Biphenyl Hydroxylation by Vole Hepatic Microsomes

Moheb H. Makary
Utah State University

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BIPHENYL HYDROXYLATION BY VOLE HEPATIC MICROSONES

by

Moheb H. Makary

A thesis submitted in partial fulfillment
of the requirement for the degree
of
MASTER OF SCIENCE
in
Toxicology

Approved:

Major Professor

Committee Member

Committee Member

Dean of Graduate Studies

UTAH STATE UNIVERSITY
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1981
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Moheb H. Makary
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ABSTRACT

Biphenyl Hydroxylation by Vole Hepatic Microsomes

by

Moheb H. Makary, Master of Science
Utah State University, 1981

Major Professor: Dr. William A. Brindley
Department: Biology

Metabolism of biphenyl by meadow voles (Microtus montanus) liver microsomes was studied by gas liquid chromatography. The microsomal biphenyl hydroxylation of Swiss Webster mice (outbred) was also investigated to provide a species comparison. 4-hydroxybiphenyl (major metabolite), 3-hydroxy biphenyl (minor) and smaller amounts of 2-hydroxybiphenyl were identified as metabolites of biphenyl incubated with vole liver microsomes. Liver microsomes from Swiss mice formed 4-hydroxy biphenyl (major metabolite) and almost equal amounts of 2- and 3-hydroxy biphenyl (minor metabolite). The amount of the hydroxy biphenyls formed by vole liver microsomes was considerably less per milligram of microsomal protein than for mice.

There was NADPH/NADH synergism with the formation of biphenyl hydroxylations in voles. Phenobarbital treatment of voles induced 2-, 3- and 4-hydroxy biphenyl formation, whereas only slight
increases were observed after administration of 3-methylcholanthrene or B-naphthoflavone. No changes in the amount of biphenyl 2-hydroxylation of mice followed pretreatment with phenobarbital. However, 3-hydroxylation showed a small increase and 4-hydroxylation was induced. Mice pretreated with 3-methylcholanthrene or B-naphthoflavone were preferentially induced in biphenyl 2-hydroxylation. The reduced hemoprotein difference spectra of microsomal fractions were studied. Only 0.8 nm shift of the Soret peak occurred in liver microsomes from voles pretreated with 3-methylcholanthrene. The induction and spectral data indicate that 2-, 3- and 4-hydroxylation are associated principally with cytochrome p-450 in voles with little evidence for a substantial role of cytochrome p-448. There is, therefore, a significant species difference between the hepatic microsomal mixed function oxidase system of meadow voles and white mice.

(68 pages)
INTRODUCTION

Most investigations of hepatic monooxygenase (mixed function oxidase, MFO) activities in mammals have been performed with rats and mice and their inbred strains. Less emphasis has been placed upon other rodents or wild animals though monooxygenases are among the most important detoxication systems. Many classes of xenobiotics are metabolized by monooxygenases of vertebrate and invertebrate animals. The principle physiological role of the enzyme is detoxication, however, recent data indicate that these same enzymes can convert certain polycyclic aromatic hydrocarbons to active carcinogenic derivatives (Kato, 1979).

Monooxygenase activities have been characterized in insecticide resistant pine vole Microtus pinetorum using aniline, ethylmorphine and benzo[a]pyrene as substrates (Hartgrove et al., 1977) and in grey tailed voles, M. canicaudus, using NADPH cytochrome C reductase, p-nitroanisole O-demethylase and aniline hydroxylase as substrates (Brindley, 1979); but in M. montanus, monooxygenases have not been characterized.

An EPA sponsored study group (Gillett and Witt, 1977) recommended the continued use of voles as the "top predator" in studies of xenobiotic distribution in model environment "...while conducting an active search for information on its biochemistry, physiology and behavior."

Biphenyl may be a useful probe for investigating the cytochrome P-450 mediated monooxygenase enzymes. Biphenyl is metabolized to
to 2-, 3-, and 4-hydroxy biphenyl by the monooxygenase.

The purpose of this research was to characterize biphenyl metabolism in vitro by vole liver microsomes. By comparing the metabolite pattern of biphenyl hydroxylation with and without treatment with enzyme inducing agents, it might be possible to determine how closely the monooxygenases of voles resemble those of mice and rats.
REVIEW OF LITERATURE

Cytochrome P-450 Mediated Monooxygenase Activity

Foreign compounds (xenobiotics) may enter the body through ingestion in food or drink, by inhalation, or by absorption through the skin. Excretion of foreign compounds occurs by way of urine, bile, feces, perspiration, vomitus, milk, hair, or expired air. Since many excretions are hydrated fractions the ease with which xenobiotics are excreted largely depends on their water solubility. Compounds that are more soluble in lipid-like materials (lipophilic) tend to accumulate in lipid rich tissues. Accumulation in the body may affect cellular processes, leading to a toxic response, or to an adaptation-like induction of monooxygenase.

A number of enzymes in animals are capable of metabolizing lipophilic compounds in such a way as to render them more water soluble and thus more easily excreted. Williams (1959) has proposed that these enzymes are either phase I reactions, involving oxidation, reduction, and hydrolysis or phase II reactions, consisting of conjugation or synthesis. Phase I reactions generally convert foreign compounds to derivatives that can undergo phase II reactions. The phase II conjugation products of the hydroxylated metabolites of biphenyl (phase I products) are examples of these (see "Biphenyl Metabolism In Vivo"). The conjugates are sufficiently polar to be excreted from the cell and from the body.

The cytochrome P-450 mediated monooxygenases are a collective example of phase I enzymes or enzyme systems. These membrane bound
multicomponent enzyme systems requiring molecular oxygen and NADPH (Mason, 1957), are also known as mixed function oxidases. They metabolize polycyclic hydrocarbons such as benzo[a]pyrene (ubiquitous in city smog, cigarette smoke, and charcoal-cooked foods), 3-methylcholanthrene, and biphenyl; halogenated hydrocarbons such as polychlorinated and polybrominated biphenyls, insecticides, and ingredients in soap and deodorants; strong mutagens such as N-methyl N'-nitro-N-nitrosoquinidine and nitrosamines; aminoazo dyes and diazo compounds; N-acetylarylamines and nitrofurans; numerous aromatic amines, such as those found in hair dyes, nitro aromatics, and heterocyclics; wood terpenes; epoxides; carbamates; alkylhalides; safrole derivatives; certain fungal toxins and antibiotics; many of the chemotherapeutic agents used to treat human cancer; most drugs; ethanol; naturally occurring and synthetic steroids, and other endogenous substrates such as biogenic amines, indoles, thyroxine and fatty acids (Nebert and Jensen, 1979; Goldstein et al., 1974).

Cytochrome P-450 denotes, collectively, all forms of membrane-bound hemoprotein associated with NADPH-dependent monooxygenase activities that are also capable of complexing with carbon monoxide. Difference spectra of these reduced complexes show an absorption peak at about 450 nm. In some cases, the absorption peak is shifted, for example to 448 nm, indicating the presence of another cytochrome.

The hemoprotein, cytochrome P-450, is the terminal oxidase in the phase I metabolism of many foreign compounds. The cytochrome interacts with molecular oxygen and the xenobiotic to be metabolized (substrate). The cytochrome-substrate-oxygen complex and two sequential reductions result in insertion of one oxygen atom into the substrate
(xenobiotic) and conversion of one oxygen atom to water or, possibly, peroxide. The reductions are mediated by NADPH through a membrane-bound flavoprotein called NADPH-cytochrome P-450-reductase. It may also be possible for NADH, via cytochrome b\textsubscript{5} to contribute to the reaction rate, but NADH itself cannot support the monooxygenase reaction. The redox changes that occur during substrate hydroxylation can be characterized according to the simplified Scheme 1, where R is substrate, Fe\textsuperscript{2+}(3+) is the heme iron of cytochrome P-450 with valence state, and ROH is the oxygenated product. Reducing equivalents are derived from NADPH through an intermediate flavoprotein, NADPH-cytochrome C reductase (Fp\textsubscript{1}). The first step in the sequence of events leading to substrate oxidation is the binding of substrate to the oxidized form of cytochrome P-450. The complex is then rapidly reduced, presumably by way of a one-electron transfer, and this reduction has been suggested as rate-limiting to the overall monooxygenation of numerous substrates (Davis et al., 1969). The cytochrome-substrate complex then interacts with molecular oxygen to form a ternary reduced cytochrome substrate-oxygen complex. A second electron is subsequently accepted, and after the uptake of two protons, the complex dissociates into oxidized cytochrome, H\textsubscript{2}O, and product. The origin of the two sequential electrons donated to the cytochrome-substrate complex is uncertain; it is not clear whether both electrons are delivered via the NADPH-NADPH-cytochrome c reductase redox system under all conditions. Cohen and Estabrook (1971) and Hildebrant and Estabrook (1971) have suggested that in the presence of limiting NADPH, the second electron might be derived from cytochrome b\textsubscript{5} via the NADPH-cytochrome b\textsubscript{5} reductase (Fp\textsubscript{5}). It appeared that NADH had a
synergistic effect on NADPH-dependent oxidative reactions (Hildebrant and Estabrook, 1971). However, cytochrome b$_5$ is not an obligatory component of the reconstituted monooxygenase system (Mannering et al., 1974), and its importance as an electron donor for cytochrome P-450 under normal circumstances must be minimized.

Figure 1. Electron flow pathway in the microsomal drug-oxidizing system.
(Adopted from Short, et al., 1976)
The cytochrome P-450-containing monooxygenases are localized in the endoplasmic reticulum, a complex network of membranes within the cell that is continuous with the outer nuclear membrane. It is perhaps most reasonable to assume that xenobiotic oxygenation occurs at or near the outside surface of the lipoidal membrane and that the more polar intermediates or products are then repelled from the hydrophobic membrane surface. It is feasible however, that oxygenation of certain substrates occurs within the membrane and that the conjugated polar product is repelled from the inside surface of the membrane (Nebert, 1979).

When a cell is homogenized, the endoplasmic reticulum degrades to small vesicles known as microsomes. Most in vitro studies with the cytochrome P-450-containing monooxygenases are carried out using microsomes.

The cytochrome P-450-containing monooxygenases are found predominantly in the endoplasmic reticulum of the liver; however, kidney, lung, intestine, brain, and skin also contain measurable activities of these enzyme systems. Cytochrome P-450-containing monooxygenases are also found in liver mitochondria, mitochondria of the adrenal cortex and in the endoplasmic reticulum of cells from testes and ovary. However, these latter enzyme systems are rarely, if ever, involved in the metabolism of foreign compounds (Testa and Jenner, 1976; Gram and Gillette, 1971).

