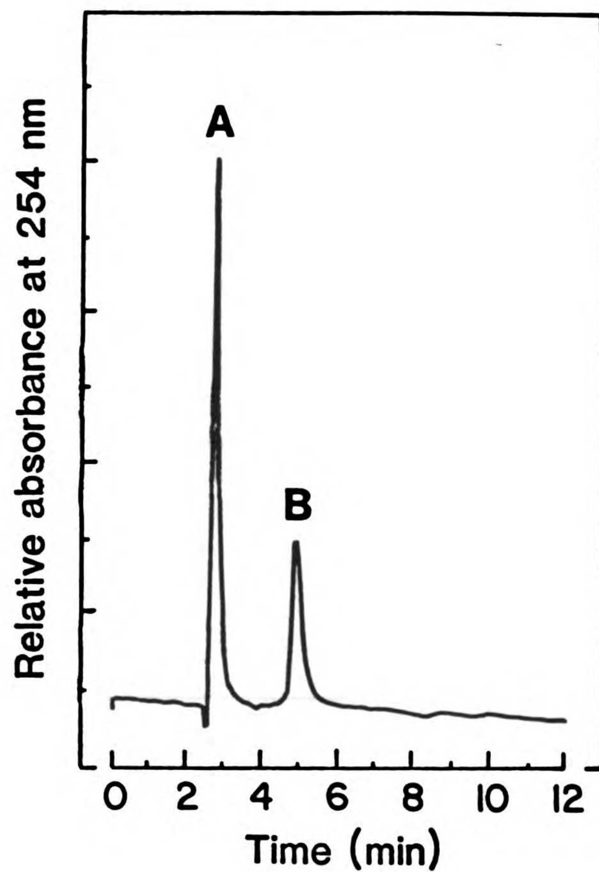
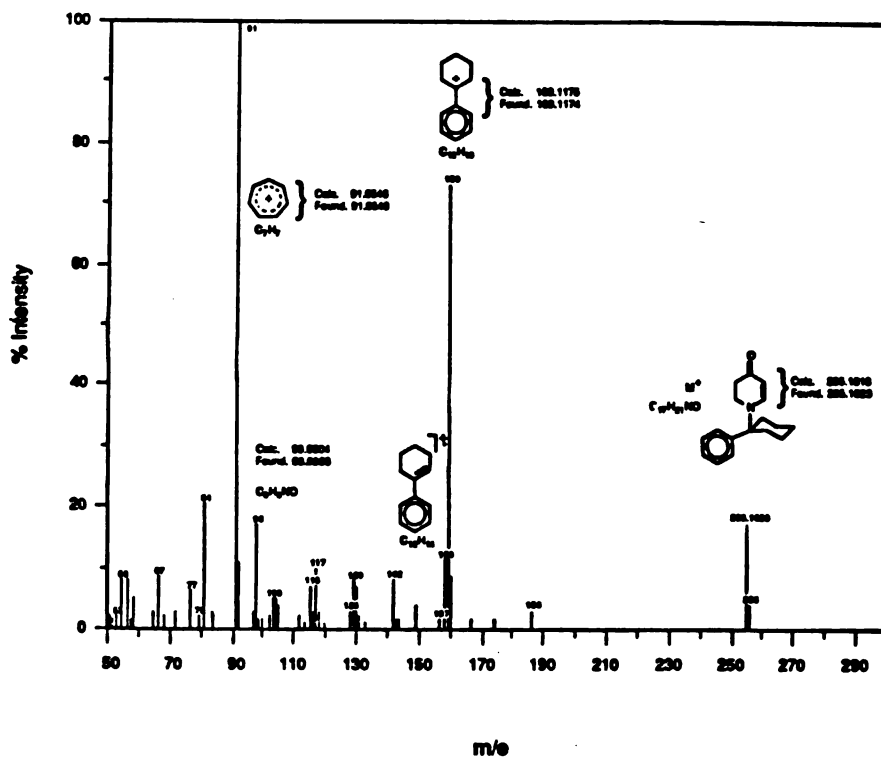


Figure 24. HPLC profile of isolated PCP-Im$^+$ metabolite(s).

Normal phase HPLC separation of isolated metabolite.
A: solvent front; B: metabolite(s).



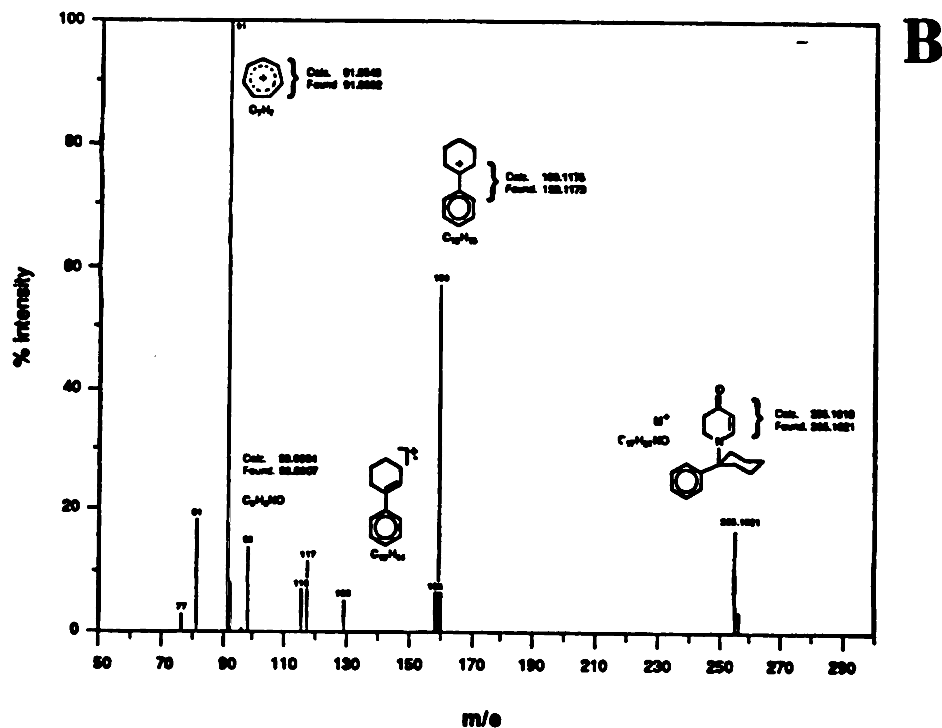
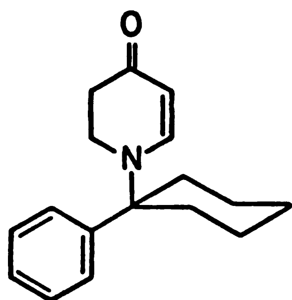


Figure 25. High resolution EI-MS of isolated PCP-Im^{+} metabolite(s) (A) and synthetic 1-(1-phenylcyclohexyl)-2,3-dihydro-4-pyridone (B).

represented by a dihydropyridone type structure involving the net introduction of one carbonyl group into the starting piperidiene moiety. Six isomeric compounds (28, 31-35) are consistent with the mass spectral data.

In contrast to the relatively simple and readily assignable chemical ionization and electron impact mass spectra that were obtained with the crude biological isolate, the 500 MHz ^{1}H-NMR spectrum was quite complex and difficult to interpret (Fig. 26). In an effort to distinguish the "background signals" present in the spectrum from signals associated with the metabolite, a background sample was prepared by duplicating the procedure used for metabolite purification but omitting PCP-Im^{+} from the incubation mixture. Signals which were present in the metabolite spectrum and absent in the background included those at δ 5.29 (s), 4.05 (d), and 2.64 (m) ppm. From a review of the literature, the anticipated NMR spectra for compounds 28, 32, 33, and 34 were established (Hubert et al., 1975; Liberatore et al., 1975; Chen et al., 1984; Guerry and Neier, 1984). Of these, the metabolite spectrum most closely resembled that expected from compound 34.

