# MODULATION OF MIXED FUNCTION OXIDASE ACTIVITY BY VITAMIN K

by

Ivan Sadowski

A Thesis Submitted to the Faculty of Graduate Studies in Partial Fulfillment of the Requirements for the Degree of

Master of Science

The University of Manitoba

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To my wife, Shelley, for her encouragement, love,
and support for the duration of this work.

ABSTRACT

Polycyclic aromatic hydrocarbons (PAHs) such as benzo(a)pyrene (BP) are ubiquitous in the environment. These compounds are very hydrophobic, and once ingested could remain in the body indefinitely were it not for the drug metabolizing system. Phase I of drug metabolism, consisting of NADPH-dependant cytochrome P-450 reductase and cytochrome P-450, introduces a polar oxygen moeity onto the hydrophobic substrate. The Phase II enzymes conjugate these intermediates with glucuronic acid, glutathione or sulfate. These conjugated products can then be excreted. Although the primary function of the drug metabolizing system is the detoxification and excretion of non-polar xenobiotics, many compounds are activated by the same enzyme system. In this thesis it is proposed that the biphasic nature of drug metabolism results in a fine balance between detoxification and activation. Since activation generally occurs during Phase I metabolism of polycyclic aromatic hydrocarbons, and conjugation detoxifies these activated intermediates, regulation and modulation of the two phases are important in determining the fate of the parent compound. Menadione (Vitamin K3) and Phylloquinone (Vitamin K1) are shown here to alter Phase I metabolism of benzo(a)pyrene. These

alterations have profound effects on BP tumorogenesis in mice.

Menadione is shown here to inhibit Phase I metabolism of BP in the H4IIE rat hepatoma cell line, normal hepatocytes, microsomes, and a purified reconstituted mixed function oxidase (MFO) system consisting of cytochrome P-450 and cytochrome P-450 reductase. A permeabilized cell assay system was developed to assess the role of NADPH as a rate limiting component in cellular BP metabolism. NADPH is shown here to be a key limiting component in cellular mixed function oxidation of BP. Menadione is a better inhibitor of microsomal BP metabolism in the presence of a saturating NADPH supply than in whole cells where NADPH was found to be limiting. Menadione therefore does not inhibit by a direct depletion of NADPH. Kinetic experiments show that menadione and cytochrome P-450 compete for reduction by cytochrome P-450 reductase. This competition results in K3 being a potent inhibitor.

Phylloquinone was found to enhance BP metabolism in H4IIE cells, normal hepatocytes, microsomes and the purified MFO system. Three hypotheses are proposed as to the mechanism of this activation. The first is that the lipid soluble Vitamin K1 may enhance binding of BP to the enzyme. Secondly, K1 may operate to enhance the interactions of P-450 with P-450 reductase. A third hypothesis

is that K1 may function as an electron carrier between an NADPH-dependant reductase and P-450 to stimulate P-450 activity. The role of phylloquinone in modulating mixed function oxidase activity is a novel and previously unrecognized function of the Vitamin, probably independant of its known role in the synthesis of active clotting proteins.

Earlier studies have shown that menadione slows the rate of tumor formation in mice given BP, while phylloquinone enhances the rate of tumor formation. The K analogs are shown in this thesis to modulate this effect by altering the balance between activation and detoxification.

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# INTRODUCTION AND HISTORICAL

In the environment there are thousands of foreign organic molecules (xenobiotics). The origin of these compounds is as diverse as their molecular structure. Some have been synthesized by man in the search for new and better pesticides, herbicides, drugs, dyes, food attitives and other chemicals with industrial or pharmacological importance. A large number of these xenobiotics are natural products, synthesized by plants and lower animals. Others may occur, emanate or are generated from industrial wastes and by-products, burning of refuse and oil contamination. Many of these compounds are very hydrophobic, and if ingested could remain in the body indefinitely were it not for the drug metabolizing enzymes (Pelkonen and Nebert, 1982). This system consisting of both cytoplasmic and endoplasmic reticulum bound components is responsible for the excretion of these molecules by rendering them water soluble. The major enzymes involved in the initial oxidation include cytochrome P-450, NADPH-dependant cytochrome P-450 reductase and a lipid membrane component, phosphatidyl choline. Cytochrome b5 and NADH-dependant cytochrome b5 reductase may also be involved in some oxidations. NADPH and NADH provide the energy and reducing equivalents to drive the reactions, and are

important cofactors. These processes are referred to as drug oxidations, detoxification reactions or simply drug metabolism.

Many environmental xenobiotics are active in producing mutagenic, teratogenic, toxic, carcinogenic and pharmacological effects. Although some of these compounds are biologically operative in their original state, many others are unreactive until metabolized by this system. Metabolism may result in a more active molecule (Pelkonen and Nebert, 1982). Detoxification is the conversion of an active or toxic parent compound into an inert water soluble, excretable metabolite. Activation is the production of a reactive molecule from a previously inactive compound. The primary purpose of the drug metabolizing enzymes is detoxification. However, many compounds are activated by the same enzymes that detoxify other compounds (Lu and West, 1980). A particular drug metabolizing enzyme may detoxify one compound, while activating another. Activation is an important mechanism in the induction of chemical carcinogenesis. The production of reactive molecules by drug metabolism may lead to the formation of covalent linkages of these intermediates to DNA, RNA, and protein (Nebert et al, 1981). Studies by Bruce Ames in the 1970s showed that there is a strong correlation between mutagenic and carcinogenic effects

(Ames et al, 1975). Adducts in macromolecules can lead to genetic and epigenetic alterations in cell processes, thus producing toxicity, mutagenesis, carcinogenesis and teratogenesis.

Drug Metabolism

Drug metabolism consists of two distinct phases. Phase I, also known as functionalization, is responsible for the introduction of a polar group, mainly oxygen, into the molecular structure of the lipophilic parent compound to form a more hydrophilic metabolite (Pelkonen and Nebert, 1982). This polar daughter compound can then act as a substrate for the Phase II or conjugation enzymes which conjugate the molecule with glucuronic acid, sulfate, or glutathione. The link is usually made with the newly added oxygen or polar moiety formed during Phase I metabolism. This mechanism renders hydrophobic xenobiotics water soluble, which is a prerequisite for excretion. During this process, while some toxic agents are rendered non-toxic, others form active toxic intermediates. Depending on various factors, the same compound can be either activated or detoxified.

These factors governing the production of toxic or detoxified metabolites are subjects of extensive investigation. It is proposed in this thesis that a delicate

balance exists between detoxification, and activation. This equilibrium is a consequence of the biphasic characteristic of drug metabolism. Frequently, the Phase I product is more reactive than the parent compound. Conjugation, however, renders this product non-toxic (Wiebkin et al, 1979; Hesse et al, 1982). If the reaction rate of either of the two phases is altered, the equilibrium between activation and detoxification will be shifted. Therefore effectors of either conjugation or activation may alter the fate of the parent compound (Burke et al, 1977). Factors which regulate the enzyme systems involved thus have a signifigant effect in determining this fate. There are, however, some examples of toxic products formed by the Phase II conjugation enzymes. These include aromatic amines, dichloroethane and procainamide (Pelkonen and Nebert, 1982). In general, activation occurs during Phase I. The products are more reactive with intracellular components because of the newly added polar group. Once the conjugate is added to these polar moieties, the compound is effectively detoxified (Glatt and Oesch, 1977). The crucial period then is the interim between the two phases. If conjugation is able to proceed at a rate sufficient to conjugate all of the functionalized products, then the balance will be in the favor of detoxification. However, if Phase I proceeds at a rate exceeding the

capabilities of conjugation, then a signifigant mass of active daughter compounds will accumulate. This accretion can result in adduct formation in macromolecules. Regulatory factors that stimulate functionalization or inhibit Phase II will shift the balance in favor of activation. Detoxification will dominate in a situation where Phase I is inhibited or conjugation is increased (Sparnins et al, 1982).