**Biphenyl**

Biphenyl is a hydrocarbon of two phenyl rings (C\textsubscript{12}H\textsubscript{10}). It has been used as a heat transfer agent and a fungistat for preserving citrus fruit during transport and storage. Biphenyl forms the
nucleus of the polychlorinated and polybrominated biphenyl, two
groups of industrial chemicals that pervade our environment. In
pesticide application, biphenyl is usually impregnated into wrapping
paper so the air space between the packaging and the fruit becomes
saturated with biphenyl vapor (Anonymous, 1964). It is the protective
packaging of fruit that leads to most interest with biological and
toxicological properties of biphenyl. Data on the toxicology of
biphenyl have not been extensive. The lack of data, especially in
humans, has given the impression that its toxicity is fairly low.
However, Booth et al. (1961), fed biphenyl to rats and found polyuria
and increased volumes of solids in the urine. In addition, the
kidneys of some rats experienced damage in the form of several
cysts and dilated tubules in the medulla and inner cortex.

Häkkinen et al. (1973) offered the first clinical account of the
death of one man and the poisoning of eight other workers who worked
in the production of biphenyl-impregnated fruit wrapping paper under
poor hygienic conditions. The biphenyl concentration in the air had
been much in excess of the present threshold limit value of 1 mg/m³.
The clinical picture of biphenyl poisoning was characterized by
central and peripheral nerve damage and liver injury. The cause of
death in the fatal case was acute yellow liver atrophy. In three
of eight men poisoned, there was histological evidence of liver injury.

Biphenyl may be a useful probe for investigating the cytochrome
P-450 mediated monooxygenase enzymes which metabolize drugs, steroids,
and carcinogens. Biphenyl is oxidatively metabolized in vivo and
in vitro by the hepatic microsomal monooxygenase enzyme system.
Biphenyl metabolic data are often cited as comparisons between the ratios of formation of the primary hydroxylation products of biphenyl with or without treatment with specific enzyme inducing agents. Examples are discussed in the following sections. Billings and McMahon (1978) concluded that biphenyl was an "ideal" substrate for use in assessing the overall capabilities of the standard microsomal preparation used in many standardized toxicology tests."

Biphenyl Metabolism In Vivo

Biphenyl was reported by Klingenberg (1891) to be converted to 4-hydroxy biphenyl in the dog and excreted as an ethereal sulfate. Stroud (1940), reported that biphenyl is also converted by rabbits to this metabolite but that four-fifths is excreted as the free form.

West et al. (1956) found the major metabolite in rats fed with biphenyl was 4-hydroxy biphenyl. The free phenol accounted for 29.93% of the biphenyl administered, while the glucuronide accounted for an additional 18.4%. 4,4'-dihydroxybiphenyl, 4-phenylcatechol, and mercapturic acid were minor metabolites representing 5.26, 2.06, and 1.27%, respectively, of the biphenyl fed.

Block and Cornish (1959) studied the metabolism of biphenyl and 4-chlorobiphenyl in the rabbit after administration into the stomach of either 1 gram biphenyl or 1 gram of 4-chlorobiphenyl. The urine contained 26% of the biphenyl as the glucosiduronic acid derivative but 50% of the ingested 4-chlorobiphenyl was excreted in this manner. Quantities of free phenolic compounds in the urine differed for the ingested biphenyl (24%) and the 4-chlorobiphenyl (3%). Ethereal sulfate excretion accounted for 13 and 11% respectively,
of the ingested biphenyl and 4-chlorobiphenyl. Neither biphenyl nor 4-
chlorobiphenyl seemed to be excreted as mercapturic acid derivatives.

Creaven and Parke (1966) gave to weanling Wister albino rats and
weanling albino mice (I.C.I. strain) oral or intraperitoneal adminis-
trations of biphenyl. The rat urine contained 18-20% of the dose as 4-
hydroxy biphenyl and its conjugates, and 2% as 2-hydroxy biphenyl and
conjugates. The urinary excretion of the mice contained 25% of the dose
as 4-hydroxybiphenyl and 5.5% as 2-hydroxy biphenyl. The 4-/2- ratios
for the in vivo experiments agree with those obtained in this study for
the in vitro experiments in mice and female rats, as will be reviewed
in a later section.

In experiments with $^{14}$C-biphenyl, Meyer et al., (1976) were able to
show both an acidic and a phenolic urinary fraction from male albino
rats. Extraction and fractionation of the 1 day urine samples showed
that the largest fraction consisted of conjugated phenolic metabolites
and acidic metabolites, amounting to a quarter of the dose.

Meyer and Scheline (1976); Meyer et al., (1976), Meyer (1977), and
Meyer and Bakker (1977), investigated the phenolic fraction from male
albino rats, male and female pigs, male guinea pigs and white land rabbits,
and some marine organisms by gas chromatography and combined gas chroma-
tography/mass spectrometry. In rats, the main route of excretion was
via the urine and the metabolites detected were conjugates of mono-,
di- and trihydroxy derivatives of biphenyl as well as the meta- and
para methyl ethers of catecholic compounds. The two main urinary
metabolites were 4-hydroxybiphenyl and 4,4' dihydroxybiphenyl (Meyer
and Scheline, 1976).
In both male and female pigs, biphenyl was metabolized mainly to mono-hydroxylated biphenyl, and in small amounts to di- and tri-hydroxylated biphenyls. (Meyer et al., 1976).

In male guinea pigs and rabbits (White Land Rabbit) biphenyl was hydroxylated to biphenylols with minor amounts of biphenyldiols. The main route of body clearance appeared to be by way of the urine in both species. In the urine of guinea pigs and rabbits, the major metabolites were 4-hydroxy biphenyl (25.5% and 35.3% of the dose, respectively) (Meyer, 1977).

In some marine organisms, namely the crustacean Cirolana borealis Liljeborg (Isopoda), the gastropod Buccinum undatum L. (mollusca) and the ophiuroid Ophiocoma nigra (Abildgaard) (Echinodermata), 2-hydroxy biphenyl was the most prominent metabolite were found. In these marine organisms, metabolites of biphenyl were found both in the sea water from the aquaria and in the tissue of the respective marine organisms (Meyer and Bakker, 1977).

Halpaap et al. (1978) studied the metabolism of biphenyl in male Sprague-Dawley rats using gas chromatography and mass spectrometric methods. Eight new metabolites were isolated from the urine. A dihydro diol and two hydroxy dihydrodiols were thought to be characteristic of an epoxide-diol pathway. There were two dihydroxy biphenyls, a trihydroxy biphenyl, a trihydroxymethoxy biphenyl and 4,4'-dihydroxy-3-methylthiobiphenyl found.

**Biphenyl Metabolism In Vitro**

The use of in vitro monooxygenase assays to predict events in vivo deserves consideration. In vitro activities might be expected
to give a reasonable amount of activity in vivo without introducing complications of secondary metabolism conjunctions or distribution to various organs. This being the case, a more clear picture of primary hydroxylations and mechanisms may be obtained. This would be a valuable circumstance if species differences are suspected and are being studied.

Creaven et al. (1965) studied the enzymic hydroxylation of biphenyl by liver microsomal preparations of 11 species of animals. They used a fluorescence method for the microestimation of the hydroxylation products, 2- and 4-hydroxy biphenyl. Fluorescence spectroscopy does not distinguish 3-hydroxy biphenyl. Liver from all species examined produced 4-hydroxy biphenyl, but only those from mice, hamsters, cats, coypus and frogs produced 2-hydroxy biphenyl as well. Adult rat and rabbit livers produced only the 4-isomer, but livers from the young of these species also produced the 2-isomer. Therefore, the 2- and 4-hydroxylating systems are probably different.

Willis and Addison (1974) found that biphenyl was metabolized in vitro by tissue preparations of some marine organisms, to 4-hydroxy biphenyl and, to a lesser extent, 2-hydroxy biphenyl. The formation rate of 4-hydroxy biphenyl ranged from 400 to approximately 2 n moles per g tissue per hour and were generally much slower than those reported for several terrestrial species.

Burke and Bridges (1975) studied the metabolism of [14C]-biphenyl by hamster liver microsomes by qualitative thin layer chromatography and quantitative fluorimetry. They found that 4-hydroxy biphenyl (major metabolite) accounted for at least 83% of the total biphenyl metabolism. Small quantities of 2,2'- and 4,4'-
dihydroxybiphenyl metabolites were also tentatively identified. With respect to the effect of NADH on the metabolism of biphenyl. Burke and Bridges (1975) found that hepatic microsomal biphenyl 4-hydroxylation proceeded significantly faster when supported by an optimal concentration of NADPH (0.25 mM) than by a four-fold greater concentration of NADH, whereas the rates of 2-hydroxylation were almost equally effective with either cofactor. NADH is known to synergistically enhance NADPH supported microsomal demethylation reaction (Cohen and Estabrook, 1971). Likewise there was NADPH/NADH synergism with biphenyl 4-hydroxylation. The amount of 2-hydroxylation obtained with NADPH plus NADH was, however, considerably less than the expected additive effect although it was greater than the rate supported by either cofactor alone.

Burke and Bridges (1975) found also that biphenyl 2- and 4-hydroxy- and 2,2'-dihydroxy biphenyl gave type I spectral interactions with liver microsomes from hamster.

Billings and McMahon (1978) studied the metabolism of biphenyl incubated with liver microsomes or hepatic 9000×g supernatants from male Sprague-Dawley rats, white Swiss mice, Golden Syrian hamsters and New Zealand rabbits using gas liquid chromatography and gas chromatography-mass spectrometry. 3-hydroxy biphenyl was formed by rabbits, hamsters and mice in addition to the 2- and 4-hydroxy biphenyl. With the rat, 2.1 µg of 3-hydroxy biphenyl were formed along with 3.0 µg of 2-hydroxy biphenyl and 160 µg of 4-hydroxy biphenyl when the 9000×g supernatant fraction from 10 g of liver was incubated with biphenyl for 30 min. Hamsters, mice and rabbits formed 3-hydroxy biphenyl along with 2-hydroxy biphenyl and 4-hydroxy biphenyl. The
ratio of 2-hydroxy biphenyl to 3-hydroxy biphenyl was about 2:1 with hamster and rabbit microsomes, and 1:1 with mouse microsomes. 4-hydroxy biphenyl was the major microsomal metabolite of biphenyl in all species that have been studied. The formation of 2-, 3-, and 4-hydroxy biphenyl required NADPH. Addition of NADH stimulated the hydroxylations. In addition to the monohydroxylated products of biphenyl, the microsomal oxidation of biphenyl formed the catechol, 3,4-dihydroxybiphenyl, by incubation of either 3- or 4-hydroxy biphenyl.