However, when a new metabolite sample was purified for analysis, the NMR spectrum obtained was quite different (Fig 27A). Repeated attempts, by purification of several



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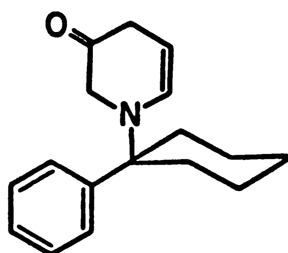

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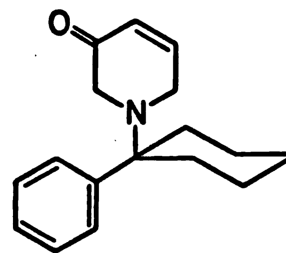

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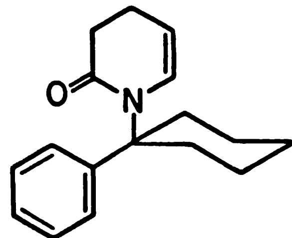

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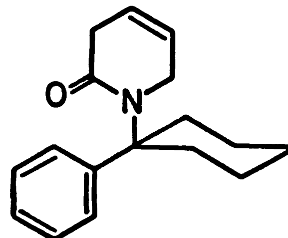

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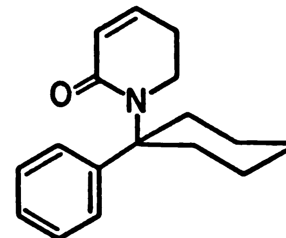

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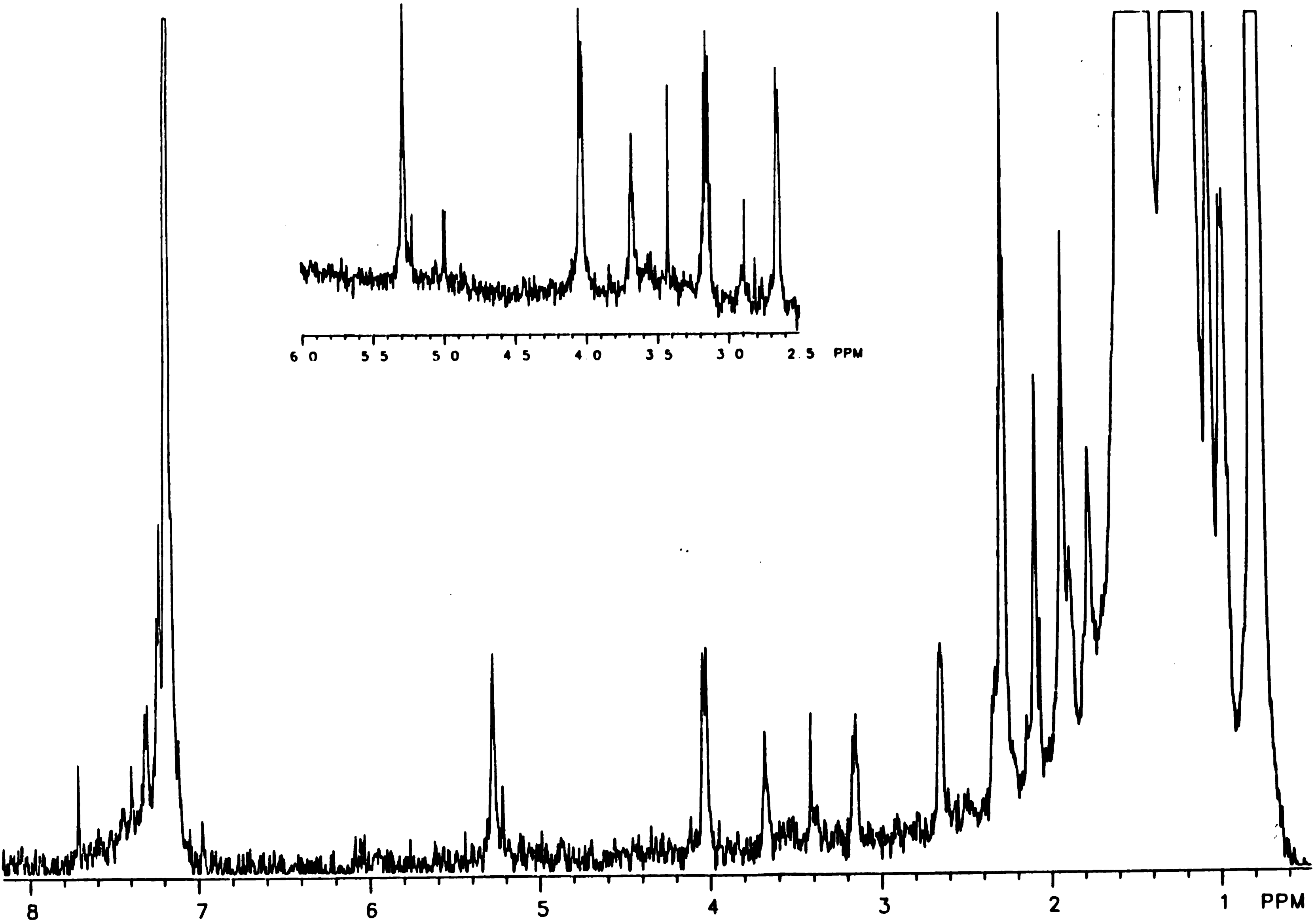
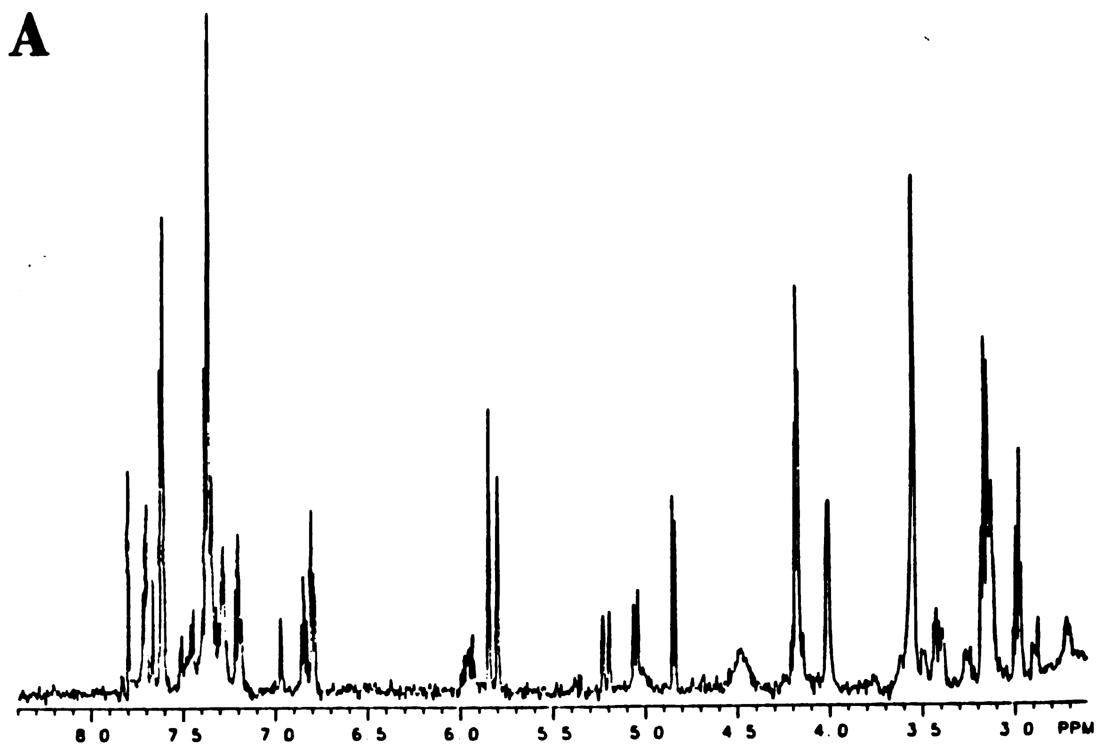