Cytochrome P-450

A key enzyme in Phase I metabolism is cytochrome P-450. The term P-450 is derived from the early work on the enzyme demonstrating that it was the carbon monoxide binding microsomal pigment which absorbed light strongly at 450 nm. It was later shown to be an atypical hemoprotein, capable of binding substrate (Omura and Sato, 1964a and b). This hemoprotein has a molecular weight of about 55,000 daltons and is present in the endoplasmic reticulum. In the late 1960s, P-450 was shown to be the substrate binding site, and terminal oxidase of the drug oxidation system. The catalytic ability of this enzyme is astounding as the number of different compounds which function as substrates is enormous. Examples include xenobiotics such as polycyclic hydrocarbons, anthracenes, polychlorinated biphenyls, defoliants, insecticides, antibiotics, drugs, nitrosamines, aminoazo and diazo

compounds, and many others (Nebert et al, 1982). There is evidence that P-450 can metabolize at least 120 drugs and environmental chemicals (Burke et al, 1981). Endogenous molecules are also substrates, these include steroids, fatty acids and prostaglandins. Cytochrome P-450 is the major enzyme mediating Phase I drug metabolism. At least nine different reactions are performed, most of these involve the addition of a single oxygen atom. The reactions include aliphatic oxidation, aromatic hydroxylation, N-dealkylation, O-dealkylation, S-demethylation, oxidative deamination, sulfoxide formation, N-oxidation and N-hydroxylation (Nebert and Jensen, 1979). In most cases the parent compound is more hydrophobic than the daughter compound. These Phase I reactions all serve the same end in providing a substrate for the Phase II enzymes. This substrate must have a polar group to which a conjugate can be linked. Because of the number of chemicals that can be oxidized by the drug metabolizing system, the process has loosely been termed the Mixed Function Oxidase (MFO) System.

Cytochrome P-450 monooxygenase activity has been found in almost all living organisms, including bacteria. The enzyme structure is highly conserved from species to species, as there are extensive sequence homologies between the rat, mouse and rabbit genes (Chen et al, 1982).

Similarly, the activity has been located in many tissues of the body including the lung, liver, intestinal tract, lymphocytes, placenta, and is thought to be ubiquitous. The activity is highest in the liver. Cytochrome P-450, as is the case with many drug metabolizing enzymes, is inducible. Exposure of an organism to xenobiotics, particularily planar aromatic hydrocarbons causes induction of P-450s to a level 5-10 fold higher than the basal or uninduced activity (Nebert et al, 1981). Considering the diverse catalytic ability of the enzyme, it was not surprizing to find that a whole family of cytochrome P-450s exist. The important question that has generated a current controversy is how many P-450s exist in any given organism (Lu and West, 1980). Using rat liver as an example there have been at least eight and possibly as many as 20 different P-450s identified on the basis of polyacrylamide gel electrophoresis, spectral properties, catalytic activity, immunological reactivity, peptide mapping and partial amino acid sequences (Warner et al, 1978). Recently the entire amino acid sequences of several P-450s have been published (Heinemann and Ozols, 1983). Six of the eight identified have been shown to be inducible and parts of their genes have been cloned (Atchison and Adesnik, 1983).

Any one particular P-450 has the capability to

metabolize a wide variety of substrates. Different isozymes of P-450 may utilize the same compound as substrate, however the metabolites could be different because of different stereospecificities and affinities toward the molecule. Overlapping substrate specificity could explain the diverse spectrum of xenobiotics that are metabolized by the P-450s. This is the view taken by Lu and West. They postulate that there is a large but finite number of P-450s. An absence of complete substrate specificity allows a wide range of compounds to be metabolized (Lu and West, 1980).

On the other side of the controversy is the idea that there may be hundreds or even thousands of P-450s. The theory proposed by Nebert suggests that every xenobiotic may induce its own P-450, much like the immune system responds to an antigen (Nebert et al, 1981; Nebert and Negishi, 1982). The hypothesis is that every organism has the genetic capacity to produce as many forms of P-450 as there are inducers in the environment. More than 200 environmental xenobiotics are presently known to be capable of inducing P-450. In the 1960s two major classes of inducers were identified, those inducing a cytochrome with an absorbance maximum at 448 nm, as induced by 3-methylcholanthrene (P-448), and those inducing a cytochrome with an absorbance maximum at 450 nm, as induced by pheno-

barbital (P-450). These two forms are the major inducible isozymes, and have been studied extensively. Their catalytic abilities are dissimilar and their molecular weights slightly different. However it is now known that compounds previously thought to induce a P-448 or a P-450 isozyme, actually induce a slightly different enzyme (Nebert et al, 1981). These new variant isozymes were discovered using improved methods of chromatography and electrophoresis. Many laboratories are now studying other inducers, and the types of enzyme produced.

Recent data has shown that responsiveness to xenobiotics is controlled by a cytosolic receptor protein. This receptor is the structural gene product of the Ah (aromatic hydrocarbon) locus (Negishi and Nebert, 1981; Tukey et al, 1981). The Ah locus was characterized in C57BL/6 mice (Gielen et al, 1972; Nebert and Bausserman, 1970), which were demonstrated to be capable of synthesizing a mRNA for P-448 in response to 3-methylcholanthrene exposure, whereas DBA/2 mice were not (Tukey et al, 1982). DBA/2 mice are therefore unable to elevate P-450 in response to aromatic hydrocarbons (Nebert et al, 1982). At least two dozen monooxygenase activities are under the control of the Ah receptor. These include polycyclic hydrocarbon and aflatoxin hydroxylations, O-demethylation of 7-ethoxycouramin, and O-demethylation of p-nitroanisole.

Other inducible enzymes of the drug metabolism system that appear to be under Ah control are UDP-glucuronyl-transferase (conjugation) and cytosolic NADP oxidoreductase (quinone reductase) (Lind and Ernster, 1974; Kumaki et al, 1977). P-450 mediated activities which are not associated with the Ah locus include metabolism of phenobarbital, estrogen, benzphetamine and aniline. Other drug metabolizing enzymes not regulated by the Ah locus are NADPH cytochrome P-450 reductase, epoxide hydrolase and glutathione transferase (Gonzalez and Kasper; 1982).

The reaction cycle of cytochrome P-450 during the oxidation of drugs (Figure 1) begins with the binding of the substrate to the enzyme (step 1). Substrate binding alters the electron configuration of the iron porphyrin prosthetic group of the molecule and subsequently causes a spectral change. Hemoproteins typically produce such spectral changes upon substrate binding, acceptance and transfer of an electron, and binding or displacement of a ligand from the heme iron (Schenkman et al, 1981; Jefcoate, 1978; Jansson, 1980; Backes, 1981). Binding of substrate can be measured by monitoring the difference spectra of the enzyme without substrate against enzyme with substrate. After substrate binding, the $Fe^{3+}$ group is reduced to $Fe^{2+}$ via electron transfer from NADPH-dependant cytochrome P-450 reductase (step 2). The reduced hemoprotein substrate