Smith et al., (1980) studied the hydroxylations of biphenyl by fungi. Among 66 species of fungi, particularly interesting were Cunninghamella echinulata, ATCC 9244, which produced 4-hydroxy biphenyl as the major metabolite, Helicostylum piriforme, O2 M 6945, which produced exclusively 2-hydroxy biphenyl, and Aspergillus parasiticus, ATCC 15517, which produced a good yield of 4,4'-hydroxy biphenyl. Biphenyl hydroxylation has also been studied in vitro in avocado pear microsomes (McPherson et al., 1975a).

Induction of Biphenyl Hydroxylation

Conney (1967) classified microsomal enzyme inducers as being of two major types: "3-methycholanthrene-like" and "phenobarbital-like". Polycyclic hydrocarbon carcinogens and barbituates are generally considered to be divided into these two classes. Empirical data, measuring the induction of microsomal enzymes appear to be quite different between "activities" and these "two classes" of inducers. However, this simple classification in some cases has been ineffective in identifying drugs as being neither precisely "3-methycholanthrene-like" nor precisely "phenobarbital-like."

Caffeine, Pregnenolone-16α-carbonitrile, styrene (ug inhalation) and
2,2',4,4', 5,5'-hexabromobiphenyl are examples of these "atypical" enzyme inducers (Nebert, 1979).

Reports by Burke and Bridges (1975) and Creaven and Parke (1966) provide a useful perspective on the effect of inducing agents upon the ratio of 4-hydroxylation to 2-hydroxylation of biphenyl. Pretreatment of rats or hamsters with phenobarbital selectively induced the microsomal 4-hydroxylation of biphenyl whereas pretreatment with chemical carcinogens, such as 3-methylcholanthrene, induced both the 2- and the 4-hydroxylation reaction.

Arcos et al. (1961) found that some of the most potent compounds showing selective inductive effects are carcinogenic polycyclic hydrocarbons. However, many non-carcinogenic compounds produce similar stimulation of the microsomal enzymes so the relationship between carcinogenesis and induction, if any, is obscure.

Pretreatment of rats with phenobarbital produces a moderate stimulation of the 4-hydroxylation of biphenyl, as evidenced by recovery of the metabolite in the urine. The ratio of 4-hydroxylation to 2-hydroxylation increased from about 10 to 13. Benzopyrene administration resulted in stimulation of biphenyl-2-hydroxylation only, although the 4/2-hydroxylation ratio was lowered from the normal value of 10 to about 6. In mice no similar stimulation was observed with phenobarbital. On the contrary, a slight inhibition of both 2- and 4-hydroxylation was observed, with no significant change in the ratio. Pretreatment of mice with benzopyrene resulted in a marked stimulation of 2-hydroxylation, with some inhibition of 4-hydroxylation and a lowering of the ratio from 4.6 to 1.3. This shows close correlation to in
in vitro metabolism in which the ratio dropped from 6.5 to 2.2 (Creaven and Parke, 1966).

The same authors (Creaven and Parke, 1966) did these experiments in vitro, pretreating rats and mice with a series of polycyclic hydrocarbons and the drugs phenobarbitone, meprobamate and nikethamide. In rats, the drugs increased the 4-hydroxylation of biphenyl without having any effect on 2-hydroxylation. Of the hydrocarbons, 20-methylcholanthrene (also known as 3-methylcholanthrene) and 3,4-benzpyrene stimulated only 2-hydroxylation, but 22-methylcholanthrene (also known as 5-methylcholanthrene) stimulated both 2- and 4-hydroxylation.

When the ratios of 4-hydroxylation/2-hydroxylation were considered, it was seen that on pretreatment with 20-methylcholanthrene or 3,4-benzpyrene, which stimulate only 2-hydroxylation, the normal rate of 5 is reduced to 2. The polycyclic hydrocarbons which stimulate both 2- and 4-hydroxylation led to ratios of 4-hydroxylation/2-hydroxylation ranging from 2 to 5. Phenobarbitone increased the ratio to values of 9-13. In mice a similar pattern was seen. Benzpyrene increased only the 2-hydroxylation of biphenyl and lowered the ratio from 6.5 to 2, whereas phenobarbitone increased 4-hydroxylation and raised the ratio to 16 (Creaven and Parke, 1966).

Burke and Bridges (1975), injected adult (14-18 week) male Syrian hamsters once daily with sodium phenobarbitone (10 mg ip as a 1% aqueous soln. per 100 g body wt.) for 5 days while controls were injected with saline (0.5 ml 0.9% NaCl). Other hamsters were injected with 3-methylcholanthrene (5 mg as a 1% suspension in ground nut oil per 100 g body wt.; once only) with controls receiving ground nut oil
Phenobarbitone induced 4-hydroxylation 5.3-fold and 2-hydroxylation 3.3-fold. 3-methylcholanthrene slightly induced both reactions to nearly an equal extent. The authors found also that phenobarbitone and 3-methylcholanthrene induced cytochrome P-450 and cytochrome P-448, respectively.

Burke and Mayer (1975) studied the metabolism of biphenyl by liver microsomes from male, corn oil pretreated, Long-Evans rats (60 gm body weight). The only metabolite measured was 4-hydroxybiphenyl so that 2-hydroxy biphenyl was not formed. Pretreatment with phenobarbital induced biphenyl 4-hydroxylation by over 5-fold. A slow but measurable rate of biphenyl 2-hydroxylation was then observed. The predominant cytochrome in the liver microsomes of corn oil or phenobarbital pretreated rats was cytochrome P-450, as judged by the wavelength of the Soret band absorbance maximum of carbon monoxide-gassed, dithionite-reduced microsomes. Pretreatment of rats with 3-methylcholanthrene induced biphenyl 4-hydroxylation by slightly more than 3-fold and promoted a relatively large 2-hydroxylation activity. The predominant cytochrome of the liver microsomes of 3-methylcholanthrene pretreated rats was cytochrome P-448. A monooxygenase system reconstituted with the monooxygenase's flavoprotein (NADPH-cytochrome P-450 reductase) and cytochrome P-450 catalyzed NADPH-supported biphenyl 4-hydroxylation. Biphenyl 2-hydroxylation or ethoxyresorufin deethylation activities were negligible with this system. The flavoprotein, for convenience, is often characterized by its reduction of cytochrome c. A monooxygenase system reconstituted with partially purified NADP-cytochrome c reductase and cytochrome P-448 catalyzed NADPH-supported biphenyl 2- and 4-hydroxylation and
ethoxyresorfin deethylation. Therefore, the patterns of biphenyl hydroxylation and ethoxyresorufin deethylation observed with liver microsomes are due largely to the inherent enzymic specificities of their cytochromes P-450 and P-448. The unique ability of solubilized cytochrome P-448 to catalyze biphenyl 2-hydroxylation and ethoxyresorfin deethylation seems, indeed, to be a characteristic metabolic capacity which resides in the cytochrome itself. By the same token, biphenyl 4-hydroxylation activity is inherent in both cytochromes P-450 and P-448.

Burke and Prough (1976) measured liver and lung microsomal activities by using biphenyl and benzo[a]pyrene as substrates. In liver microsomes of corn oil-treated hamsters, biphenyl 4-hydroxylation was the most active. Biphenyl 2-hydroxylation was the least active and benzo[a]pyrene hydroxylation was intermediate in activity. Hamsters pretreated with 3-methylcholanthrene (20 mg/kg of body weight as a 2% solution in corn oil, once daily for 4 days, with the final injection being given 24 hr before sacrifice) were induced in hepatic biphenyl 2- and 4-hydroxylase activities 3.4 and 2.3 fold, respectively, but were not greatly induced in benzo[a]pyrene hydroxylase activity. The predominant activity of lung microsomes from corn oil-treated hamsters was biphenyl 4-hydroxylation which was 70-80% of the level in liver microsomes.

Biphenyl 2-hydroxylase and benzo[a]pyrene hydroxylase activities were approximately 1-3% as active as biphenyl 4-hydroxylase in lung microsomes. Hamsters pretreated with 3-methylcholanthrene were induced in 2-hydroxylation and benzo[a]pyrene hydroxylation but not 4-hydroxylation in lung microsomes.
Atlas and Nebert (1976) studied the genetic expression of induced biphenyl hydroxylase in responsive (C57BL/6) and non-responsive (DBA/2) strains of inbred mice. 2-hydroxy and 4-hydroxy biphenyl were induced by 3-methylcholanthrene in responsive mice. The production of 4-hydroxy biphenyl was increased two to three-fold whereas 2-hydroxy biphenyl, having lower basal levels than the 4-hydroxy biphenyl, was induced four to five-fold. There were no significant differences between 3-methylcholanthrene-treated and non-responsive mouse controls. Atlas and Nebert (1976) also studied the induction due to B-naphthoflavone, 2,3,7,8-tetra-chlorodibenzo-p-dioxin (TCDD) and phenobarbital in responsive and non-responsive strains of mice. B-naphthoflavone has a similar inductive effect as 3-methylcholanthrene. 2-hydroxy and 4-hydroxy biphenyl were induced by B-naphthoflavone to levels which were comparable to those induced by 330-methylcholanthrene in responsive mice.

Non-responsive mice were not induced. TCDD was unique. TCDD was capable of fully expressing induction in non-responsive as well as responsive mice. Phenobarbital induced 4-biphenyl hydroxylation in responsive as well as non-responsive mice but not 2-biphenyl hydroxylation which was not significantly increased above control levels in either strain.

Boobis et al. (1977) compared B-naphthoflavone and 3-methylcholanthrene as inducers of hepatic cytochrome P-448 and aryl hydrocarbon (benzo[a]pyrene) hydroxylase activity in the responsive (C57BL/GN) strain of mice. A single intraperitoneal injection with 3-methylcholanthrene (80 mg/kg of body weight) induced hepatic aryl hydrocarbon hydroxylase about 2.7 fold. Maximal levels of the
activity were maintained from the second through the seventh day after injection. The same dose of B-naphthoflavone induced the hydroxylation about 4-fold. The maximal level occurred at 40 hr. The activity returned to basal levels by 72 hr, following a single injection of B-naphthoflavone.