Figure 26. Original NMR spectrum of isolated PCP-Im^+ metabolite(s).

new samples, to reproduce the first NMR spectrum were unsuccessful. The mass spectra, on the other hand, were consistently unchanged. The new NMR spectrum, which was quite reproducible over the various samples, consistently displayed the following signals: δ 7.53 (d), 4.85 (d), and 3.18 (m) ppm. These signals would be expected from compound 28 (Guerry and Neier, 1984). In addition, at least 4 other signals could be distinguished in the olefinic proton region of the spectrum at δ 5.45, 5.25, 5.15, and 4.65 ppm. In comparing the "old" and "new" spectra, it was noted that the doublet at 4.85 ppm in the new spectrum was present in the earlier spectrum but was of a much lesser intensity relative to the other signals (Fig. 26, inset). In the new spectrum, the doublet at 4.85 ppm was a prominent signal (Fig. 27A). A further review of the literature revealed that compound 28 would be expected to have a chromophore absorbing at about 324 nm (Guerry and Neier, 1984), compound 34 would not be expected to absorb between 220-300 nm (Sundberg et al., 1967) and compound 35 would be expected to exhibit 2 maxima at about 206 nm and 240 nm (Sundberg et al., 1967). Based on the NMR and UV data, a tentative assignment of compound 28 as the structure of the metabolite was made and efforts were directed to the synthesis of this material in collaboration with Dr. Michael Schmidt-Peetz.

Following previously described procedures (Fig. 5;



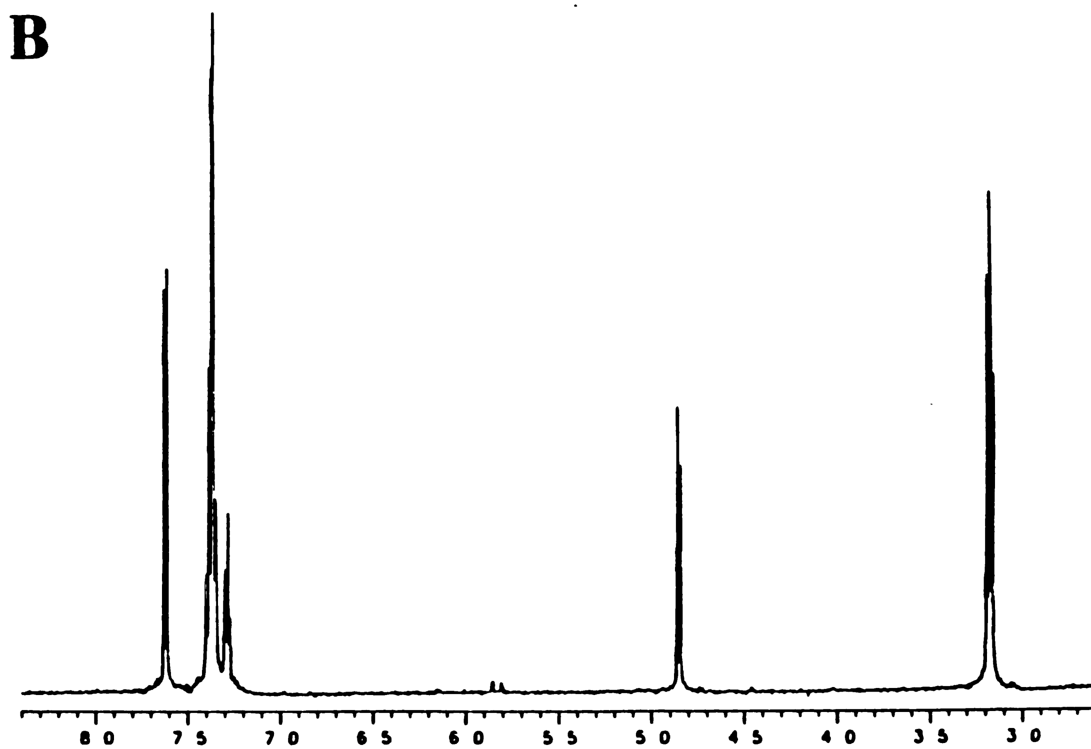


Figure 27. New NMR spectrum of isolated PCP-Im$^+$ metabolite(s) (A) and NMR spectrum of synthetic 1-(1-phenylcyclohexyl)-2,3-dihydro-4-pyridone (B).

Leonard and Cook, 1959; Maddox et al., 1965), condensation of the dioxalan of 4-piperidone (24) with cyclohexanone (25) in the presence of potassium cyanide led to the corresponding alpha-cyanoamine (26). Reaction of 26 with phenylmagnesium bromide resulted in intermediate 27 which upon treatment with mercuric acetate in the presence of acetic acid yielded the desired dihydropyridone (28). The product was characterized spectrally and by microanalysis. The UV and chemical ionization mass spectral features of the synthetic molecule were essentially identical to those of the metabolic isolate. Additionally, the high resolution EI mass spectrum of the synthetic material and the crude metabolic isolate were essentially identical (Fig. 25B) In addition to a strong molecular ion (M^+ 225), fragment ions at m/z 159, 158, 98 and 91 (corresponding to structures indicated in Fig. 25B) provide very strong evidence for the assigned structure. Proton NMR analysis of the synthetic compound led to the unambiguous characterization of the product. Two doublets centered at 4.85 (C_3-H) and 7.53 (C_2-H) ppm were shown to be coupled as required by structure 28. No evidence of additional coupling, as would be expected for all of the isomeric species, was apparent at 500 MHz. Analysis of the ^{1}H-NMR spectrum obtained with the crude metabolic isolate revealed the presence of all of the key signals displayed by the synthetic compound (Figs. 27A and 27B). A rough estimate based on the amide metabolite represents

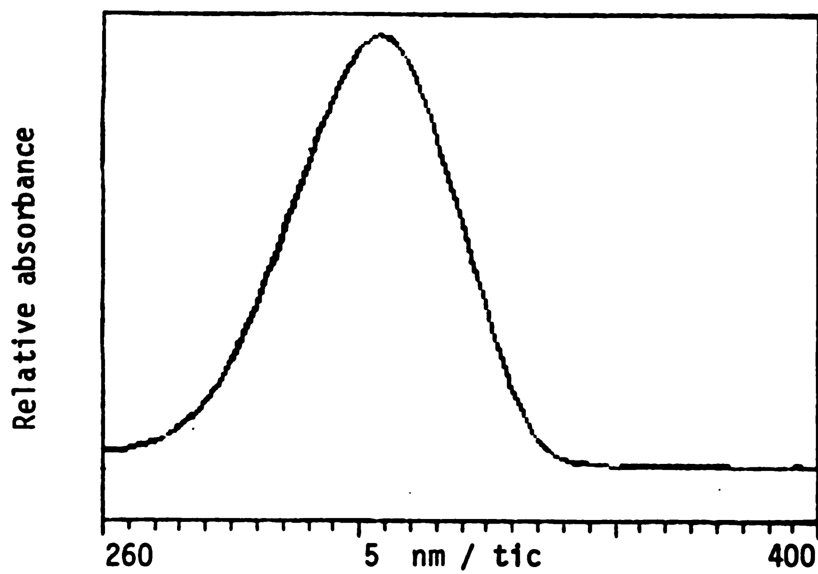


Figure 28. UV spectrum of a mixture of isolated PCP-Im$^+$ metabolite(s) and synthetic 1-(1-phenylcyclohexyl)-2,3-dihydro-4-pyridone.