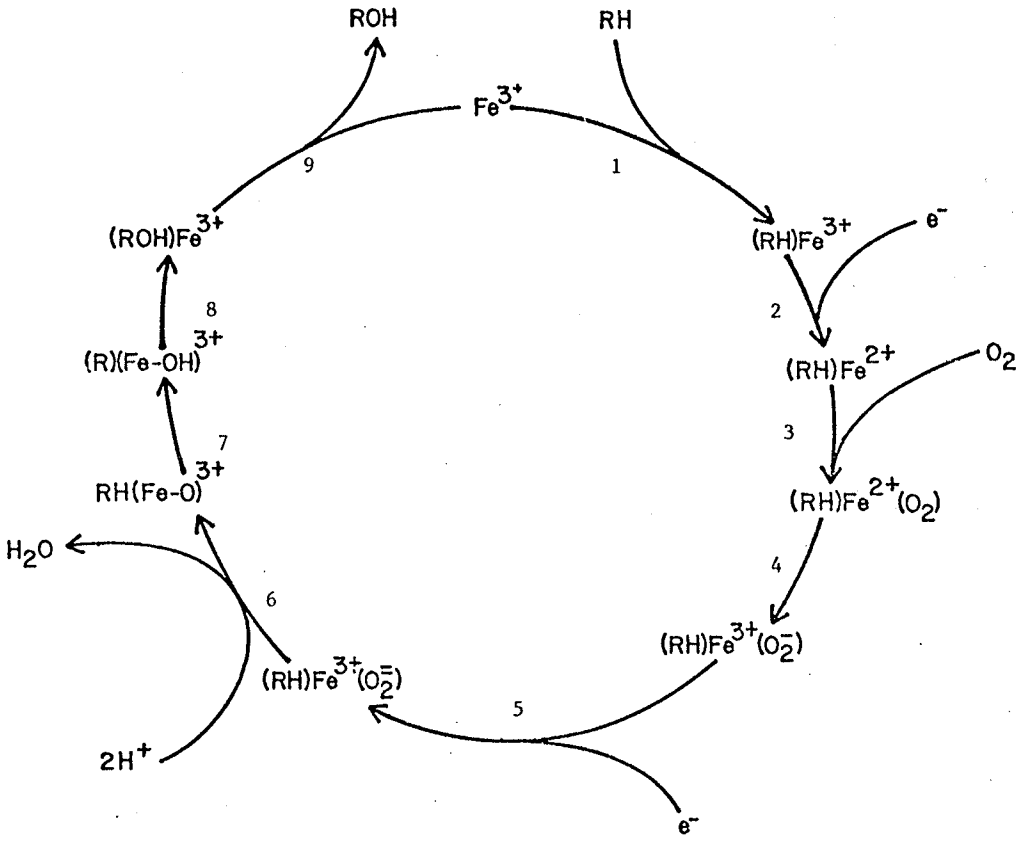
complex then binds oxygen (step 3). A second electron is transferred from P-450 reductase or cytochrome b5 (step 5). The reduced oxygenated hemoprotein substrate complex dissociates to yield water and hydroxylated substrate, regenerating the oxidized hemoprotein (steps 6-9). Cytochrome P-450 is known to be organized into clusters of proteins in the microsomal membrane. These clusters could be arranged around a P-450 reductase molecule, or randomly interact in the fluid membrane with reductase. Most evidence suggests that the enzymes are not rigidly arranged, and free lateral mobility is possible to allow for random interactions between the proteins (Yang, 1977).

Cytochrome P-450 Reductase and Cytochrome b5

NADPH-dependant cytochrome P-450 reductase, or NADPH cytochrome c reductase is a flavoprotein containing one molecule of both flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) per molecule of enzyme. This enzyme catalyzes the two single electron transfers to cytochrome P-450 from the two electron donor NADPH. Depleting the enzyme of its flavins and subsequent reconstitution have shown that the two nucleotides have separate roles in the catalysis. FAD accepts electrons from NADPH, and FMN transfers the electrons to cytochrome P-450 (vermilion et al, 1981; Iyanagi, 1980). The hypothesis is that FAD accepts two electrons from NADPH in a single two

FIGURE 1: Cytochrome P-450 reaction cycle. Proposed scheme for mechanism of action of cytochrome P-450 in hydroxylation reactions. RH represents a substrate and ROH the corresponding hydroxylated product. The oxidation reduction state of the heme iron is shown for all steps. See accompanying text for specific details.

FIGURE 1


ROH
RH
$Fe^{3+}$
9
1
$(ROH)Fe^{3+}$
$(RH)Fe^{3+}$
$e^-$
2
8
$(R)(Fe\text{-}OH)^{3+}$
$(RH)Fe^{2+}$
$O_2$
3
7
$RH(Fe\text{-}O)^{3+}$
$(RH)Fe^{2+}(O_2)$
$H_2O$
4
6
$(RH)Fe^{3+}(O_2^-)$
$(RH)Fe^{3+}(O_2^=)$
5
$2H^+$
$e^-$

electron step and FMN donates two electrons to P-450 in successive one electron steps. The reductase probably contains a single binding site for the reduced dinucleotide. P-450 reductase is also capable of reducing artificial one and two electron acceptors such as cytochrome c, ferricyanide, and dichlorophenolindophenol (Masters et al, 1965; Powis and Appel, 1980). Another microsomal hemoprotein, Cytochrome b5, can also accept electrons from reductase. NADH as well as NADPH has been shown to be an electron donor for cytochrome P-450 reductase. The Km for NADH is very high however (Noshiro and Omura, 1978), and probably does not reduce the enzyme to any extent in vivo. Studies done using incorporation of reductase into microsomal membranes have shown that it is the rate limiting enzyme component of the mixed function oxidase system. Therefore, it is a key enzyme in the regulation of xenobiotic metabolism (Miwa et al, 1978). There may be as many as 30-100 molecules of P-450 per molecule of P-450 reductase following induction. The enzyme has been purified to apparent homogeneity from rat and rabbit liver (Yasukochi and Masters, 1976; Dignam and Strobel 1977; Vermilion and Coon, 1974). Enzymes isolated from 3-methlycholanthrene and phenobarbital treated animals are indistinguishable. There is no evidence for multiple isozymes. Purification data revealed that the enzyme contains a hydrophobic amino acid

chain which is responsible for anchorage of the molecule to the microsomal membrane (Knapp et al, 1977; Black and Coon 1982). This chain can be cleaved from the enzyme with trypsin. A trypsinized preparation of reductase is able to reduce artificial electron acceptors, but not P-450. An untrypsinized preparation requires a detergent in the reaction buffering system, and is able to reduce cytochrome P-450 in vitro (Black et al, 1979). The anchorage peptide chain is probably required for complex formation between the two enzymes.

When the microsomal mixed function oxidase system was first resolved into its separate components, it was discovered that besides cytochrome P-450 and the reductase a third heat stable factor was required for activity. This factor was determined to be the lipid component phosphatidyl choline (Strobel et al, 1970). The role that phosphatidyl choline plays in stimulating enzyme activity is still not clear, however a number of mechanisms have been suggested. A primary function of the lipid may be to facilitate the formation of a complex between P-450 and the reductase (Lu et al, 1980). Conformational changes of P-450 may be easier in the presence of the phospholipid (Tsong and Yang, 1978). A fluid environment provided by the lipid may be necessary for electron transfer between the two proteins (Ingelman-Sundberg, 1977). The lipid

probably accomodates all three of these activities, but the physical role of optimizing interactions of the two proteins is probably more important than a direct chemical function. Microsomal membranes have a complex assortment of phospholipids. Altering the fatty acid composition of the membrane has been shown to profoundly affect P-450 activity (Wills, 1980). A large number of lipids are able to stimulate activity in vitro, however dilaurylphosphatidylcholine generates the greatest activity.