Burke and Prough (1977) studied biphenyl hydroxylation in male Sprague-Dawley rats and New Zealand rabbits using high-pressure liquid chromatography. Microsomes of an untreated rat formed 4-hydroxy biphenyl (1.5 nmol/mg of microsomal protein/min.). A hardly significant amount of 2-hydroxy biphenyl appeared and there was no detectable amount of 3-hydroxy biphenyl. Conversely, 2-hydroxy biphenyl, 3-hydroxy biphenyl and 4-hydroxy biphenyl (1.9, 0.7 and 3.3 nmol/mg microsomal protein/min., respectively) were all important metabolites with microsomes of 3-methylcholanthrene-treated rats. Neither 2- nor 3-hydroxy biphenyl formed upon incubation of biphenyl with liver microsomes of untreated rabbits. Biphenyl 4-hydroxylation was detectable but in no case was 2- or 3-hydroxy biphenyl formed.

Billings and McMahon (1978) studied the effect of cytochrome P-450 induction on biphenyl hydroxylation in male Sprague-Dawley rats and white Swiss mice. Phenobarbital (50 mg/kg i.p.) was given once daily for 4 days. Animals were treated with B-naphthoflavone (80 mg/kg i.p.) or 3-methylcholanthrene (20 mg/kg i.p.) 48 hr before death. 3-methylcholanthrene or B-naphthoflavone treatment preferentially induced 2-biphenyl hydroxylation 3-fold. The amount of 4-hydroxy biphenyl increased slightly. Biphenyl 4-hydroxylation was induced in rats and mice by phenobarbital, while 3-hydroxy biphenyl
production was induced in rats. The authors concluded that there was no increase in 3-hydroxylation in mice but the data show 3-hydroxylation was increased 50% by the drug treatment.

Toftgard et al. (1980), characterized the different forms of liver microsomal cytochrome P-450 in male Sprague-Dawley rats by SDs-polyacrylamide gel electrophoresis after induction with 16α-cyanopregnenolone, phenobarbital, and 3-methylcholanthrene. The correlation between the induced forms of cytochrome P-450 and the metabolism of biphenyl, benzo[a]pyrene and steroids was investigated. Rats treated with 16α-cyanopregnenolone demonstrated a significant (50%) decrease in the amount of 4-hydroxy biphenyl formation while no significant effects were observed after phenobarbital or 3-methylcholanthrene treatment. Phenobarbital and 3-methylcholanthrene significantly stimulated 2-hydroxylation of biphenyl (about four and twenty times, respectively) while no effect was obtained with 16α-cyanopregnenolone. Biphenyl 3-hydroxylation was detected only after induction with phenobarbital and 3-methylcholanthrene while a significant stimulation of more than three and ten times. SDs-polyacrylamide gel electrophoresis of rat liver microsomes showed that phenobarbital treatment increased three different bands with apparent molecular weights of 50,000, 54,000 and 56,000. Methylcholanthrene treatment increased in two bands with apparent molecular weights of 55,000 and 58,000 while treatment with 16α-cyanopregnenolone increased one band with an apparent molecular weight of 54,000 (which was also induced by phenobarbital). Toftgard et al. (1980) reported a comparison of the metabolite patterns of biphenyl hydroxylation and the different bands resolved by SDs-gel electrophoresis and that RLwMε P-450₅₅ (an
accepted abbreviation for "rat liver microsomal cytochrome P-450 molecular weight 55,000") and/or RlvM Corey-50 found after 3-methylcholantherene treatment are involved in the formation of 2- or 3-hydroxy biphenyl. Treatment with phenobarbital also caused an increased formation of 2- and 3-hydroxy biphenyl but the increase was much smaller than following 3-methylcholantherene treatment.

RlvM Corey-50, induced by phenobarbital, may to some extent, be capable of hydroxylating biphenyl in the 2- and 3-positions while RlvM Corey-54 is probably not involved in the formation of these metabolites since no increase in their formation was seen after 16α-cyanopregnenolone treatment. In contrast to Creaven and Parke's results (1966), Billings and McMahon (1978) observed no induction in the formation of 4-hydroxy biphenyl after phenobarbital treatment.

Haugen (1981) studied the effect of the inducers phenobarbital and 3-methylcholantherene, inhibitors, and solvents on the 2-, 3-, and 4-hydroxylation of biphenyl by male rat (CD strain) liver microsomes. Phenobarbital pretreatment primarily induced 2- and 3-hydroxylation, the latter most dramatically. Methylcholantherene pretreatment induced 2- and 3-hydroxylation to a similar extent. Biphenyl 4-hydroxylase was induced 1.9- and 2.2-fold with 3-methylcholantherene and phenobarbital, respectively.

Voles

Traditional Classification

Order Rodentia

Super family Muroidae

Family Cricetidae
Cole et al. (1976) used the prairie vole, *Microtus ochragaster*, in laboratory terrestrial model ecosystems containing both plant and invertebrates, in comparing environmental fates of aldrin and DDT with those of fonefos and methoxychlor. The individual organs of the voles were analyzed separately for insecticide residues. In the vole levels of DDE were greater than DDT, indicating that the animal had the selective metabolic capability of readily converting DDT to DDE.

Gile and Gillett (1977) studied the disposition of $^{14}$C-dieldrin in a terrestrial microcosms chamber (TMC) model ecosystem consisting of synthetic soil medium, agricultural crops, numerous invertebrates and the grey-tailed vole, *M. canicau dus*. Residues were determined in air, soil, water, plants, invertebrates, and tissues of the vole. No detoxication studies of the vole were made in these experiments; however, Gile and Gillett (1977) found the vole to accumulate some insecticide residues.

In a symposium on terrestrial microcosms and environmental chemistry Witt et al., (1977) stated that the vole (sometimes referred to as a field mouse) was the animal of choice by EPA because of many reasons. These included a supposition that voles would be the top consumers
in the microcosms. Voles can also be reared conveniently. Voles may have behavioral traits and sizes which are favorable in the compact microcosm environment. The EPA group recommended the continued use of this animal as the "top predator" of the terrestrial microcosm system, "...while conducting an active search for information on its biochemistry, physiology and behavior." In terms of detoxication by the liver, this goal has not been realized or seriously approached except for an unpublished study of *M. canicaudus* (Brindley, unpublished data) and several studies of *M. pinetorum* (Hartgrove et al., 1977).

Webb and Horsfall (1967) reported the first instance of the development of resistance to a pesticide by mammals in their natural habitat. Wild pine mice, *M. pinetorum*, with a history of exposure to endrin, exhibited a 12-fold greater tolerance to endrin than did mice having no history of endrin exposure. Webb and Horsfall (1967) assumed that resistance could be attributed either to a genetically controlled mechanism to detoxify endrin or to the induction of detoxifying enzymes.

Webb et al. (1972) found benzpyrene hydroxylase activity in hepatic microsomes from endrin resistant pine mice to be higher than in microsomes from susceptible mice. Pretreatment of resistant pine mice with dieldrin resulted in a further increase in hydroxylase levels. They attributed resistance to heritable as well as to adaptive aspects.

Hartgrove and Webb (1973) studied the effect of age on the development of benzpyrene hydroxylase activity in endrin-susceptible and -resistant pine mice. Both strains appeared to develop maximal benzpyrene hydroxylase activity between 4 and 8 weeks of age. The
activity then slowly declined to the levels observed at about 20 weeks of age in both strains.

Hartgrove et al. (1974) found an increase in microsomal activity determined by ethylmorphine-\(N\)-demethylation, aniline hydroxylation and cytochrome P-450 content estimated after voles were injected (i.p. for 3 consecutive days) with 50 mg/kg of endrin. Both endrin-susceptible and -resistant pine voles were treated. However, endrin, as it appeared to cause no increase in microsomal metabolism of ethylmorphine. The cytochrome P-450 content was decreased. Aniline hydroxylation gave variable results. Hartgrove et al. (1974) suggested that endrin did not induce microsomal activity.

Hartgrove et al. (1977) characterized the hepatic mixed function oxidase system in endrin-resistant and -susceptible pine voles by measuring basal and endrin-inducible levels of microsomal activity by \textit{in vitro} incubations with ethylmorphine, aniline, and benzo[a]pyrene. Species difference were investigated by characterizing the hepatic microsomal system of the laboratory mouse. Basal levels of microsomal activity were not significantly different, by statistical tests, between endrin-resistant and -susceptible pine voles for ethylmorphine, \(N\)-demethylation, aniline hydroxylation and \(\text{[14C]}\)-benzpyrene disappearance and benzpyrene 3-hydroxylation, although levels were consistently lower in the susceptible animals. There were no significant strain differences attributable to endrin treatment except there was a 23\% lower rate of ethylmorphine \(N\)-demethylation for the susceptible animals after treatment of both strains with 0.5 mg of endrin/kg. Endrin treatment decreased the rate of ethylmorphine
N-demethylation in pine voles, but in contrast, increased N-demethylation in white mice. The rate of aniline hydroxylation was increased in the pine vole and an even greater increase was found in white mice with endrin treatment. The rates of formation of fluorescent products from benzo[a]pyrene were decreased significantly in pine voles after endrin exposure; in contrast, benzo[a]pyrene metabolism of white mice was increased significantly.
MATERIALS AND METHODS

Animals

Adult virgin female montane meadow voles, Microtus montanus, 9-14 weeks of age, were obtained from Dr. Norman Negus, Department of Biology, University of Utah, Salt Lake City, UT. These were allowed to remain in the Utah State University animal holding facility in groups of 5 voles each for at least one week prior to the start of experimentation. The cages were polypropylene boxes measuring 48.5 X 26.7 X 17.8 cm provisioned with cotton batting and wood chips. The voles were fed commercial rabbit food pellets ad libitum, they had a constant water supply, and were kept at about 24°C with a 14 h photoperiod.

Swiss Webster mice (outbred) 9 weeks of age were obtained from Simonsen Laboratories, Inc., Gilroy, CA, through the Utah State University animal facility.

Chemicals

Biphenyl, 2-hydroxy biphenyl, 4-hydroxy biphenyl and B-naphthoflavone, were obtained from the Aldrich Chemical Company, Milwaukee, WI; 3-hydroxy biphenyl was obtained from ULTRA-Scientific, Hope, RI; NADP⁺, NADH, Sodium D, L-isocitrate, isocitrate dehydrogenase, Tris buffer (pH 7.7), Coomassie Brilliant Blue R, and bovine serum albumin were supplied by the Sigma Chemical Company, St. Louis, MO; sodium phenobarbital was obtained from Merck & Co., Inc., Rahway, NJ; 3-methylcholanthrene was a gift from Dr. Joseph C. Street, Utah State University; Varaport-30® with a mesh size of 100/120 was purchased
from Varian Aerograph, Walnut Creek, CA; REGISIL® (BSTFA) and OV-1®
were supplied by the Regis Chemical Co., Morton Grove, IL; all solvents
were Nano-grade® and supplied by Baker Chemical Co., Phillipsburg, NJ,
and Fisher Scientific Company, Fair Lawn, NJ. Distilled water was
used throughout the procedures. Helium, oxygen and hydrogen were
supplied by Chemical Corporation-National Cylinder Gas Division,
Chicago, IL. Carbon monoxide was provided by Matheson Gas Products,
Newark, CA.