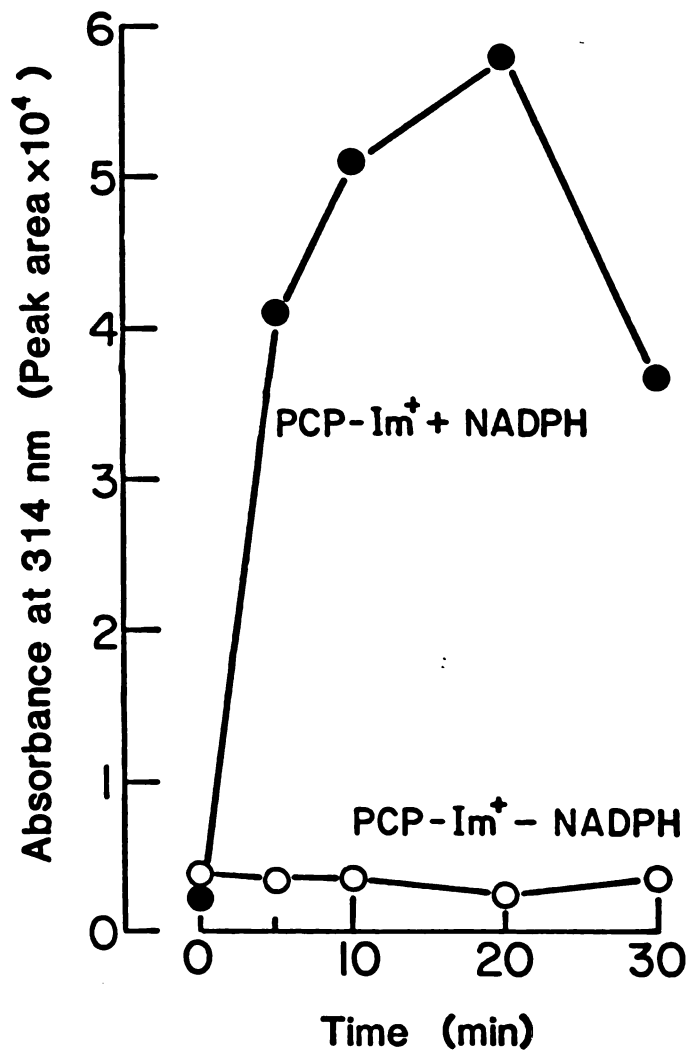


Figure 29. Formation of metabolite(s) from $PCP-Im^+$.

$PCP-Im^+$ (0.5 mM) was incubated with or without NADPH and liver microsomes from phenobarbital-treated rabbits for 0-30 min. Metabolite formation was measured by HPLC monitoring absorbance at 314 nm. (N=4)

approximately 30% of the olefinic signals present in the metabolite spectrum (Fig. 27A). Analysis of the synthetic product by HPLC with diode array detection (HPLC-DAD) showed the chromatographic retention time and UV spectrum to be identical to those of the metabolic isolate. Upon HPLC-DAD analysis of a mixture of the metabolite and the synthetic material, a single peak eluted which had a retention time and a UV spectrum (Fig. 28) identical to that of each the metabolite and the synthetic material separately.

Measurement of the dihydropyridone concentration with respect to incubation time revealed an increasing concentration from 0-20 min followed by a decrease (Fig. 29). This observation suggested that the dihydropyridone species is unstable in the microsomal incubation mixture and possibly undergoes further metabolism. In an effort to examine the possibility of further metabolism of the dihydropyridone species, microsomal incubations were carried out for 0 or 30 min either in the absence or presence of NADPH. HPLC analysis of extracts of incubation mixtures was used to assess the concentration of dihydropyridone remaining. Qualitative analysis of the resulting chromatograms indicated no observable change in dihydropyridone concentration. However, from a very rough estimation by comparison of the dihydropyridone peak area with that of a background peak, it appears that the

dihydropyridone concentration decreased by approximately 10%.

To study the possible role of the newly identified metabolite in the PCP dependent inactivation of cytochrome P-450, the effect of synthetic dihydropyridone on liver microsomal metabolism was examined. Preincubation of the dihydropyridone for 30 min with microsomes (prepared from livers of phenobarbital-treated rabbits) in the presence of NADPH resulted in a 10% decrease in microsomal N-demethylase activity compared to control values (Table 4). Preincubation of the dihydropyridone without NADPH resulted in no change in N-demethylase activity. In control samples, microsomes were incubated with or without NADPH in the absence of dihydropyridone and then carried through the same washing procedure described in the Methods section. Since PCP inactivates 45% of microsomal N-demethylase activity, it appears that this metabolite, which inactivates only 10% of the enzyme activity, is not responsible for the observed inhibitory effect of PCP.

DISCUSSION

The tentative identification, by HPLC coelution with the synthetic standard, of the 4-hydroxypiperidinyl metabolite (2) of PCP in NADPH supplemented rat brain microsomal incubations of PCP provides evidence that brain tissue is

Table 4

Effect of 1-(1-phenylcyclohexyl)-2,3-dihydro-4-pyridone on ketamine N-demethylase activity

Incubation	nmol HCHO/mg protein
-NADPH/ -dihydropyridone	14.07
	14.90
+NADPH/ -dihydropyridone	14.79
	14.75
-NADPH/ +dihydropyridone	14.52
	13.42
+NADPH/ +dihydropyridone	12.80
	12.72

1-(1-phenylcyclohexyl)-2,3-dihydro-4-pyridone (0.5 mM) was incubated according to method B for 30 min with liver microsomes from phenobarbital-treated rabbits.

capable of metabolizing this drug. Although a more complete metabolic profile of PCP in brain tissue remains to be established, the observation of metabolic activity is significant since the ability to metabolize would be a prerequisite for bioactivation of PCP to occur in brain. Because previously obtained results suggest that PCP-Im^+ is an obligatory intermediate in the bioactivation of PCP in liver microsomal preparations, the question was raised of whether the iminium species is generated by brain metabolism of PCP. In an attempt to answer this question, tritium-labeled PCP was incubated with brain microsomes and NADPH. Analysis of the post-incubation mixture by HPLC showed no peak of UV absorbance or radioactivity which corresponded with PCP-Im^+. This result was somewhat surprising in light of previous evidence that, in 30 min, approximately 29% of PCP is metabolized by liver microsomes to PCP-Im^+ (Ward et al., 1982b). Consequently, efforts turned toward examining the fate of PCP-Im^+ in brain tissue. The time-dependent decrease of PCP-Im^+ in brain tissues which were heat treated as well as untreated indicates that enzymatic processes are not involved in the disappearance. It is possible that the disappearance of PCP-Im^+ simply represents a slow conversion of PCP-Im^+ to its corresponding eneamine. In buffer alone, however, PCP-Im^+ was stable for up to 90 min. Furthermore, incubation of PCP-Im^+ for up to 75 min with the 100,000g supernatant fraction resulted in no decrease in PCP-Im^+

concentration. Thus, it seems unlikely that the disappearance of $PCP\text{-}Im^+$ is due only to its spontaneous conversion to the eneamine. These observations suggest that $PCP\text{-}Im^+$ molecules themselves may interact chemically with brain constituents. The results of these experiments are consistent with the proposal that phencyclidine neurotoxicity is mediated by reactive metabolites, generated either peripherally or in the brain, which interact with and modify the function of brain molecules. The interaction of PCP generated $PCP\text{-}Im^+$ with brain macromolecules might preclude solvent extraction of the iminium species from the mixture and thereby prevent detection by HPLC. The use of sodium cyanide in a microsomal incubation of PCP perhaps could lead to trapping of any generated $PCP\text{-}Im^+$ prior to its interaction with brain constituents. Identification of the corresponding alpha-aminonitrile would provide evidence for formation of $PCP\text{-}Im^+$ from PCP in brain. While studies in liver indicate that further metabolism of $PCP\text{-}Im^+$ is necessary for covalent binding to occur, it is possible that brain constituents which are not present in liver can be bioalkylated directly by $PCP\text{-}Im^+$. This possibility could be examined by studies on covalent binding of $PCP\text{-}Im^+$ to brain macromolecules.