Some early microsomal data suggested that not only NADPH was required for P-450 activity, but also NADH. Controversy surrounded this topic for years until it was discovered that some, but not all,subtrates required cytochrome b5 for their oxidation (Werringloer and Kawano, 1980). Cytochrome b5 is a hemoprotein which receives electrons from either NADH cytochrome b5 reductase, or NADPH P-450 reductase. Cytochrome b5 donates the second electron to P-450. An important finding concerning b5 is that the introduction of this second electron in the P-450 reaction cycle (Figure 1) may be the rate limiting step in that process (Bjorkhem, 1977). If this is so, depending on the substrate, cytochrome b5 could be an important regulatory step in mixed function oxidase catalytic activity. Complex formation between the two hemoproteins b5 and P-450 have been shown to alter the spin state of the

latter. It is suggested that these interactions may result in an alteration of the catalytic ability towards different substrates (Tamburini and Gibson, 1983; Fujita and Pelsach, 1977). Other recent data suggests that various isozymes of P-450 have different requirements for cytochrome b5 (Kamataki et al, 1983; Waxman and Walsh, 1983).

Conjugation Enzymes

Phase II metabolism includes a series of conjugation enzymes such as UDP-glucuronyltransferase, arylsulfotransferase, and glutathione S-transferase. Other enzymes which are partially involved in Phase II metabolism are epoxide hydratase and cytosolic DT diaphorase (Gelboin, 1980).

Conjugation with glucuronic acid represents the major excretion mechanism for xenobiotics. UDP-glucuronyltransferase is microsomal and has several different isozymes. The multiple forms have not been completely studied, but it is speculated that they have different substrate specificities. This would enable them to handle a wide variety of primary metabolites (Burchell and Weatherill, 1981). UDP glucuronic acid is the source of the carbohydrate, glucuronic acid is conjugated to the xenobiotic and UDP is released.

The Aryl Sulfotransferases are a group of cytosolic enzymes which conjugate sulfate from AMP-5 -phosphosulfate to a functionalized compound to form an aryl sulfur group and adenosinemonophosphate. At least four isozymes have been isolated and are grouped into those that have a specificity for phenols, and those that are specific for hydroxylamines (Sekura et al, 1981). A number of conjugated sulfur metabolites are more active than the parent compound, or the primary derivative. One example of these is 2-acetylaminoflourine (Miller, 1970).

The third conjugation enzyme is glutathione S-transferase. This enzyme is also cytosolic and conjugates glutathione to a polar group such as halogens, sulfur, nitrogen or oxygen moieties. Transferase is present in high concentrations in the liver and represents a large part of the conjugating ability of the cell (Habig and Jakoby, 1981). In regard to the more lipophilic molecules encountered by the drug metabolizing enzymes, GSH transferase, as well as aryl sulfotransferase, are rather limited in their detoxification abilities. These molecules remain closely associated with lipid membranes and are not readily available to the cytosolic conjugation enzymes. Xenobiotics which possess polar moieties in the original state can be conjugated with glucuronic acid or sulfate without Phase I metabolism given sufficient solubility in the cytosol.

Regulation of Drug Metabolism

A very important aspect of drug metabolism that has been largely ignored is the metabolic regulation of the activities of the enzymes involved. Because of the balance between detoxification and activation, regulation is an important topic in relation to chemical carcinogenesis. In a whole cell system,regulation can occur at a number of different levels and is very complex. This complexity is drived from the fact that there is a multiple array of enzymes that are involved, and metabolism is dependant upon a supply of reduced NADPH. NADPH itself is generated by other multienzyme systems which also have complex control mechanisms. Regulation of the system can be imposed by induction, substrate and cofactor supply, activators and inhibitors and by competing reactions with endogenous substrates (Thurman and Kauffman, 1980).

Induction, although a slow means of regulation, can have a profound effect on xenobiotic metabolism. Exposure to xenobiotics results in a specific profile of P-450s in the endoplasmic reticulum by the induction and supression of various forms (Parkinson et al, 1983). Different inducers are not only capable of inducing unique forms of P-450, but also induce other enzymes as well. These secondary enzymes are either directly or indirectly involved in drug metabolism. A different array of enzymes

is induced for each inducer. For example 3-methylcholanthrene induces UDP-glucuronyltransferase, NADPH generating enzymes, cytosolic DT diaphorase (Conway et al, 1983; Fagan et al, 1983; Reinke et al, 1981; Kauffman and Evans, 1980), and glutathione S-transferase (Pickett et al, 1982), whereas phenobarbital induces glucuronyltransferase as well as NADPH cytochrom P-450 reductase and several NADPH generating systems (Gonzales and Kasper 1980 and 1982). Induction by xenobiotics also results in a proliferation of smooth endoplasmic reticulum (Orrenius and Ericsson, 1966). The metabolic capabilities of the drug metabolism system are altered to accomodate the excretion of the specific inducer. Metabolism of a particular compound will be altered by induction with different inducers (Alexandrov, 1977).

When discussing activation and inhibition, it is very important to consider rate limiting factors in the metabolic process. Two rate limiting factors of Phase I metabolism have been mentioned earlier, these were the introduction of the second electron in the P-450 reaction cycle, and NADPH cytochrome P-450 reductase as a rate limiting enzyme component. Many inhibitors and activators of mixed function oxidation have been found. Activators generally act by altering the spin state of the P-450 modecule, or enhance the binding capabilities of the

substrate (Cinti, 1978). Inhibitors of P-450 have also been extensively studied. Some act directly on the enzyme by blocking substrate binding sites or iron cation interaction with the porphyrin ring structure. These include phenols, quinones and isocyanides. Others act in an indirect manner and require conversion to a reactive molecule, often mediated by P-450. These include amines, SKF-525A and hydrazines. A third group of P-450 inhibitors are those which are irreversible. These bind covalently to the enzyme and as a result destroy the catalytic activity (Testa and Jenner, 1981). P-450 reductase has been shown to be inhibited by metabolic intermediates such as ATP, ADP, a-ketoglutarate and oxaloacetate (Dombrowski et al, 1980). This gives support to the hypothesis that mixed function oxidation and energy metabolism are coupled in vivo. No intermediatory metabolites have been found that inhibit P-450, hence intracellular regulation of drug metabolism is probably mediated by reductase activity. Specific antisera to P-450 reductase inhibits mixed function oxidation (Masters et al 1973) lending further support to the hypothesis that reductase is a key rate limiting component, and a possible target for regularory mechanisms.

Inhibitors of eigher P-450 or P-450 reductase would certainly lower the rate of drug metabolism, but what is

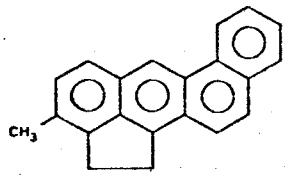
the absolute rate limiting factor in a whole cell system? Studies by Thurman and Kauffman (1977) using a liver perfusion technique have shown that the supply of reduced dinucleotide is probably limiting. A decline in NADPH supply resulted in decreased mixed function oxidation. Also NADPH:NADP ratios were correlated directly with MFO activity (Thurman et al, 1977). Therefore, factors which deplete or enhance the supply of reduced dinucleotides in the cell would cause an alteration in the rate of xenobiotic metabolism. This would include factors such as nutritional state, inducing agents and agents affecting NADPH production. The Pentose phosphate shunt is thought to be a primary source for reduced dinucleotide for drug metabolism (Junge and Brand, 1975). Recent data suggests that mitochondria are an important source of NADPH for drug oxidations as well as or instead of the pentose phosphate shunt (Reinke et al, 1983). The NADPH supply is also closely regulated by induction. When mixed function oxidase enzymes are induced the capacity of the cell to produce NADPH is also greatly increased (Conway et al, 1983). Cofactors can also be limiting for Phase II reactions. This is a major factor in conjugation regulation, which is dependant upon a supply of UDP-glucuronic acid, glutathione, and inorganic sulfur (Reinke et al, 1981).