Instrumentation

Chemical analyses of biphenyl hydroxylation products by vole and
mouse liver microsomes were carried out by using a Varian 204-1C gas
liquid chromatograph with flame ionization detection. An 8-foot
stainless steel column with 3% OV-1 on Varaport-30 (mesh-size 100/120)
was used. The chromatograph was operated under the following parameters:
helium carrier gas flow rate, 30 ml/min; hydrogen 30 ml/min; oxygen
400 ml/min; column temperature 160° C; injector, 200° C. The oven
temperature was raised from 160° C to 200° C, starting 4 minutes after
the sample was injected. Hydroxylated biphenyls formed in
the incubation were quantified from a standard curve constructed by
adding authentic compounds to incubation mixtures and carrying these
samples through the entire analytical procedure.

Measurements of the carbon monoxide difference spectra for cyto-
chrome P-450 (Omura and Sato, 1964) were carried out by using microsomal
suspensions from M. montanus liver divided into 2 portions. One portion
was bubbled with carbon monoxide for ca. 30 sec. in a 1 cm cuvette.
Both portions were then reduced with a small amount of dithionite,
mixed, and difference spectra were recorded from 490 to 410 nm with a Beckman DU-8 spectrophotometer with a scattered light accessory set for total measurement of scattered and transmitted light.

Preparation of Vole and Mouse Liver Microsomes

Microsomes were isolated by the procedure of Hartgrove et al. (1977). Voles and mice were sacrificed at 8-9 a.m. through all the experiments by decapitation and the livers were immediately removed. After removal of the gall bladder, the livers were weighed, placed in beakers surrounded by ice, and kept chilled at 4° C by use of a cold table during subsequent preparative procedures. Each liver was minced and homogenized by four complete up and down strokes of a power driven Potter-Eveljhem type glass-teflon homogenizer in 5 ml of cold 1.15% KCl. The homogenate was made to 12 ml with 1.15% KCl. The homogenate was sonicated with a BIOSONIK III (Bronwill Scientific, Inc., Rochester, NY) cell disrupter to improve the yield of microsomal protein. Sonication was conducted in an ice bath using three 5-sec. bursts at a 10-W output with a 15-sec. interval between each burst. After sonication, the homogenate was centrifuged at 9000 g for 20 min. in a Beckman L5-65B refrigerated ultracentrifuge. The supernatant was centrifuged at 30,000 rpm (105,000 g) for 75 min. in a type 30 rotor to sediment the microsomal pellet. The surface of the resultant pellet was rinsed before the pellet was resuspended in 5 ml 1.15% KCl in 0.02 M Tris buffer (pH 7.7) by gentle, but motor driven, homogenizing.
Protein Determination

The determination of protein was performed by Bradford's (1976) procedure using a dye solution of 100 mg of Coomassie Brilliant Blue G, dissolved in 50 ml 95% ethanol to which had been added 85% (w/v) phosphoric acid. This was shaken very vigorously for 1/2 hour. The resulting solution was diluted to a final volume of 1 liter. The reagent was always filtered just prior to use.

Aliquots of 0.1 ml of protein solution were pipetted into test tubes and brought up to 0.5 ml with 1.15% KCl in 0.02 M Tris buffer (pH 7.7). Five milliliters of dye solution were added to each tube. Absorbance was measured against a cuvette containing 0.1 ml buffer and 5 ml of dye solution at 595 nm on a Zeiss PMQ-11 spectrophotometer. The optical densities were then compared with a standard curve prepared with known concentrations of bovine serum albumin.

Enzyme Assay

Each incubation (5 ml) consisted of 2-2.5 mg microsomal protein, 4 mM MgCl₂, an NADPH-generating system and 1 mM biphenyl in 25 ul of dimethyl sulfoxide. The NADPH-generating system consisted of 0.25 mM NADP⁺, 10 mM sodium DL-isocitrate, and 0.1 mg of isocitrate dehydrogenase (from porcine heart with an activity of 4.3 units/mg protein). In some experiments, 0.25 mM NADH were added to study the stimulation of the hydroxylation. Incubations were conducted for 10 min with 2 mg microsomal protein at 37°C in a water bath shaker at 120 oscillations/min. The formation of all three biphenyl hydroxy isomers was linear with time up to 15 min and with protein concentrations up to 4 mg protein. All incubations were conducted in duplicate.
Extraction and Analysis

The biphenyl hydroxylase assays were performed with a slight modification of the method of Billings & McMahon (1978). Incubations were terminated by addition of 0.25 ml of 5N HCl and the solutions were then extracted twice with 10 ml diethylether by vigorous shaking for 10 min in 125 ml Erlenmeyer flasks on an equipoise shaker. The combined extracts were evaporated using a rotary evaporator with a water aspirator for vacuum. The residues, after evaporation, were rinsed with 1 ml of n-butylchloride and extracted with 5 ml of 0.1 N NaOH. The NaOH was acidified with 0.6 ml of 5 N HCl and extracted twice with 4 ml of n-butylchloride. The combined extracts were then evaporated to dryness with the rotary evaporator. The dried n-butylchloride extracts were dissolved in 0.1 ml of Regisil [bis(trimethylsilyltrifluoroacetamide]. After standing at room temperature for 1 hr, the solutions were analyzed by gas chromatography.

Special Treatments

Voles and mice were given phenobarbital by intraperitoneal injections at one of two doses (50 mg/Kg or 20 mg/Kg) dissolved in 0.25% NaCl. The animals were killed 24 hr after the last dose. Animals were treated also by intraperitoneal injection, with B-naphthoflavone (80 mg/Kg) or 3-methylcholanthrene (20 mg/Kg) dissolved in corn oil, and the animals were killed 48 hr after the last dose. The volume of solution injected was 10 mg/Kg body weight for both mice and voles. Control animals received similar volumes of saline or corn oil only. To minimize the possibility of any variations in
the activities of the enzymes due to changes in time, all treatments were made between 8 and 9 a.m. Food and water were provided ad libitum during the 24 hr preceding sacrifice.
RESULTS

Effects on Animals

Mice injected with phenobarbital quickly went to sleep. Voles were not sedated by phenobarbital and those injected with phenobarbital were more active than voles injected with saline. The drug doses used did not cause mortality or obvious weight loss. Mice injected with 3-methyl-cholanthrene had yellow colored livers by the time of sacrifice but this was not noticed in voles. All animals appeared to be healthy before and after administration of the inducers. Average weights of animals, average liver weights and average milligrams of microsomal protein per gram liver shown in Table 1.

Gas Chromatographic Analysis of Biphenyl Hydroxylation

Figures 1 and 5 show the gas chromatography of a silylated extract from incubation of biphenyl with vole and mouse liver microsomes, respectively. Typical elution times were determined with analytical standards to be for biphenyl 1.17 min.; 2-hydroxy-biphenyl 2.20 min.; 3-hydroxy biphenyl 3.25 min. and 4-hydroxy-biphenyl 3.57 min. These standards were > 98% pure as determined by the supplier and judged by their individual chromatograms in the experiments.

Biphenyl metabolite quantities were measured with a standard curve constructed by adding 250-500 ng authentic compound to incubation mixtures containing no NADP⁺. These were carried through the entire analytical procedure. The percent recovery of authentic hydroxy
biphenyls taken through the complete assay (incubation, extraction, and gas chromatography) was 85% for 4 experiments. Peak heights were used for a quantitative base. Microsomal suspensions from livers of control animals did contain a component which had a retention time identical to 4-hydroxy biphenyl. Before the recovery data were calculated, the recorder response of this component was subtracted from the 4-hydroxy biphenyl response obtained from the incubation mixture plus authentic compound. The net peak height was used to calculate quantitative levels and recovery. There was another peak from the incubation mixture between 2-hydroxy biphenyl and 3-hydroxy biphenyl with a retention time of 2.5 min., but this never interfered with any hydroxy biphenyl. A peak appeared just beyond the 4-hydroxy biphenyl which was not identified and it did not affect the determination of the 4-hydroxy biphenyl because there was no effect upon the metabolite's peak height.

Effects of Nicotinamide Cofactors

Biphenyl 2-hydroxylation as well as the formation of 3- and 4-hydroxy biphenyl requires NADPH in voles (Table 2). However, addition of NADH stimulated the hydroxylation. NADH is known to synergistically enhance NADPH support of microsomal demethylation reaction (Cohen and Estabrook, 1971) and increase the formation of 2-, 3-, and 4-hydroxy biphenyl to about 50% (Billings and McMahon, 1978). Likewise, in voles, there was NADPH/NADH synergism with biphenyl 4-, and 3-hydroxylation. The amount of 2-hydroxylation obtained with NADPH plus NADH was, however, considerably less than the more-than-additive effects observed with 3- and 4-hydroxylation.
Studies on Spectral Changes in Vole Liver Microsomes

Carbon monoxide difference spectra of vole liver microsomes were obtained using sodium dithionite, a reducing agent (Fig. 6). Pretreatment of voles with 3-methylcholanthrene or B-naphthoflavone usually led to a hypsochromic shift in the soret peak of the reduced hemoprotein carbon monoxide complexes of liver microsomes but only of 0.8 mm. Pretreatment of the animals with phenobarbital and the controls showed an absorption at 450.4 nm.

Effect of Protein Concentration on Formation of Biphenyl Hydroxylation

The relationship between the amount of microsomal protein present in the incubation and the rate of formation 2-, 3-, and 4-hydroxy biphenyl is illustrated in Figure 7. The rate of biphenyl hydroxylation increased linearly as the amount of protein was increased up to 4 mg protein, after which concentration the rate of the enzyme reaction increased more slowly.

Effect of Incubation Time on Formation of Biphenyl Hydroxylation

A similar relationship was observed with regard to incubation time (Fig. 8); that is, the rate of formation of 2-, 3-, and 4-hydroxy biphenyl was linear up to 15 minutes, while a further increase in the incubation time did not appreciably increase the reaction product. Based upon these findings, a standard assay condition of 2-2.5 mg of microsomal protein and 10 minutes for each incubation was adopted for the remaining portions of this study.

Effect of Drugs Treatment on Vole and Mouse Liver

Changes produced in mouse liver weights by phenobarbital and
B-naphthoflavone relative to their respective controls and also changes in liver weight per body weight by phenobarbital, B-naphthoflavone and 3-methylcholanthrene or liver weight per body weight are shown in Table 1. In voles, there were no changes in liver weights. Phenobarbital induced a 60% increase in microsomal protein content of liver (mg protein/g liver) compared with controls. No increase following 3-methylcholanthrene or B-naphthoflavone was observed in voles or mice.