Peripherally administered PCP has been shown to enter and persist for relatively long periods of time in rat

brain. The concentrations of PCP in rat brain 1, 2 and 3 weeks after a single intraperitoneal injection of PCP (25 mg/kg) were approximately 12, 6, and 5 ng/g tissue, respectively, and those of metabolites of PCP were 390, 230, and 74 ng-equivalents/g tissue, respectively (Misra et al., 1979). The brain to plasma ratios for PCP between 0.5 h to 48 h after injection ranged between 6 to 8.8. The ability of peripherally administered $PCP-Im^+$ to enter the brain and to be metabolized in the brain remain to be studied.

In summary, the results of these preliminary experiments indicate that metabolism of PCP by the brain is possible. The possibility that $PCP-Im^+$, generated in liver or other peripheral organs, may be directly reactive with brain components also exists. However, many questions, including those regarding the ability of the brain to generate $PCP-Im^+$ from PCP, the ability of $PCP-Im^+$ to enter the brain from the periphery, and the fate of $PCP-Im^+$ in the brain, remain to be answered.

The results of liver studies indicate that $PCP-Im^+$ is metabolized by cytochrome P-450-dependent enzymes as evidenced by the NADPH dependence of the reaction and its inhibition by SKF 525A as well as by carbon monoxide, both inhibitors of P-450. The inhibitory effect of N-octylamine on microsomal metabolism of PCP indicates that the

flavin-containing monooxygenase does not play a significant role in PCP metabolism. This observation is consistent with reports of failure to detect the metabolic formation of PCP N-oxide (12, Cho et al., 1983), the product expected from flavin-containing monooxygenase-catalyzed oxidation of a tertiary amine (Cashman and Ziegler, 1986). The enhancement by N-octylamine of PCP-Im^+ disappearance from microsomal incubations would suggest that the iminium species is a substrate for the flavin-containing monooxygenase. However, observation of the same increased rate of PCP-Im^+ disappearance with N-octylamine regardless of whether NADPH was present indicates that this enzyme is not involved since it requires NADPH as a cofactor. No explanation for this puzzling observation has yet been found. Incubation of PCP iminium perchlorate with the purified flavin-containing monooxygenase, rather than microsomes, might provide an answer to the question of whether PCP-Im^+ is a substrate for this enzyme.

In studies of liver microsomal incubations of PCP-Im^+ the metabolism-dependent formation of a new species was noted. This metabolite was isolated and characterized. From the results of HPLC-DAD, high resolution EI-MS, and NMR analyses, the metabolite was tentatively assigned the dihydropyridone structure (28) which subsequently was characterized fully by comparison with an authentic synthetic sample. The presence of additional NMR signals,

particularly in the olefinic region of the spectrum, indicates that other species are present. The possibility that such species may include isomers of the dihydropyridine (28) which would be consistent with the observation of only one molecular ion at m/z 255 in the EI and CI mass spectra. The absence of UV absorbance by such isomers between 220-400 nm, as expected for compound 34 (Sundburg et al., 1967), might account for the observation of only one chromophore. The original NMR may have represented such an isomer(s). The identification of other species in the metabolite sample and the interpretation of the original NMR spectrum have not been achieved.

The dihydropyridone metabolite was formed also in microsomal incubations with PCP. The relative rates of dihydropyridone generation from PCP and from PCP-Im^+ suggest that formation of this metabolite proceeds via the iminium ion (Fig. 23). Sodium cyanide inhibition of dihydropyridone formation from PCP would provide further support for this suggestion. Metabolism of PCP to the iminium ion is cytochrome P-450-dependent (Ward et al., 1982b) as is the further metabolism of PCP-Im^+. Approximately 23% of PCP-Im^+ (0.5 mM) is converted to the dihydropyridone after a 30 min incubation with liver microsomes (1.5 mg protein/ml). Formation of the dihydropyridone from PCP-Im^+ is NADPH-dependent and time-dependent, suggesting that this also is a cytochrome

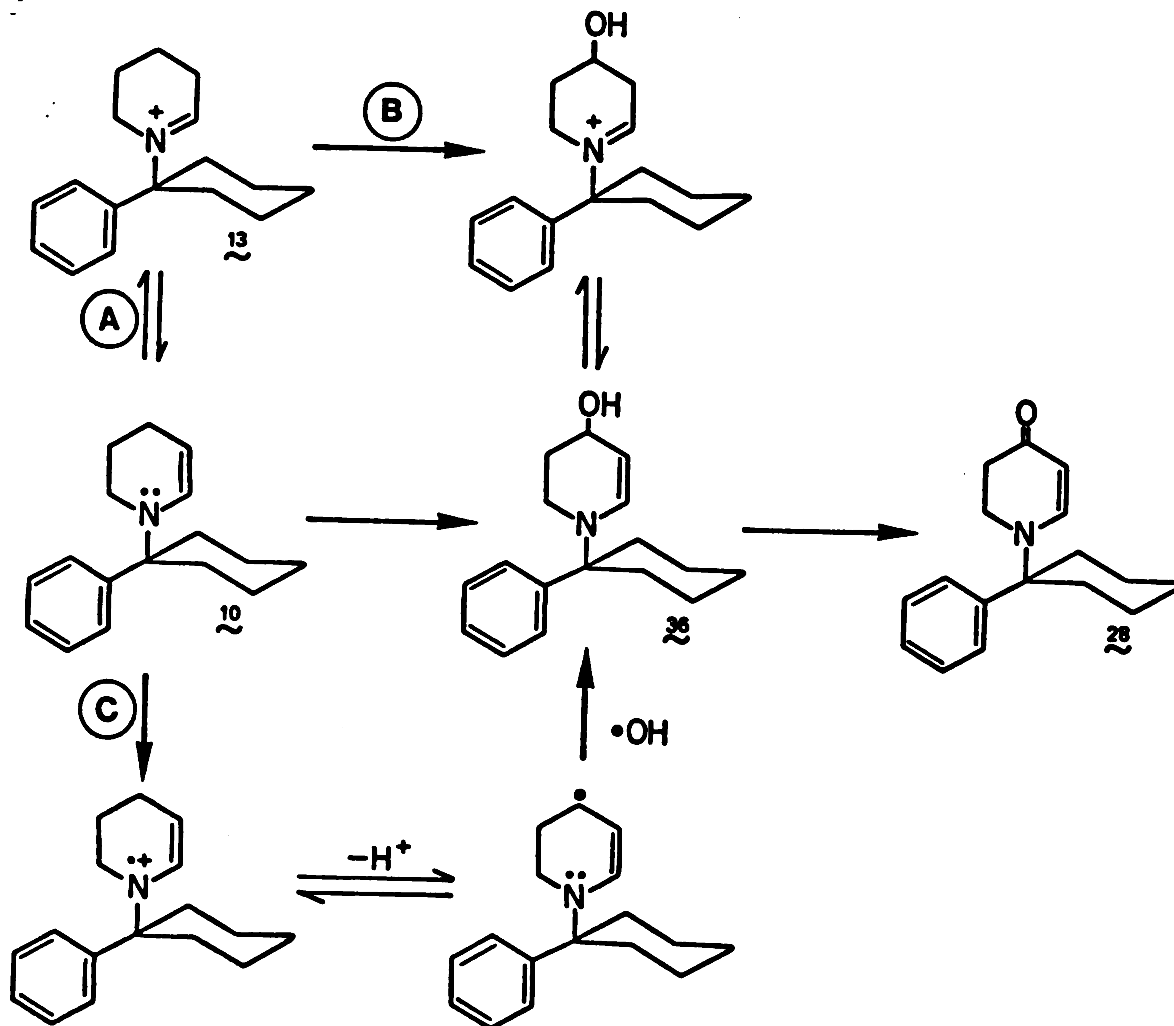


Figure 30. Proposed pathway for metabolic formation of 1-(1-phenylcyclohexyl)-2,3-dihydro-4-pyridone.