Benzo(a) Pyrene

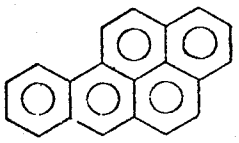
Polycyclic Aromatic Hydrocarbons (PAHs) are some of the most important xenoboitics and chemical carcinogens in the environment (Figure 2). Some of the sources of polycyclic aromatic hydrocarbons are emissions from automobiles, industrial processes, energy production by hydrocarbons, cooking and smoking. Cigarette smoke is a rich source of PAHs, which may be a cause of the high incidence of lung cancer in smokers (Doll and Hill, 1964). One of the most widely studied PAH is benzo(a)pyrene (BP). This compound is a potent carcinogen and commonly found in the environment (Phillips, 1983). Very sensitive techniques have been developed to assay its metabolism and subsequently it has been used as a model carcinogen for many years (Nebert and Gelboin, 1968). Like almost all PAHs benzo(a)-pyrene requires activation before it becomes mutagenic and carcinogenic. Benzo(a)pyrene is a good inducer as well as a good substrate for the mixed function oxidase system. The isozyme of P-450 that has the greatest affinity for BP is cytochrome P-448, or the major 3-methylcholanthrene inducible form. Other forms of P-450 will metabolize BP, but the various types of the enzyme have very different steriospecificities toward the molecule (Brunstrom and Sundberg, 1980). Because of the multiple sites at which cytochrome P-450 can act upon a polycyclic aromatic hydro-

FIGURE 2: Polycyclic aromatic hydrocarbons.

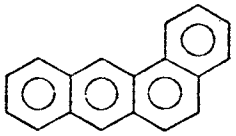
FIGURE 2



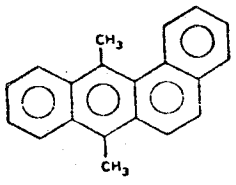

3-METHYLCHOLANTHRENE



BENZO(a)PYRENE



BENZO(a)ANTHRACENE



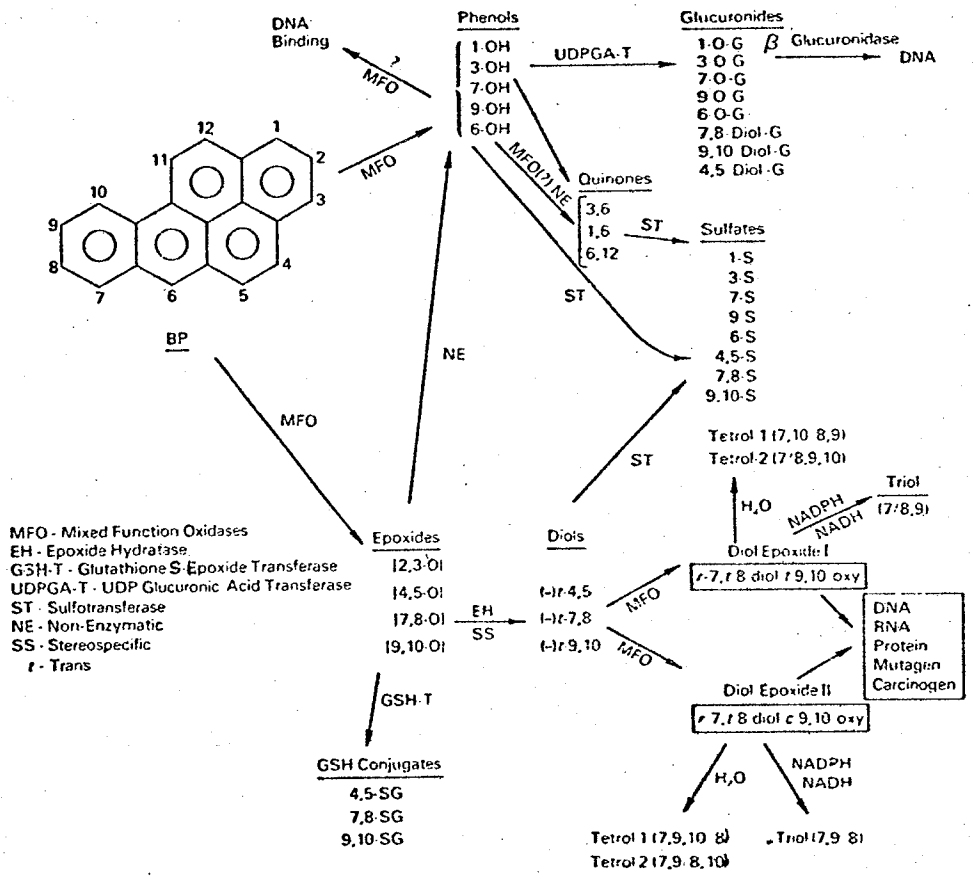

7,12-DIMETHYLBENZO(a)ANTHRACENE

carbon, a large number of different metabolites are formed (Figure 3). For benzo(a)pyrene there are over 40 different metabolites identified, which include phenols, quinones and diols. These metabolites are conjugated to glucuronic acid, sulfur, and glutathione prior to excretion.

The primary metabolites of Phase I metabolism are the 4, 5-epoxide, 7, 8-epoxide, and the 9, 10-epoxide. A 2, 3-epoxide is a theoretical intermediate which is converted to phenols. Phenols can be formed directly from P-450, or through non-enzymatic reactions from the epoxides. Phenols that have been identified are the 1-OH, 3-OH, 7-OH, 9-OH, and 6-OH forms. Phenols and epoxides are conjugated directly to glucuronic acid or glutathione respectively via UDP-glucuronic acid transferase or GSH-transferase. The phenols often act as substrates for P-450 again to form the 3, 6, 1, 6, and 6, 12 quinones. The epoxides are converted by epoxide hydratase to the t4, 5, t7, 8, and t9, 10 diols (Weinstien et al, 1976; Fahl, 1982). These diols may also act as substrates for the MFO system to form Diol epoxide I and Diol epoxide II which are potent mutagens and carcinogens (Geacintov et al, 1982; Kinoshita et al, 1982). The diol epoxides can undergo further reactions to form triols and tetrols (Osborne et al, 1976). The diols, quinones and phenols are conjugated to sulfates by arylsulfotransferases.

FIGURE 3: Metabolism of benzo(a)pyrene by cytochrome P-450. Figure taken from Gelboin (1980), Physiolog. Rev., 60; 1107.

FIGURE 3


DNA Binding
?
MFO
Phenols
1-OH
3-OH
7-OH
9-OH
6-OH
UDPGA-T
Glucuronides
1-O-G
3 O G
7-O-G
9 O G
6 O-G
7,8-Diol-G
9,10 Diol-G
4,5 Diol-G
β Glucuronidase
DNA
MFO
MFO(?) NE
Quinones
3,6
1,6
6,12
ST
Sulfates
1-S
3-S
7-S
9 S
6-S
4,5-S
7,8-S
9,10-S
ST
BP
NE
MFO
ST
Tetrol 1 (7,10 8,9)
Tetrol-2 (7'8,9,10)
H₂O
NADPH
NADH
Triol
(7'8,9)
MFO - Mixed Function Oxidases
EH - Epoxide Hydratase
GSH-T - Glutathione S-Epoxide Transferase
UDPGA-T - UDP Glucuronic Acid Transferase
ST - Sulfotransferase
NE - Non-Enzymatic
SS - Stereospecific
t - Trans
Epoxides
1,2-Ol
4,5-Ol
7,8-Ol
9,10-Ol
EH
SS
Diols
(-)-t-4,5
(-)-t-7,8
(-)-t-9,10
MFO
MFO
Diol Epoxide I
r-7,t 8 diol t 9,10 oxy
DNA
RNA
Protein
Mutagen
Carcinogen
Diol Epoxide II
r 7,t 8 diol c 9,10 oxy
GSH-T
GSH Conjugates
4,5-SG
7,8-SG
9,10-SG
H₂O
NADPH
NADH
Tetrol 1 (7,9,10 8)
Tetrol 2 (7,9 8,10)
Triol (7,9 8)