**Effect of Microsomal Induction by Phenobarbital, B-naphthoflavone and 3-Methylcholanthrene in Voles and Mice**

Liver microsomes of voles and mice pretreated with saline or corn oil (controls in the case of voles), phenobarbital, B-naphthoflavone and 3-methylcholanthrene were studied for their biphenyl hydroxylation activities (Table 3). The microsomal activities of Swiss Webster mice were investigated to provide a species comparison. Pretreatment of voles with phenobarbital prior to preparation of liver microsomes caused a significant alteration in the rate of biphenyl hydroxylation. The change in microsomal hydroxylation was profound; that is, pretreatment with phenobarbital (50 mg/kg) induced 2-hydroxy biphenyl by 5.45 fold, 3-hydroxy biphenyl by 9.65 fold and 4-hydroxy biphenyl by 4.24 fold relative to controls (saline injection). At the same time, pretreatment of mice with phenobarbital did not raise the amount of 2-hydroxy biphenyl above control levels, but increased 3-hydroxy biphenyl by 50% and induced 4-hydroxy biphenyl by about 3-fold above the control levels. Control mice did not receive any treatment.

Pretreatment of voles with B-naphthoflavone (80 mg/kg; three injections) increased the formation of 2-hydroxy-, 3-hydroxy- and 4-hydroxy biphenyl by 1.8, 1.8 and 1.6 fold, respectively. Pretreatment
with 3-methylcholanthrene (20 mg/kg/three injections) increased the metabolites by 1.8, 1.6 and 1.4 fold relative to controls (corn oil), respectively. On the other hand, pretreatment of mice with B-naphthoflavone and 3-methylcholanthrene significantly induced 2-hydroxy biphenyl and increased 3- and 4-hydroxy biphenyl slightly.
Table 1. Effect of drug injection upon liver weight, liver weight to body weight ratios, and microsomal protein of female meadow voles and white Swiss mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Pretreatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>Vole</td>
<td></td>
</tr>
<tr>
<td>Body wt (g)</td>
<td>30.4 ± 4.7</td>
</tr>
<tr>
<td>Liver wt (g)</td>
<td>1.165 ± 0.31</td>
</tr>
<tr>
<td>Microsome wt per liver wt (mg/ protein/g)</td>
<td>16.17 ± 2.6</td>
</tr>
<tr>
<td>Liver wt per body wt</td>
<td>0.038 ± 0.005</td>
</tr>
<tr>
<td>Mouse</td>
<td></td>
</tr>
<tr>
<td>Body wt (g)</td>
<td>36.09 ± 2.9</td>
</tr>
<tr>
<td>Liver wt (g)</td>
<td>1.73 ± 0.04</td>
</tr>
<tr>
<td>Microsome wt per liver wt (mg/ protein/g)</td>
<td>14.16 ± 2.63</td>
</tr>
<tr>
<td>Liver wt per body wt</td>
<td>0.048 ± 0.004</td>
</tr>
</tbody>
</table>

* Indicates significant difference (P < 0.01)
Table 2. Cofactor requirements for hydroxylation of biphenyl (ng metabolite/mg microsomal proteins/min) by vole liver microsomes

<table>
<thead>
<tr>
<th>Cofactor</th>
<th>2-hydroxy biphenyl</th>
<th>3-hydroxy biphenyl</th>
<th>4-hydroxy biphenyl</th>
</tr>
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<tbody>
<tr>
<td>None</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>NADPH</td>
<td>30.8</td>
<td>55.8</td>
<td>383.9</td>
</tr>
<tr>
<td>NADPH/NADH</td>
<td>34.31</td>
<td>75.92</td>
<td>514.9</td>
</tr>
<tr>
<td>NADH</td>
<td>0</td>
<td>0</td>
<td>15</td>
</tr>
</tbody>
</table>

2 mg of microsomal protein of voles liver were incubated for 10 min with 1 mM biphenyl. The concentrations of all cofactors was 0.25 mM. Means for 2 experiments.
Table 3. Effect of Cytochrome P-450 induction on biphenyl hydroxylation by hepatic microsomes fraction of voles or mice (ng metabolites/mg microsomes protein/min)

<table>
<thead>
<tr>
<th>Treatment of Animals</th>
<th>2-hydroxy biphenyl</th>
<th>3-hydroxy biphenyl</th>
<th>4-hydroxy biphenyl</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mice</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None (4)</td>
<td>118 ± 21.4</td>
<td>91.76 ± 8.7</td>
<td>1010.46 ± 71</td>
</tr>
<tr>
<td>B-naphthoflavone one (3)</td>
<td>247.67 ± 43**</td>
<td>144.33 ± 49*</td>
<td>1414.2 ± 83.29**</td>
</tr>
<tr>
<td>injection 80 mg/kg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-methylcholanthrene (3)</td>
<td>325.9 ± 81**</td>
<td>129.85 ± 13**</td>
<td>1322 ± 68**</td>
</tr>
<tr>
<td>three injection 20 mg/kg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenobarbital three (3)</td>
<td>116.45 ± 11.97</td>
<td>142.7 ± 5.9**</td>
<td>3143.4 ± 48**</td>
</tr>
<tr>
<td>injection 50 mg/kg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Voles</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None (10)</td>
<td>28.84 ± 3.4</td>
<td>49.14 ± 10.6</td>
<td>359.32 ± 79.5</td>
</tr>
<tr>
<td>Corn oil (6)</td>
<td>28.53 ± 9.3</td>
<td>43.8 ± 18</td>
<td>356.9 ± 116</td>
</tr>
<tr>
<td>B-naphthoflavone one (6)</td>
<td>52.45 ± 8.3**</td>
<td>81.61 ± 26.7**</td>
<td>585.7 ± 140**</td>
</tr>
<tr>
<td>injection 80 mg/kg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B-naphthoflavone one (3)</td>
<td>48.67 ± 1.05**</td>
<td>70.95 ± 20*</td>
<td>559.49 ± 59*</td>
</tr>
<tr>
<td>injection 80 mg/kg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-methylcholanthrene (6)</td>
<td>51.51 ± 8.8**</td>
<td>73.58 ± 33.56*</td>
<td>529 ± 170*</td>
</tr>
<tr>
<td>three injection 20 mg/kg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenobarbital three (6)</td>
<td>29.77 ± 4.4</td>
<td>50.46 ± 13</td>
<td>382.4 ± 127</td>
</tr>
<tr>
<td>injection 50 mg/kg</td>
<td>162.51 ± 11.5*</td>
<td>487.3 ± 29.6**</td>
<td>1621.93 ± 181.6**</td>
</tr>
<tr>
<td>Phenobarbital three (4)</td>
<td>73.76 ± 25.6**</td>
<td>181.92 ± 37.8**</td>
<td>895.4 ± 247.4**</td>
</tr>
<tr>
<td>injection 20 mg/kg</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The number of animals is shown in parentheses. Results are mean ± standard deviation
* Indicates significant difference (P < 0.05)
** Indicates significant difference (P < 0.01)
Figure 2. Gas liquid chromatographic (GLC) analysis of trimethylsilyl ether of hydroxy biphenyl isomers formed by vole liver microsomal hydroxylation of biphenyl. Biphenyl was incubated for 15 min. with 2.2 mg of microsomal protein from vole liver. Peaks 1, 2, and 3 correspond, respectively, to authentic samples of 2-, 3-, and 4-hydroxy biphenyl.
GLC
Response

FUT 6 LUT
Figure 3. Gas liquid chromatographic (GLC) analysis of trimethylsilyl ether of control vole liver microsomes.
Figure 4. Gas liquid chromatographic (GLC) analysis of trimethylsilyl ether of hydroxy biphenyl isomers. Peaks 1, 2 and 3 are, respectively, authentic samples of 2-, 3-, and 4-hydroxy biphenyl.
Figure 5. Gas liquid chromatographic (GLC) analysis of trimethylsilyl ethers of hydroxy biphenyl isomers formed by mouse liver microsomal hydroxylation of biphenyl.

Biphenyl was incubated for 10 min. with 2 mg microsomal protein from mouse liver. Peaks 1, 2, and 3 correspond, respectively, to authentic samples of 2-, 3-, and 4-hydroxy biphenyl.
GLC Response
Figure 6. Carbon monoxide difference spectra of reduced microsomes from voles treated with a) no injections, b) 3 injections, 20 mg/kg, 3-methylcholanthrene, and c) 3 injections of 20 mg/kg phenobarbital with absorption maxima of a) 450.4 nm, b) 449.6 nm and 450.4 nm
Figure 7. Effect of protein concentration on formation of 2-, 3-, and 4-hydroxy biphenyl by vole liver microsomes.
Figure 8. Effect of incubation time on formation of 2-, 3-, and 4-hydroxy biphenyl by vole liver microsomes
DISCUSSION

Monooxygenases in microsomes can be characterized by the fluorometric measurement of the 2- and 4-hydroxylation of biphenyl (Creaven et al., 1965). Only recently has the simultaneous formation of 3-hydroxy biphenyl been measured (Burke and Prough, 1977; Billings and McMahon, 1978). The present work used a slightly modified gas liquid-chromatographic assay based upon Billings and McMahon's technique (1978). The three primary biphenyl metabolites were described as were the effects of inducers and cofactors on the distribution of metabolites from vole liver microsomes.

Gas Chromatographic Analysis of Biphenyl Hydroxylation in Hepatic Microsomes Preparation

The recovery of 2-, 3-, and 4-hydroxy biphenyl in the analysis of hepatic microsomal preparations was very acceptable. There was a small interfering peak present in the liver microsomes just beyond the 4-hydroxy biphenyl (Fig. 3). This interference did not affect the determination of the 4-hydroxy metabolite because quantitation was based on peak height of a known standard (Fig. 4). The minimum amounts detectable when processed through the entire procedure were 12.5, 19 and 19 ng for 2-, 3- and 4-hydroxy biphenyl, respectively. Peak heights were directly proportional to concentrations up to 500 ng for 2-hydroxy biphenyl and 700 ng for 3- and 4-hydroxy biphenyl. Billings and McMahon (1978) reported a flame ionization gas chromatography assay capable of measuring a minimum detectable amount of 0.25 ug for 2-hydroxy biphenyl. The added sensitivity of the present method may be due to improved
recovery because of extraction of the whole microsomal incubation rather than after discarding of sedimentable protein, and by using 2-2.5 mg microsomal protein instead of using equivalent to 500 mg liver.