P-450-dependent step in the metabolism of PCP. Further evidence to establish the role of cytochrome P-450 might be provided by experiments to determine whether dihydropyridone formation is inhibited by SKF 525A and carbon monoxide. More definitive evidence might be obtained through the use of purified cytochrome P-450 in metabolism experiments.

The pathway by which PCP-Im^+ is converted to the dihydropyridone is far from clear. By analogy with the previously characterized biotransformation of PCP to the 4-hydroxypiperidinyl metabolite (2, Wong and Biemann, 1975; Wong and Biemann, 1976), the iminium species might undergo a cytochrome P-450 mediated hydroxylation at the 4 position of the piperidine ring (Fig. 30). The hydroxy group then might be oxidized further to the corresponding ketone (28). Equilibrium between the iminium ion and its corresponding eneamine might occur either before (Fig. 30, pathway A) or after (Fig. 30, pathway B) hydroxylation, resulting in the product 1-phenyl-(1-cyclohexyl)-1,2,3,4-tetrahydropyridin-4-one 28. Alternatively, the allylic alcohol (36) might be formed via a radical mechanism (Fig. 30, pathway C). Studies on the extent of formation of the dihydropyridone metabolite showed that its concentration increased with incubation time up to 20 min after which the concentration decreased. This observation suggests that the dihydropyridone species is unstable in the microsomal

incubation mixture and perhaps undergoes further metabolism. Preliminary studies to examine the metabolic fate of synthetic dihydropyridone in microsomal incubations suggested a time-dependent decrease in its concentration. Qualitatively, the decrease was not as dramatic as might have been expected from studies on dihydropyridone formation and subsequent disappearance in microsomal incubations of $PCP\text{-}Im^+$. This apparent difference in rates of dihydropyridone disappearance may have been due to differences in its concentrations in the 2 experiments. In one case, the dihydropyridone was generated from $PCP\text{-}Im^+$ (0.5 mM), whereas, in the other experiment, the starting concentration of the dihydropyridone itself was 0.5 mM. When dihydropyridone is present at an initial concentration of 0.5 mM, its relative disappearance may be less than when 28 must be generated from $PCP\text{-}Im^+$, and therefore is present at a lower concentration.

Incubation of the chemically synthesized dihydropyridone with liver microsomes resulted in a 10% decrease in N-demethylase activity. It appears, therefore, that this metabolite is not responsible for the 45% inhibition of enzyme activity which is observed after incubation with PCP. Nevertheless, characterization of this $PCP\text{-}Im^+$ metabolite may be a step toward elucidating the pathway of bioactivation of PCP. The dihydropyridone represents the product of a 4 electron oxidation of the iminium species, a

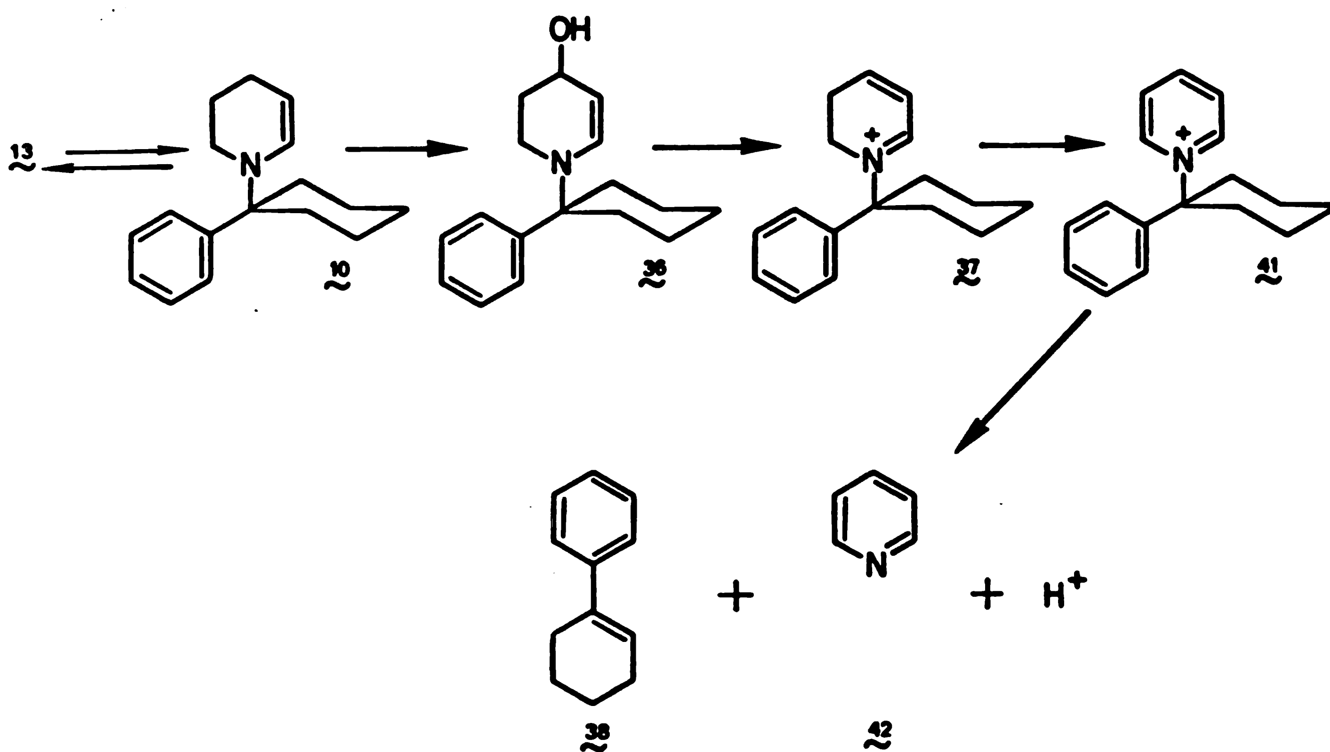


Figure 31. Proposed pathway for metabolic formation of potentially reactive intermediates of PCP.

process which presumably involves the formation of an intermediate 2 electron oxidation product(s). The existence of such intermediates must be considered and may lead to formation of reactive species. The allylic alcohol, for example, is a possible intermediate which might undergo loss of hydroxide ion to form the dihydropyridinium species (37; Fig. 31). This species is structurally similar to $MPDP^+$ (23), an intermediate formed in the bioactivation of the neurotoxin MPTP by MAO-B (Chiba et al., 1985).

Thus, liver studies have led to the characterization of a new metabolite of PCP and indications that other metabolites, possibly structural isomers, also are formed. Although the characterized metabolite appears not to mediate the PCP-induced inactivation of liver microsomal ketamine N-demethylase, precursors of this metabolite may be involved directly or may lead to formation of reactive species which would, in turn, inhibit enzyme activity. It should be noted also that evidence exists for the formation of other metabolites of PCP iminium perchlorate. This evidence includes the observation of 2 new peaks of radioactivity upon reverse phase HPLC separation of $PCP\text{-}Im^+$ incubation mixtures (Fig. 20). The identity of these 2 peaks is yet unknown, though it is likely that one represents the dihydropyridone. Nevertheless, the presence of 2 new peaks indicates that more than one metabolite was formed. The NMR spectrum of the purified metabolite sample

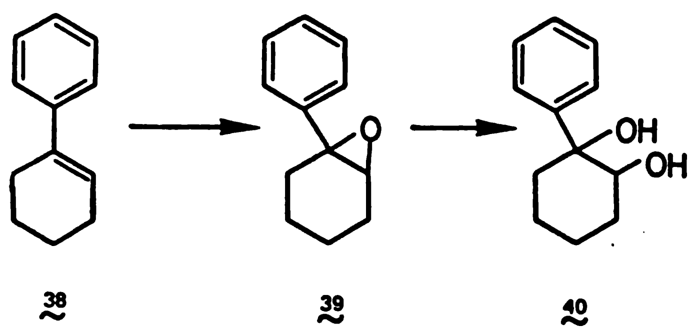
(Fig. 27A) suggests the presence of other species, possibly structural isomers of the dihydropyridone, and provides further evidence for the existence of more than one microsomal metabolite of $PCP\text{-}Im^{+}$.