A number of metabolites have the capacity to bind to DNA and are therefore potential mutagens (Owens et al, 1979; King et al, 1975). Recycling of metabolites through the enzyme system also occurs at a number of points (eg. phenols, diols). The more potent mutagens and carcinogens are generally those that have been recycled, or the more polar (secondary) metabolites. The amount of recycling that occurs depends on a number of factors. Large amounts of BP as substrate results in the formation of more primary metabolites and fewer recycled products (Shen et al, 1979). Small amounts of BP allows for greater availability of catylitic sites for primary metabolites (Bowden et al, 1982). Conjugation enzymes also affect the amount of secondary product formation. Conjugation of primary products lowers the availability of oxygenated metabolites for recycling. Benzo(a)pyrene metabolism is a good example of the delicate balance between detoxification and activation as mediated by functionalization and conjugation. Butylated Hydroxyanisole (BHA) is known to stimulate the glutathione transferases and inhibit mixed function oxidation (Benson et al, 1978). Dietary BHA results in an inhibition of the formation of BP induced DNA adducts in mouse liver, lung and forestomach (Adriaenssens, 1983). This example with BHA demonstrates the importance that regulators of drug metabolism can have on benzo(a)pyrene

metabolism, and mutagenicity. Prevention of secondary metabolite formation and accumulation by conjugation of primary products reduces the liklihood of adduct formation.

A sensitive assay system for BP metabolism has been developed using a fluorimetric technique (Nebert and Gelboin, 1968). 3-OH BP is a very fluorescent major phenol metabolite that is easily extracted from a reaction mixture. The extent of total metabolism can be derived from the amount of 3-OH formed. A second assay system measures total BP metabolism using a radiolabled substrate (De Pierre et al, 1975). The amount of BP remaining in the aqueous phase of the reaction after hexane extraction determines the extent of metabolism. Both of these assays have their advantages, which include the sensitivity of the fluorescent assay and the convenience of the radioactive assay. Probably the best assay for BP metabolism is by High Pressure Liquid Chromatography (HPLC). Techniques have been developed that can separate the primary, secondary, or the more polar metabolites (Yang et al, 1977). Using HPLC it has been shown that various forms of P-450 have different catalytic activities for BP as substrate. The various forms exhibit preferential hydroxylation at different points on the BP molecule (Gozukara et al, 1982). Thus induction of P-450s in an animal by various chemicals can alter the profile of benzo(a)pyrene metabolites (Yang

et al, 1975). This alteration is mediated not only by the isozyme induced but also by other factors such as activity of conjugation enzymes and NADPH supply.

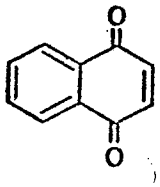
Vitamin K

Vitamin K was first discovered in the 1930's and was shown to be an essential factor in the coagulation process. The term Vitamin K is used to describe all quinone analogs of 2-methyl-1, 4napthoquinone (Figure 4). Vitamin K1 or 2-methyl-3-phytyl-1, 4-napthoquinone (phylloquinone) is lipid soluble, and a close analog to Vitamin K2 (menaquinone). Vitamins K1 and K2 have direct antihemorrhagic activity, unlike the unalkylated (2-methyl-1, 4-napthoquinone) Vitamin K3 (menadione). In general the form synthesized by plants is phylloquinone, whereas menaquinones are produced by bacteria. It is difficult to assay the spectrum of K analogs in animals as the concentrations are low. However a number of menaquinones (K2s) have been identified (Suttie, 1980).

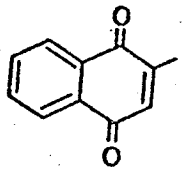
An important aspect to consider when studying the K vitamins is the concentrations at which they occur in the body. Vitamin K is found only in very low concentrations, even in the liver where most of the K is concentrated the total content is about 0.06 ug/g (Duella and Matschiner, 1972). The majority of this is located within the micro-

FIGURE 4: The Vitamins K1, K2, K3 and related compounds. The vitamin K antagonist, warfarin.

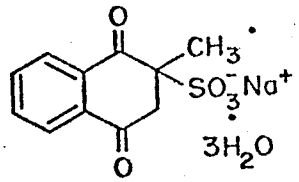
FIGURE 4



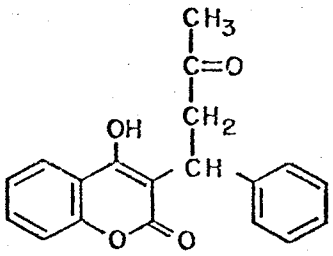
1,4-NAPTHOQUINONE



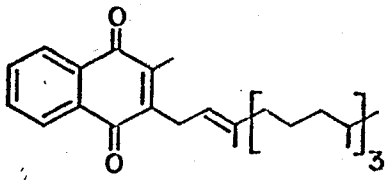
MENADIONE



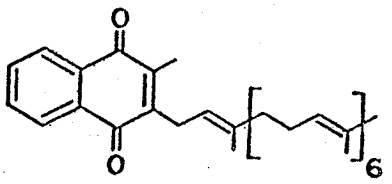
MENADIONE BISULFITE



WARFARIN



PHYLLOQUINONE



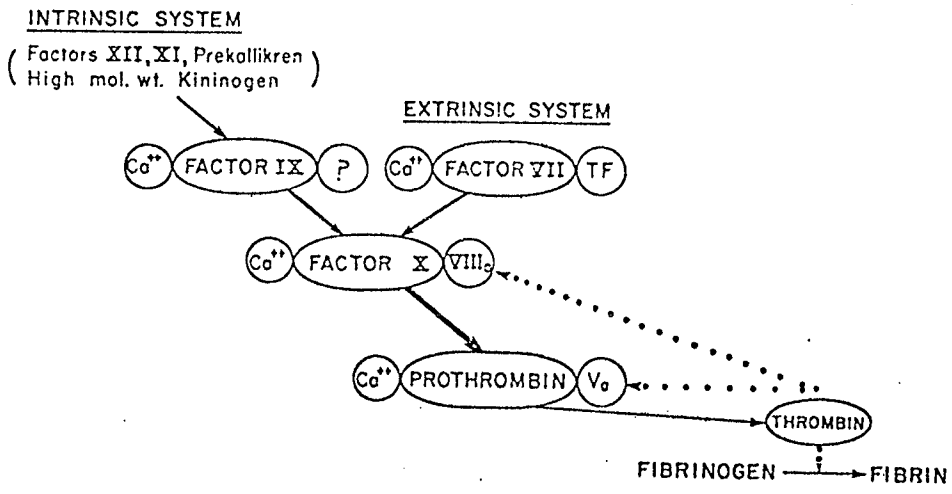
MENAQUINONE

somal membrane, as well as a small amount in the mitochondria. Animals are not able to synthesize the 2-methyl-1, 4-napthoquinone ring structure, but side chains are added to compounds such as menadione, and interconversions of dietary K1 components occur (Suttie, 1978).

The blood clotting mechanism is a complex cascade reaction ultimately leading to the formation of fibrin. Triggering of the process results in a series of proteolytic reactions which activate subsequent steps (Figure 5). A key step in the process is the conversion of prothrombin to thrombin. Thrombin activates the conversion of fibrinogen to fibrin. There are two pathways leading to the formation of the fibrin clot. The intrinsic pathway involves components present only in plasma whereas the extrinsic pathway involves plasma factors as well as tissue factors. The intrinsic pathway is initiated when factor XII interacts with a collagen surface, resulting in the conversion of XII to XIIa. Factor XIIa activates factor XI which in turn activates factor XX conversion to IXa. An interaction of factors IXa and VIII forms a complex which acts as a catalyst in the activation of factor X. In the presence of calcium ions and phospholipid, the activated X interacts with factor V to form a complex capable of converting prothrombin to thrombin. This involves cleavage of a large glycopolypeptide from

FIGURE 5: Cascade model for the whole blood coagulation system. The four components enclosed in ellipses are the Vitamin K-dependant clotting proteins. Taken from Jackson and Suttie (1977), Prog. Hemat., 10;333.