Characterization of Biphenyl Hydroxylation in Voles

Many vertebrates have been studied for their ability to hydroxylate biphenyl. In each case, 4-hydroxy biphenyl is the major metabolite as it is in voles (Table 3) (Creaven et al., 1965; Creaven and Parke, 1966; Burke and Bridges, 1975; Prough and Burke, 1975; Burke and Mayer, 1975; Burke and Prough, 1976; Billings and McMahon, 1978; Thorgeirsson et al., 1979, Toftgard et al., 1980 and Haugen, 1981). However, in voles, 3-hydroxy biphenyl is produced in greater quantities than 2-hydroxy biphenyl. This is not the same as for Swiss mice (Table 3) which were investigated in this study to provide a direct species comparison. Biphenyl 2-hydroxylation approximately equaled 3-hydroxylation in mouse liver microsomes in agreement with results of Billings and McMahon (1978). In other mammals like hamsters or rabbits, 2-hydroxy biphenyl exceeds 3-hydroxy biphenyl (Billings and McMahon, 1978).

The amount of the hydroxy biphenyls formed by vole liver microsomes was considerably less per milligram microsomal protein than for mice (Table 3). In general, the quantitative production of hydroxy biphenyl by hepatic microsomes are in the order of mouse> hamster> vole> rabbit> rat (Thorgeirsson et al., 1979; and this study).
In vivo biphenyl has been shown to produce 19 metabolites (Halpaap et al., 1978), but use of a cell-free microsomal fraction in vitro simplifies the metabolite pattern to the three primary hydroxylations (Prough and Burke, 1975; Billings and McMahon, 1978).

**Microsomal Nature of Biphenyl Hydroxylase**

Biphenyl has been shown to be metabolized by enzymes located in the microsomal fraction of vole liver. The microsomal fraction almost certainly consists of fragments of endoplasmic reticulum. Vole liver was homogenized and the homogenate was first centrifuged to remove mitochondria and larger organelles or unhomogenized cells. Then the supernatant was centrifuged to obtain the microsomes (Siekevitz, 1963). The microsomal fraction thus obtained, contained a hemoprotein, which has been shown to act as a terminal oxidase for a variety of lipophilic drugs including biphenyl. The carbon monoxide complex of the hemoprotein reduced with dithionite yielded a complex with an absorption peak at about 450 nm (Fig. 6). These monooxygenase (mixed function oxidase) enzyme systems require molecular oxygen and NADPH or a cofactor. While an oxygen requirement was not tested in this study, a reduced pyridine nucleotide (NADPH) was shown to be essential (Table 2). All of these factors strongly suggest that the vole biphenyl hydroxylase is a microsomal monooxygenase.

Biphenyl is a toxicologically important xenobiotic in its own right, since it is used as a fungistat on fruit and forms the nucleus of polychlorobiphenyl plasticizers. Billings and McMahon (1978) have concluded that biphenyl appears to be "an ideal substrate for use in assessing the overall capabilities of standard microsomal preparations used in many standardized toxicology tests."
Cofactor Requirements

It was shown in this study that the metabolism of biphenyl by vole liver microsomes required NADPH. However, 3- and 4-hydroxy biphenyl hydroxylation exhibited the NADPH-NADH synergism reported for other drug metabolism reactions (Cohen and Estabrook, 1971). The synergist effect was far less prominent for biphenyl 2-hydroxylation. Adult Syrian hamsters also had low NADH synergism of biphenyl 2-hydroxylation (Burke and Bridges, 1975). Billings and McMahon (1978) showed higher NADPH-NADH synergism for 2-, 3-, and 4-hydroxy biphenyl in Swiss mice than hamsters or voles, both of which exhibit less prominent synergism for 2-hydroxy biphenyl production.

Effects of Enzyme Induction on Liver

Today there are many reports indicating that the metabolism of a certain substrate by one form of induced P-450 is different from that by another form of induced P-450. Some drugs that are metabolized differently (but at specific chemical positions on the molecule) by more than one form of P-450 include biphenyl, benzo[a]pyrene and warfarin (Thorgeirsson and Nebert, 1977).

Pretreatment of voles with phenobarbital (50 mg/kg) showed increases of about 60% of microsomal protein per gram liver weight relative to their respective controls (saline injection) (Table 1) and that is in agreement with Burke and Bridges (1975) who injected hamsters with phenobarbital. Injection of 3-methylcholanthrene into hamsters did not change microsomal protein yield per gram liver. Injection of voles with 3-methylcholanthrene or B-naphthoflavone did not show significant changes relative to control (corn oil...
injection) liver weight and did not increase the microsomal protein per gram liver weight. Hamsters pretreated with 3-methylcholanthrene showed some increase in liver weight but no significant increase in microsomal protein per gram liver weight (Burke and Bridges, 1975). White mice pretreated with phenobarbital or β-naphthoflavone showed only an increase in liver weight relative to controls (Table 1). Goldstein et al. (1974) mentioned that pretreatment of animals with phenobarbital, which stimulates a great many enzyme activities, produces a detectable increase in the amount of microsomal protein per gram of liver; but 3-methylcholanthrene, which stimulates fewer enzymes, does not. This generalization is not correct for all species. In voles and hamsters phenobarbital increases microsomal protein per gram liver. In mice it does not (Table 1) (Burke and Bridges, 1975).

Drug-Induced Biphenyl Hydroxylation

Voles pretreated with phenobarbital were induced 9.6 fold in 3-hydroxylation, 5.4 fold in 2-hydroxylation, and 4.2 fold in 4-hydroxylation relative to the controls (Table 3). Hamsters pretreated with phenobarbital were induced in 2- and 4-hydroxylation by 3.3 and 5.3 fold, respectively, based on a fluorometric assay which could not detect 3-hydroxylation of biphenyl (Burke and Bridges, 1975). Mice pretreated with phenobarbital did not show any significant change in the amount of 2-hydroxylation of biphenyl relative to controls. However, 3-hydroxylation showed little increase and 4-hydroxylation was induced 3-fold relative to control (Table 3) and that is in agreement with results of Billings and McMahon (1978), Atlas and Nebert (1976) and Thorgeirsson et al. (1979).
Rats pretreated with phenobarbital were selectively stimulated in 3- and 4-hydroxylation. However, 2- and 3-hydroxy biphenyl are produced in very small quantities and may not be detectable under most incubation conditions that have been reported. For example, 2.1 ug of 3-hydroxy biphenyl were formed along with 3.0 ug of 2-hydroxy biphenyl and 160 ug of 4-hydroxy biphenyl when a mitochondrial-free fraction from 10 g of liver was incubated with biphenyl for 30 minutes (Billings and McMahon, 1978).

Evidence of Species Differences

A conclusion from these studies is that voles are exceptional in the effectiveness of phenobarbital in induction of biphenyl 2-hydroxylation (Table 3). Voles pretreated with 3-methylcholanthrene or B-naphthoflavone had slightly increased 2-, 3- and 4-biphenyl-hydroxylation rather than specific induction for 2-hydroxylation (Table 3) as would be expected from previous studies (Billings and McMahon, 1978). These 3-methylcholanthrene-increased activities are indistinct from those of uninduced microsomes in terms of the ratio of one metabolite to the others.

The microsomal activity of white mice pretreated with 3-methylcholanthrene or B-naphthoflavone was studied to provide a species comparison (Table 3). A marked species difference in microsomal mixed function oxidase activities was noted between pine voles and white mice (Hartgrove et al., 1977). The present results with mice were in agreement with those of Billings and McMahon (1978) and Creaven and Parke (1966) and confirm a significant species difference between the microsomal mixed function oxidase system of meadow voles and white mice. Atlas and Nebert (1976) suggested that hepatic
biphenyl 2-hydroxylation activity in mice is associated solely with a 3-methylcholanthrene-inducible form of cytochrome P-450 (presumably P1-450 or P-448) and the absence of significant induction of this activity in the mouse by phenobarbital also supports this hypothesis. This demonstrates a significant species difference between the microsomal mixed function oxidase system of voles and mice.

Rats pretreated with 3-methylcholanthrene or B-naphthoflavone were preferentially stimulated in 2-hydroxylation (Billings and McMahon, 1978; Toftgard et al., 1980). A monooxygenase has been separated by protein isolation techniques and reconstituted with cytochrome P-448 from a liver of a rat pretreated with 3-methylcholanthrene. The reconstituted P-448 system supported biphenyl 2- and 4-hydroxylation (Burke and Mayer, 1975). Therefore, in rats and mice there is a similar pattern of induction (Creaven and Parke, 1966) with 3-methylcholanthrene and phenobarbital.

Hamsters pretreated with 3-methylcholanthrene had equal (or nearly so) induction of 2- and 4-hydroxylation (Burke and Bridges, 1975; Burke and Prough, 1976). Burke and Prough (1976) suggested that biphenyl 2- and 4-hydroxylases in hamster liver microsomes are generally identical although subtly different from one another. Burke and Prough (1976) discussed their conclusion relative to normal kinds of hydroxylation (constitutive) and those quantitatively increased by drug treatment (induced). Constitutive biphenylhydroxylase of hamster liver microsomes catalyzed both the 2- and 4-hydroxylation reactions, in contrast to rat liver microsomes wherein only a 3-methylcholanthrene inducible hydroxylase catalyzed biphenyl 2-hydroxylation (Burke and Mayer, 1975) or catalyzed 2- and 3-hydroxylation (Toftgard et al., 1980).
In hamster liver microsomes, there appeared to be two types of biphenylhydroxylase activities capable of catalyzing both the 2- and 4-hydroxylation reactions: a constitutive enzyme complex and a 3-methylcholanthrene inducible enzyme system (Burke and Prough, 1976).

A conclusion from the preceding discussion is that phenobarbital and 3-methylcholanthrene or B-naphthoflavone treatment had essentially the same effect on biphenyl 2- and 4-hydroxylation in mouse microsomes as in rat microsomes (Billings and McMahon, 1978) and these effects are different than those found in hamster microsomes.

In summary, the following conclusions are possible. There are some similarities between hamsters and voles. Hamsters pretreated with phenobarbital were induced preferentially in 4-hydroxylation (5.5 fold) as compared to 2-hydroxy (3.3 fold); 3-methylcholanthrene induced both reactions equally (Burke and Bridges, 1975). Voles pretreated with phenobarbital were induced in 2-, 3-, and 4-hydroxylation; 3-methylcholanthrene administration only slightly increased 2-, 3-, and 4-hydroxylation in ratios similar to those of control voles.