CONCLUSIONS AND DISCUSSION

Results of the experiments discussed above have provided strong evidence that the previously reported metabolism-dependent covalent binding of phencyclidine to biological proteins is accompanied by changes in microsomal enzyme function. The iminium ion metabolite of PCP was implicated both in covalent binding and enzyme inhibition by the protective effect of cyanide ion which traps $PCP\text{-}Im^{+}$ to form a stable cyano adduct. The role of the iminium species in PCP bioactivation was supported further by studies in which chemically synthesized $PCP\text{-}Im^{+}$ incubated with liver microsomes produced similar effects to those observed earlier with PCP, namely, covalent binding, inactivation of ketamine N-demethylase, and destruction of cytochrome P-450.

Although hepatotoxicity is not a prominent effect of PCP abuse in humans, liver necrosis attributed to the use of PCP has been reported (Armen et al., 1984). The necrosis was thought to be caused by malignant hyperthermia, a complication of PCP abuse. However, the possibility also exists that PCP may have played a direct role in causing some degree of toxic liver cell damage. It is not clear whether chronic PCP use in humans leads to alterations in hepatic mixed function oxidase activity. Such an effect might be significant in changing the disposition of drugs abused concurrently with PCP by altering the rates and pathways of their metabolism.

It should be noted, also, that in studies reported thus far on the covalent binding of PCP and PCP-Im^+ to tissue proteins, the radiolabel has been incorporated in the phenyl ring of the drug molecule (Law and Farquharson, 1980; Law, 1981; Ward et al., 1982b). The possibility remains, therefore, that the piperidine ring and/or the cyclohexyl moiety may not be directly involved in the covalent binding. For example, a potential breakdown product of PCP, 1-phenylcyclohexene (38, PC), might undergo further metabolism to potentially reactive species such as the epoxide (39). In fact, PC has been reported to be a minor metabolite of PCP in rabbit liver preparations (Cho et al., 1983). However, because GC-MS methods were used to identify this metabolite, it is possible that the conditions of GC led to the formation of 38 by thermal decomposition of PCP or its metabolites. If such were the case, PC may not actually have been present in the biological sample. PC is known to be a pyrolysis product when PCP is smoked (Cook et al., 1981; Freeman and Martin, 1981) and its metabolism has been studied. Irreversible binding of radioactivity to rat tissues after *in vivo* administration of ^{14}C-PC has been reported (Chakrabarti et al., 1983). Other investigators, however, were not able to observe significant binding of radioactivity after methanol extraction of *in vitro* mouse liver homogenate incubations with phenyl ring tritiated PC (Martin et al., 1982). The apparent inconsistencies between the two


OH
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studies were suggested to be due to species differences or to different metabolic conditions. The identification of 1-phenylcyclohexane-1,2-diol (40), a hydrolysis product of 39, as one of the metabolites of PC in mouse liver (Martin et al., 1982) and rat liver (Cook et al., 1984) microsomal mixtures is consistent with the hypothesis that phenylcyclohexene oxide (39) is a reactive metabolite of PC. The argument could be made, therefore, that the piperidine ring is not involved directly in covalent binding of PCP to proteins. The inhibition of covalent binding by cyanide ions has been attributed to the trapping of the nucleophilic iminium ion intermediate and blocking its further metabolism to the ultimate reactive species. The protective effect of cyanide ions is not inconsistent with the proposal that a metabolite of PC could be a reactive species. The PCP-Im^+ might be metabolized further to a species from which the piperidine moiety is lost, yielding 1-phenylcyclohexene which, in turn, could generate reactive species. Trapping of the iminium ion would effectively block its further metabolism leading to generation of PC. In addition, cyanide ions could inhibit the enzyme(s) involved in steps of the metabolism of PC leading to formation of reactive species.

Investigation of the possible role of PC in the bioactivation of PCP should begin with establishing whether PC is a metabolically generated intermediate. This could be

done by using synthetic PC as a standard in HPLC analysis of post-incubation mixtures. The question might be addressed indirectly as well. If the major reactive metabolites of PCP are derived exclusively from PC, direct involvement of the piperidine ring in covalent interactions with tissue proteins would not be expected. This question might be examined by repeating the covalent binding studies using phencyclidine, or its iminium ion, with the radiolabel incorporated in the piperidine rather than the phenyl ring. Failure to observe covalent binding of radioactivity would be an indication that the piperidine moiety was not directly involved in the binding. The presence of radioactivity associated with the microsomal proteins after repeated extractions would strongly suggest the involvement of the piperidine ring in covalent binding of PCP to macromolecules. Because of the possible loss of a piperdinyl tritium ion through proton exchange or during a metabolic reaction, the radiolabel should be incorporated at one of the carbon atoms.

Our studies of liver microsomal metabolism of $PCP\text{-}Im^{+}$ led to the characterization of a dihydropyridone metabolite which does not itself appear to be involved in the bioactivation of PCP. Identification of this 4 electron oxidation product of $PCP\text{-}Im^{+}$ suggests the existence of an intermediate resulting from a 2 electron oxidation of $PCP\text{-}Im^{+}$. One possible structure for this proposed

intermediate is the corresponding alcohol (36). The alcohol conceivably could lose hydroxide ion to form the dihydropyridinium species (37) which might in turn be oxidized to form the pyridinium species (41). Loss of the pyridine moiety from 41 would yield PC. In fact, attempts to synthesize 36 chemically did result in the isolation of 38 (Schmidt-Peetz, personal communication). Thus, while the newly identified PCP metabolite does not appear to be involved in bioactivation of PCP, it may provide clues to intermediates which themselves might be reactive or might lead to formation of reactive species. Another approach which might be used to identify reactive intermediates of PCP metabolism is to characterize covalent adducts formed by the interaction of metabolites with proteins. This method has been used to identify the cytochrome P-450-generated trichloromethyl free radical metabolite of carbon tetrachloride which is capable of binding to phospholipids in the membrane surrounding cytochrome P-450 (Trudell et al., 1982).

PHARMACOLOGICAL RELEVANCE

Although the results of experiments to examine the effects of PCP on liver microsomal enzyme activity indicate that bioactivation of PCP may lead to biochemical, functional changes, possible relationships between these effects and the prolonged PCP-induced neurotoxicity are not obvious. Many questions relating the observed *in vitro* effects to *in vivo* toxicity have not been answered. Since the PCP iminium species appears to be critical in the bioactivation pathway, either formation of $PCP\text{-}Im^+$ in the brain or the ability of $PCP\text{-}Im^+$ to enter brain must be established. Once $PCP\text{-}Im^+$ is in the brain, its reactivity with brain tissues, as evidenced by covalent binding or functional changes such as enzyme inactivation, should be studied. The next question which arises is whether modulation of factors influencing bioactivation corresponds with a parallel effect on toxicity. This question may be much more difficult to answer because it requires a working model for the neurotoxicity of PCP. Unlike the toxic effect of MPTP, which is well-characterized as destruction of neurons in the substantia nigra associated with depletion of dopamine in the striatum, the toxic effects of PCP are not well-understood. Although the PCP-induced psychosis is said to resemble schizophrenia, as MPTP neurotoxicity is said to resemble Parkinson's disease, idiopathic schizophrenia is not clearly defined on a biochemical level. The picture is further complicated by the fact that the occurrence of the PCP psychosis is

unpredictable. Not all users of the drug develop the long term toxicity and factors which might aid in determining the long term outcome of PCP exposure, such as dose, have not been established. As discussed earlier, drug dose appears not to be related to the development of chronic PCP toxicity. Because of these complexities, a behavioral model for chronic PCP toxicity may not be readily established. Until such a model is available, a less complex neurochemical or neurophysiological system might be considered.