FIGURE 5


INTRINSIC SYSTEM
( Factors XII, XI, Prekallikren
High mol. wt. Kininogen )
EXTRINSIC SYSTEM
Ca++
FACTOR IX
?
Ca++
FACTOR VII
TF
Ca++
FACTOR X
VIIIa
Ca++
PROTHROMBIN
Va
THROMBIN
FIBRINOGEN
FIBRIN

the amino terminus to form intermediate II which is cleaved again to form thrombin. Thrombin then converts the soluble plasma protein fibrinogen to fibrin, which is an insoluble gel. The extrinsic pathway bypasses a number of reactions through a tissue factor and factor VII. Factor VII enters the coagulation pathway by activating factor X directly (rev., Davie and Fujikawa, 1975).

Factors X, VII, XI and prothrombin (II) are dependant on Vitamin K for their synthesis. Prothrombin is a calcium binding protein derived rrom preprothrombin. This conversion is regulated by Vitamin K. The modification consists of post-translational carboxylation of glutamic acid residues to form Y-carboxyglutamic acid (Olson et al, 1978). The enzyme involved, Vitamin K dependant carboxylase, requires a reduced form of the vitamin (KH2), carbon dioxide, oxygen and the precursor protein preprothrombin, as well as phosphatidylcholine (Esmon and Suttie, 1976; deMetz et al, 1981). Carboxylase appears to be a very complicated enzyme, possibly consisting of a multienzyme complex (Wallin and Suttie, 1982). Success has been limited to date in attempts to purify and solubilize the enzyme. The formation of Y-carboxyglutamic acid residues (gla) also coincides with the formation of a vitamin K 2, 3-epoxide (Suttie et al, 1978). To regenerate a reduced form of the vitamin, the 2, 3-epoxide is cycled

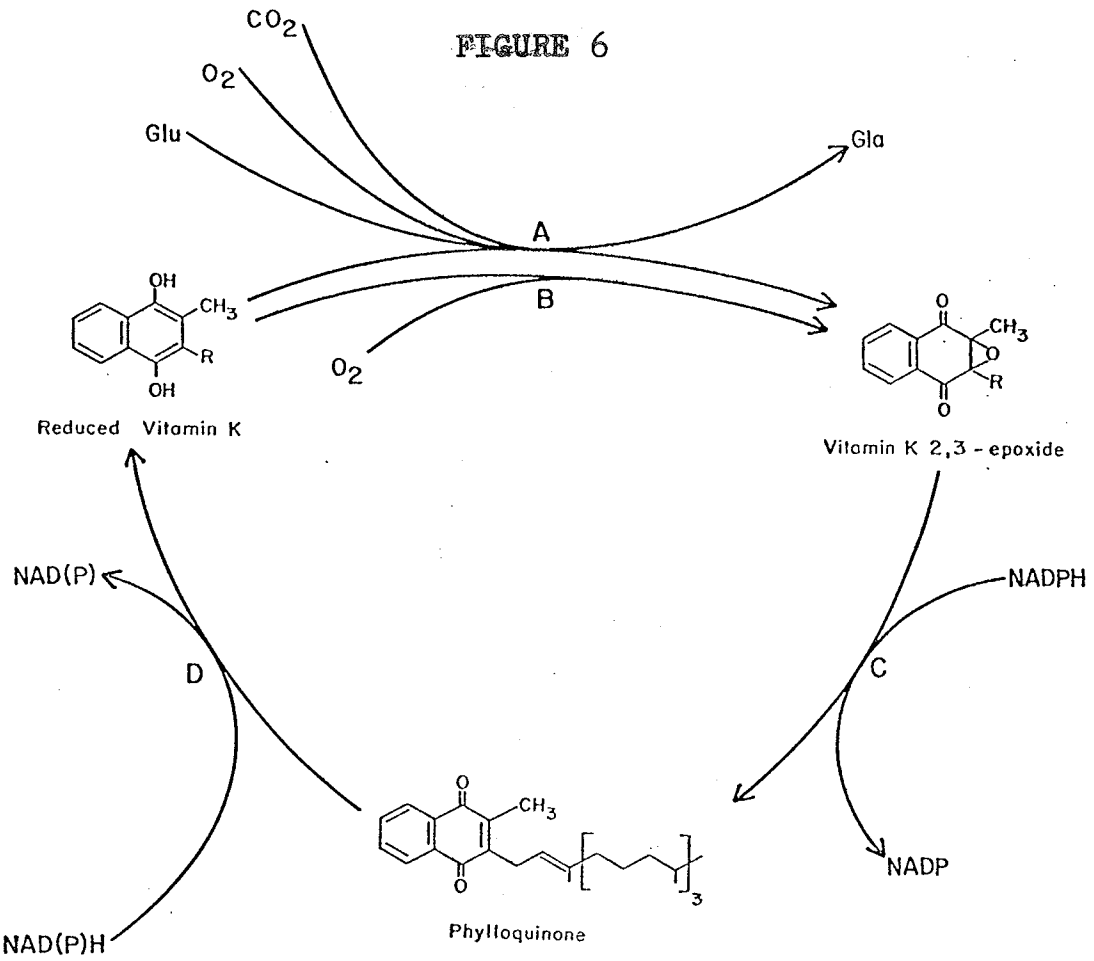
through the oxidized form by an epoxide reductase (Siegfried, 1978), then to the reduced form by a diaphorase (Wallin et al, 1978) (Figure 6). The Vitamin K cycle and the carboxylase enzymes are microsomal. NAD(P)H supplies electrons for both the epoxide reductase and diaphorase (Jackson and Suttie, 1977). There is a close association in the microsomal membrane between the K cycling and carboxylase enzymes and the drug metabolizing enzymes. This is evident from purification data which show the P-450 is a major contaminant in purified carboxylase preparations (Wallin and Suttie, 1982).

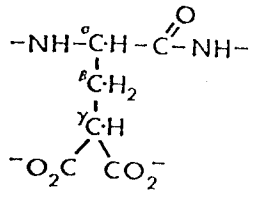
Y-carboxyglutamyl residues have also been found in factors VII, IX and X. These proteins are also Vitamin K dependant (Suttie, 1980). The gla residues confer on these proteins the ability to bind calcium which in turn facilitates binding to phospholipid matricies. In the conversion of prothrombin to thrombin a proteolysis of two peptide bonds is catalyzed by factor X. Calcium ions enhance this reaction, and when the reaction complex is able to interact with a phospolipid bilayer in the presence of calcium ions, the reaction is 50-100 times faster than in the absence of phospholipid (Jackson and Suttie, 1977).

It is still unclear as to what the role of Vitamin K is in the carboxylase reaction. Three possibilities have

FIGURE 6: Vitamin K cycle. Enzyme activities are; a) Vitamin K-dependant carboxylase, b) Vitamin K epoxidase, c) Vitamin K epoxide reductase, and d) DT-diaphorase. Note that the same enzyme, Vitamin K-dependant carboxylase probably catalyzes both reactions a and b. In lower Figure 6 the structure of the Y-carboxyglutamyl amino acid residue is shown.