The amount of increase of microsomal protein per gram of liver after pretreatment of hamsters and voles with phenobarbital was almost equal in hamsters (55%) (Burke and Bridges, 1975) and in voles (60%) (Table 1). This does relate to an accepted classification of mammals. Rats and mice are in the family Muridae and also the same subfamily, Murinae, while voles and hamsters are in the family Cricetidae, but in different subfamilies. Hamsters are in subfamily Cricetinae and voles are in subfamily Microtinae.
(Gaylord, 1945). There are differences between hamsters and voles (members of different subfamilies) in response of biphenyl hydroxylation to the inducing agents, 3-methylcholanthrene. Pretreated hamsters showed induction for 2- and 4-hydroxylation and the Soret peak of the reduced cytochrome P-450 carbon monoxide complex was shifted from 450 to 448 nm (Burke and Bridges, 1975), or from 450 to 448.5 nm (Thorgeirsson et al., 1979). In voles pretreated with 3-methylcholanthrene, there was little increase in 2-, 3-, and 4-hydroxylation and only 0.8 nm shift of the P-450 absorption was observed (Fig. 6).

The lack of induction of voles by 3-methylcholanthrene or B-naphthoflavone and the high induction observed when voles are treated with phenobarbital (especially in 2-hydroxylation which is mainly induced by 3-methylcholanthrene in rats and mice), and almost equal induction with phenobarbital or 3-methylcholanthrene in hamsters (Billings and McMahon, 1978; Burke and Bridges, 1975; Burke and Prough, 1976) suggests that 2-, 3-, and 4-hydroxylation are associated principally with cytochrome P-450. In the vole there was spectral evidence for a presence of a cytochrome with a lower absorption maximum than P-450 since inducers of cytochrome P-448 were slightly effective in inducing biphenyl hydroxylation and lowered the absorption of cytochrome P-450 in the vole by up to 0.8 nm. With administration of appropriate inducers, hamsters have shown a hyposochromic shift of 1.5 nm while mice have shown shifts of 2.0 nm (Thorgeirsson et al., 1979). In D2 mice which do not respond to 3-methylcholanthrene induction, there is no spectral shift.

Thus, the monohydroxylation of biphenyl by monooxygenase enzymes occur at three positions in rats and mice, each with its
own characteristics. For example, 2-hydroxylation appears to respond to typical cytochrome P-448 inducers, while hydroxylation at position 4 responds to typical cytochrome P-450 inducers (Billings and McMahon, 1978; Burke and Mayer, 1975). However, that is not the case in voles where phenobarbital, as a typical cytochrome P-450 inducer, induced 2-, 3-, and 4-hydroxylation (5.4, 9.6 and 4.2 fold, respectively). Also in voles, 3-methylcholanthrene, a typical cytochrome P-448 inducer, did not show specific induction for 2-hydroxy biphenyl and led only to small and almost equal increases in the three monohydroxylations of biphenyl.

**Biphenyl Hydroxylation as a Toxicological Model**

Atlas and Nebert (1976) considered biphenyl 2-hydroxylase activity to be useful as a marker for cytochrome P-448 associated with 3-methylcholanthrene induction and that is the case for mice, rats and rabbits. The idea stated some years ago by Creaven and Parke (1966) that carcinogens are inducers of biphenyl 2-hydroxylase in rats and mice while noncarcinogens are not, is an overstatement since the noncarcinogen B-naphthoflavone is very similar to the carcinogen 3-methylcholanthrene as an effective inducer of 2-hydroxybiphenyl in rats and mice (Atlas and Nebert, 1976; Billings and McMahon, 1978). However, it may prove true that inducers of biphenyl 2-hydroxylase activity enhance tumorigensis because they induce cytochrome P-448 (Atlas and Nebert, 1976). In voles that would be a particularly interesting circumstance to test since phenobarbital can induce 2-hydroxy biphenyl while 3-methylcholanthrene and B-naphthoflavone, even with high levels and extended treatment, resulted in little increase in the amount of 2-hydroxy biphenyl (Table 3).
Toxicological tests with rodents have overwhelmingly emphasized rats and mice although, of course, considerable data have been published with rabbits and hamsters. Kato (1979) and Walker (1978) have emphasized quantitative and sometimes qualitative differences between species. This study of biphenyl hydroxylation in voles and mice shows a substantial reason to further compare the Cricetid and Murid rodents. Species differences should be taken into account in the selection of animal models for toxicological research. Characterization of monooxygenases by the comparative metabolism of substrates with multiple products is one way to identify species differences important in toxicology.
SUMMARY

The liver of mammals contains a 'monooxygenase' enzyme system that consists of components of the hepatocyte endoplasmic reticulum. These components include the flavoprotein, NADPH-cytochrome P-450 reductase, and a terminal oxidase such as cytochrome P-450 or P-448. One function of the monooxygenase system is to perform primary hydroxylation on lipophilic drugs, toxicants or pollutants. This increases the drug's polarity and susceptibility for conjugation. While monooxygenase reactions may result in enhancement of toxicological properties of some drugs, most of the effects are to detoxify the xenobiotic.

Because of the central role of monooxygenases in toxicology, there is considerable interest in their presence in animals and their responses to drugs. One characteristic response is the development of a higher level of metabolic activity (induced) in the liver after drug exposure. Comparison of inductive effects can be used to provide evidence of multiple forms of cytochrome P-450.

A number of mammals, some birds, invertebrates, plants, and microorganisms have been shown to metabolize the hydrocarbon, biphenyl (C_{12}H_{10}). Biphenyl is primarily hydroxylated and the characteristic pattern can be used to identify similarities or dissimilarities between either enzyme inducing agents or species.

The montane meadow vole, Microtus montanus, is a cricetid rodent which is taxonomically related to rats and mice at the order (Rodentia)
level but not to the level of family. Hamsters and voles, however, are in the same family but different subfamilies. A review of the literature indicated that there were possible species differences in biphenyl hydroxylation or other monooxygenase activities among these rodents and that monooxygenase levels in montane meadow voles had not been characterized.

Hepatic microsomes were prepared by differential centrifugation from female meadow voles. Incubation of biphenyl with microsomes and a NADPH generating system formed 4-hydroxy biphenyl (major metabolite), 3-hydroxybiphenyl (minor) and smaller amounts of 2-hydroxybiphenyl.

The voles were injected intraperitoneally with saline, saline containing phenobarbital, corn oil, or corn oil containing either 3-methylcholanthrene or B-naphthoflavone. Controls included microsomal preparation from uninjected voles.

The results indicated that there is a phenobarbital inducible metabolism of biphenyl to 3-, 2-, and 4-hydroxy biphenyl and that the quantitative production of metabolites increased in that order. Injection of B-naphthoflavone and 3-methylcholanthrene had slightly increased 2-, 3-, and 4-hydroxylation. This pattern of biphenyl hydroxylation in relation to induction differed significantly from that of rats or mice as reported in the literature. Similar experiments were performed with mice to confirm these results. Induction of biphenyl hydroxylation is more similar in voles to that of hamster though there appear to be subtle differences between these rodents as well.
It should not, therefore, be assumed that the responses of all rodents to toxicants or enzyme inducing agents will be the same as those of rats or mice. Further investigations of the toxicological effects observed in voles should be conducted to better understand their appropriateness as animal models in toxicological research.
LITERATURE CITED


Burke, M.D. 1975. Biphenyl hydroxylation and spectrally apparent interactions with liver microsomes from hamsters pretreated with phenobarbital and 3-methylcholanthrene. Xenobiotica 6:357-376.


APPENDIX
Table 4. Effect of Cytochrome P-450 induction on biphenyl hydroxylation by hepatic microsomes fraction of voles or mice (ug/gm liver/mlin)

<table>
<thead>
<tr>
<th>Treatment of Animals</th>
<th>2-hydroxy biphenyl</th>
<th>3-hydroxy biphenyl</th>
<th>4-hydroxy biphenyl</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mice</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None (4)</td>
<td>1.67 ± 0.22</td>
<td>1.37 ± 0.26</td>
<td>15.3 ± 3.4</td>
</tr>
<tr>
<td>B-naphthoflavone one (3)</td>
<td>2.4 ± 0.7</td>
<td>1.7 ± 0.6</td>
<td>14.77 ± 3.18</td>
</tr>
<tr>
<td>injection 80 mg/kg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-methylcholanthrene (3)</td>
<td>4.02 ± 1.4**</td>
<td>1.69 ± 0.26</td>
<td>17.15 ± 0.38</td>
</tr>
<tr>
<td>three injection 20 mg/kg</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Phenobarbital three (3)</td>
<td>1.6 ± 0.17</td>
<td>1.95 ± 0.12**</td>
<td>42.91 ± 2.45**</td>
</tr>
<tr>
<td>injection 50 mg/kg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Voles</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None (10)</td>
<td>0.45 ± 0.1</td>
<td>0.733 ± 0.14</td>
<td>5.55 ± 1.3</td>
</tr>
<tr>
<td>Corn oil (6)</td>
<td>0.56 ± 0.2</td>
<td>0.904 ± 0.29</td>
<td>6.7 ± 1.34</td>
</tr>
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<td>B-naphthoflavone (6)</td>
<td>0.733 ± 0.18</td>
<td>1.339 ± 0.43*</td>
<td>9.38 ± 3.09*</td>
</tr>
<tr>
<td>three injection 80 mg/kg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B-naphthoflavone (3)</td>
<td>0.62 ± 0.07</td>
<td>0.88 ± 0.149</td>
<td>7.15 ± 0.96</td>
</tr>
<tr>
<td>one injection 80 mg/kg</td>
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</tr>
<tr>
<td>3-methylcholanthrene (6)</td>
<td>1.046 ± 0.277**</td>
<td>1.3 ± 0.48</td>
<td>10.138 ± 2.5*</td>
</tr>
<tr>
<td>three injection 20 mg/kg</td>
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<td></td>
</tr>
<tr>
<td>Saline</td>
<td>0.442 ± 0.08</td>
<td>0.76 ± 0.22</td>
<td>5.57 ± 1.7</td>
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<td>Phenobarbital three (6)</td>
<td>4.0 ± 0.66**</td>
<td>12.016 ± 1.37</td>
<td>39.64 ± 4.3**</td>
</tr>
<tr>
<td>injection 50 mg/kg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenobarbital three (4)</td>
<td>2.049 ± 0.814**</td>
<td>4.83 ± 1.55**</td>
<td>24.96 ± 8.06**</td>
</tr>
</tbody>
</table>

* Indicates significant difference (P < 0.05)
** Indicates significant difference (P < 0.01)

The number of animals is shown in parentheses. Results are a mean ± standard deviation.