As discussed previously, the PCP-precipitated psychosis consists of a profound perceptual and cognitive disturbance that resembles the primary symptoms of schizophrenia (Fauman et al., 1976; Fauman and Fauman, 1977; Petersen and Stillman, 1978; Aniline and Potts, 1982). An excess release of a variety of neurotransmitters, especially dopamine, has been proposed to underlie the symptoms of both PCP intoxication (Domino, 1980; Rappolt et al., 1980; Johnson, 1983) and schizophrenia (Snyder, 1981). The use of antipsychotic agents in the treatment of PCP-psychosis (Smith and Wesson, 1980) as well as schizophrenia (Baldessarini, 1985) is consistent with this suggestion.

Pharmacological and biochemical evidence has revealed broad effects of PCP on the dynamics of a variety of neurotransmitters including dopamine (Doherty et al., 1980;

Johnson, 1983; Bowyer et al., 1984), norepinephrine (Taube et al., 1975), serotonin (Smith et al., 1977; Nabeshima et al., 1985), histamine (Itoh et al., 1985), gamma-aminobutyric acid (Nabeshima et al., 1981) and acetylcholine (Maayani et al., 1974). These changes were observed at pharmacologically relevant concentrations of PCP. The effects of PCP on dopamine synthesis and release are quite complex and differ according to the preparation being used. PCP stimulates dopamine synthesis in synaptosomes in vitro (Vickroy and Johnson, 1981) but inhibits tyrosine hydroxylase activity (the rate determining step in dopamine synthesis) in vivo (Doherty et al., 1980). In rat striatal synaptosomes, PCP stimulates release and inhibits uptake of dopamine (Bowyer et al., 1984). The release of stored as well as newly synthesized dopamine by slices of rat brain striatum is enhanced by PCP (Vickroy and Johnson, 1983). A massive release of dopamine may underlie some of the most prominent symptoms of PCP intoxication such as the hypertensive crisis and hyperthermia (Rappolt et al., 1980). Synaptosomal uptake of norepinephrine (Taube et al., 1975) and serotonin (Smith et al., 1977) is inhibited by PCP. Administration of PCP to mice appears to increase brain histamine turnover possibly by facilitating the release of histamine (Itoh et al., 1985). Evidence obtained in vitro indicates that PCP both antagonizes the acetylcholine receptor and has potent anticholinesterase activity

(Mayaani et al., 1974).

PCP and behaviorally active congeners have been found to block potassium ion conductance (Albuquerque et al., 1983; Bartschat and Balustein, 1986). These compounds were found to block selectively a presynaptic voltage-regulated potassium ion channel, one of four characterized physiologically and pharmacologically distinct potassium channels (Bartschat and Blaustein, 1986). It has been suggested that blockage of a fraction of presynaptic potassium channels might be sufficient to prolong the action potential duration and thereby enhance calcium-dependent neurotransmitter release at central synapses involved in behavioral expression (Blaustein and Ickowicz, 1983). The rank order of potency for blockage of this class of potassium channels parallels both the relative ability of these agents to produce characteristic behavioral deficits in rats and their ability to displace radiolabeled PCP from its high affinity binding sites in brain (Bartschat and Blaustein, 1986). Further studies led to the suggestion that a previously characterized high affinity PCP binding protein is associated with the potassium channels blocked by nanomolar concentrations of PCP (Blaustein and Ickowicz, 1983).

The discovery of high affinity ^{3}H-PCP binding sites in rat brain was first reported in 1979 (Zukin and Zukin,

1979). The ^{3}H-PCP was shown to be displaceable by non-radioactive PCP and a series of more than 30 different PCP analogs, with relative potencies that correlated with their relative potencies in behavioral tests (Zukin and Zukin, 1983). Non-PCP like hallucinogens and known neurotransmitters proved inactive in the binding assay. ^{3}H-PCP binding was most enriched in crude synaptosomal subcellular fractions and was about three times higher in hippocampus (region of highest density) than in cervical spinal cord (region of lowest density). Binding sites displayed the expected characterisitcs of a protein receptor (Zukin et al., 1983; Vincent et al., 1980). ^{3}H-PCP binding sites were visualized by using tritium-sensitive film analyzed by computer densitometry and color coding (Quirion et al., 1981). The ^{3}H-PCP was found to bind most densely to cortical areas, diffusely in neocortex, and somewhat heterogeneously in the laminae of the hippocampal formation and dentate gyrus. Most of the brainstem and spinal cord showed low specific ^{3}H-PCP binding, with gray matter generally showing more binding than white. Although morphine, naloxone, and opiate peptides did not displace bound ^{3}H-PCP, psychotomimetic benzomorphans, classifed as sigma opiates, were quite potent displacers in vitro and had PCP-like behavioral properties in vivo (Quirion et al., 1981). These results suggest that PCP and the sigma opiates act at the same sites.

The concept of opiate receptor heterogeneity (Martin et al., 1976) led to the identification of four classes of opiate receptors which were distinguished on the basis of: 1) differing physiological and behavioral profiles of different opiates, 2) differing sensitivities of agonists to naloxone antagonism, and 3) differences in binding characteristics and protection potentials. Mu receptors were proposed to mediate the classical opiate actions of morphine and similar narcotics. Kappa receptors were defined as sites at which ketocyclazocine and related benzomorphans produce analgesia as well as side effects, including miosis, sedation, and ataxia. Sigma receptors were postulated to mediate the unique actions of SKF 10,047, cyclazocine, and related drugs which produce tachycardia, tachypnea, and "canine delirium", which was thought to be the equivalent of human psychotomimetic effects (Martin et al., 1976). Delta receptors exhibit a higher affinity for the naturally occurring enkaphalins relative to morphine.

While less is known about the sigma receptor than the other opiate receptors, sigma opiates are known to be psychotomimtic in man (Jasinski, 1977). Based on the insensitivity of sigma behavioral effects in animals to reversal by naloxone, these technically are non-narcotic (Teal and Holtzman, 1980). However, naloxone was shown to

displace specifically bound ^{3}H-PCP from its receptor sites (Zukin and Zukin, 1979). In rodents, primates, and pigeons, a variety of non-classical opioid derivatives have been found to elicit the PCP-appropriate stimulus in PCP-trained animals (Zukin and Zukin, 1983). The results of biochemical and pharmacological studies are thought to suggest the pharmacological relevance of the PCP/sigma receptor in mediating the psychotropic actions of PCP and the sigma opiates.

Synaptosomal potassium conductance might be a system to consider for further investigation of the possible role of PCP bioactivation in toxicity. The high affinity PCP binding protein thought to be associated with the potassium channels blocked by PCP may represent a target for covalent binding by reactive metabolites and may be modified irreversibly as a result of the interaction. Although the duration of the PCP effect on potassium ion conductance was not reported, studies on its persistence might be worthwhile to determine whether there is any possibility that the duration could correspond to neurotoxicity. If the effect of PCP itself is determined to be reversible and if covalent binding of PCP-derived reactive species can be shown to result in irreversible changes, this system might be an appropriate model for further investigation of the role of PCP bioactivation in the long lasting neurotoxic effects of the drug.

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