FIGURE 6




γ CARBOXYGLUTAMYL

been proposed (rev., Johnson, 1981). One is that K is acting as a carrier for carbon dioxide. A second is that K may somehow labilize a hydrogen on the Y-carbon of the glutamic acid residue so that $CO_2$ can attack that carbon. A third possibility is that the vitamin is required to keep the enzyme in the active state (Suttie, 1978). Recent data presented by Larson suggests that the labilization of the Y-carbon hydrogen is probably the primary function (Larson et al, 1981). Any model proposed must take into consideration the relationship between Vitamin K epoxide formation and carboxylation, since the formation of the carboxylated residues proceeds in association with the formation of the epoxide (Suttie et al, 1978). However, as the epoxide has been shown to be formed in the absence of a carboxylase reaction, this would suggest that the two processes are not concomitantly related (Suttie, 1980). Menadione is not able to catalyze the carboxylase reaction, therefore the lipophilic side chain is a necessary moiety of the K1 molecule for coagulant activity (Sadowski et al, 1976). Vitamin K3 administered to animals probably must be alkylated in vivo before it can participate in K-dependant synthesis of coagulation factors.

The coumarins are a group of compounds that are Vitamin K antagonists. There is still some controversy surrounding the mechanism of coumarin action (Suttie, 1978).

The observations that the actions of Vitamin K and coumarins such as warfarin (Figure 4) could be offset by an increased dose of the other led to the theory of competition for a receptor protein. It was suggested that since the compounds were dissimilar chemically a negative allosteric effect mediated by a second receptor site for warfarin may be operating. There are observations which suggest that both warfarin and Vitamin K interact with a ribosomal protein in the regulation of prothrombin biosynthesis (Olson, 1974). This is not consistent with the known role of Vitamin K in the carboxylase reaction. Another theory suggests that warfarin acts by blocking the transport of Vitamin K. There is only indirect evidence for this theory and no indication is given as to which membranes are involved (Lowenthal and Birmbaum, 1969). A more plausible theory proposed by Matshiner is that warfarin acts by inhibiting the Vitamin K epoxide reductase and quinone reductase reactions, thus preventing the recycling of the reduced form of K. There is much evidence to support this hypothesis. The epoxide reductase from warfarin resistant rats was shown to be less sensitive to the drug when studied in vitro (Matshiner et al, 1974; Caldwell et al, 1970). Other studies indicated that administration of warfarin resulted in an accumulation of the K epoxide (Bell and Matshiner, 1970; Matschiner et al,

1970). A problem with this theory is that when a large supply of reduced K exists, either in vivo or in vitro, warfarin is still inhibitory. Possibly, a cycling event for K is required for carboxylation regardless of the amount of available reduced Vitamin.

The role of Vitamin K in microorganisms is different. In the 1930s it was found that quinones such as Vitamin K were members of the respiratory electron transport chain. It is thought thak K transfers electrons from an NADH dependant flavoprotein to a b cytochrome (Martius, 1954). Many other quinones such as menadione were found to be able to bypass parts of the respiratory chain, thus restoring respiration in an inhibited system (Brodie and Watanabe, 1966). Vitamin K in Mycobacterium phlei and other organisms appears to function in the same manner as ubiquinone in mammalian mitochondria, linking two electron carrier flavins and the single electron carrier cytochromes (Suttie, 1978). Menaquinones were also shown to have structural importance in the cell membranes of certain bacteria (Goldenbaum et al, 1975). There was early evidence, such as that presented by Martius (Martius and Nitz-Litzow, 1954), that K was involved in mammalian oxidative phosphorylation. These claims could not be corroborated however. Other studies by Wosilait (1961), indicated that Kl could function as an electron carrier

between a vitamin K1 reductase and cytochrome c. Any further evidence that K1 is involved in oxidative phosphorylation, however, was not substantiated. Consequently the only known role of Vitamin K in mammalian organisms is the involvment in post translational modification of proteins involved in calcium binding.

Regulation of the Mixed Function Oxidase System by Vitamin K

An interrelationship between the metabolism of the K vitamins and cytochrome P-450 activity was postulated when it was shown that K3 modified the toxic effect of rapeseed meal in chickens (Israels et al, 1979). This toxicity was probably mediated by xenobiotics which were activated by the MFO system. Further investigation of this phenomenon revealed that menadione inhibited the microsomal metabolism of benzo(a)pyrene in both an induced and an uninduced state. Vitamin K1 was also shown to inhibit microsomal BP metabolism, but only at very high concentrations (100uM and greater). Metabolism of BP in tissue explants was also inhibited by Vitamin K3. Using the Ames Salmonella/microsome mutagenicity assay (Ames et al, 1975) K3 was demonstrated to inhibit the mutagenic effect of BP. Studies using whole animals showed that menadione, K-deficiency, or warfarin treated mice had a decreased incidence of tumors as compared to the control BP group. Vitamin K1

on the other hand produced a much higher death rate from tumors induced by BP than did the control group. These observations led to the conclusion that the K series of vitamins may have an important role as regulators of mixed function oxidase activity (Israels et al, 1983). Vitamins K1 and K3 were proposed to be acting at different metabolic sites, thus producing opposite effects.

The effect of Vitamin K3 on MFO metabolism has also been documented to inhibit oxidative demethylation of aminopyrene and p-chloro-N-methylaniline (Wills, 1969), cortisol metabolism (Kupfer et al, 1968), and the N-oxidation of N, N-dimethylaniline (Hlavica and Kehl, 1977). Wills suggested that menadione may be mediating oxidation of NADPH thus rendering it unavailable for mixed function oxidation (Wills, 1972). In a whole cell system, since NADPH may be limiting, a depletion of reduced dinucleotide by menadione could cause inhibition of the MFOs. The mechanism of delay of tumorogenesis and tumor death in mice given benzo(a)pyrene by menadione could be a slowing of mixed function oxidation with the prevention of polar metabolite accumulation (Israels, 1983).

The action of Vitamin K1 on tumorogenesis and tumor death in mice is opposite to that of menadione. This effect is more difficult to explain since at high concentrations in vitro K1 shows an inhibitory effect of BP

metabolism. In an in vivo situation where an NADPH supply is limiting, Vitamin K1 could be modulating BP metabolism by affecting the NADPH supply. The vitamin K cycle which also uses NADPH and is microsomal could produce its effect through competition for the reduced dinucleotide. Evidence that this K metabolizing process is modulating BP metabolism in vivo is that K-deficiency, and treatment with warfarin resulted in a decreased incidence of BP induced tumorogenesis and tumor death (Israels, 1983).

The fetus and newborn are markedly Vitamin K deficient. This is a result of the inability of the lipid soluble vitamin K to cross the placenta. The resulting deficiency of the K dependent clotting factors places the newborn at hemorrhagic risk. To prevent bleeding an injection of Vitamin K1 is routinely given to post-partum human infants to increase the K-dependant clotting factors.

The project described in this thesis in part investigates the possible signifigance of this phenomenon. Prenatal exposure to carcinogens such as polycyclic aromatic hydrocarbons, mycotoxins, aromatic amines and nitrosamines have been shown to induce tumors in a number of different species (Drukrey et al, 1969). Activation of the procarcinogen can occur in the mother, the placenta, or the fetus. Cytochrome P-450 has been shown to be induced in the fetal liver by BP exposure (Oesch, 1980) and in the

placenta of women who smoke during pregnancy (Nebert et al, 1979). The fetus is particularily at risk because of its rapid growth rate and the lack of a direct excretory pathway for water soluble metabolites. Vitamin K could be detrimental to the fetus and newborn because of its possible relationship to xenobiotic metabolism. Because of the large number of xenobiotics that cross the placenta, Vitamin K deficiency may be protective against mutagenesis and carcinogenesis.

Outline of Research

In this work the possible regulatory role of the K vitamins on mixed function oxidation is examined. The study centered around two major areas of interest. The first was the effect of the K vitamins on benzo(a)pyrene metabolism in whole cell systems. The main advantage of using whole cells rather than microsomes as an experimental model is that the intrinsic supply of NADPH can be utilized. Since this supply is nonsaturating, as it should be in an in vivo situation, the possible relationship between K metabolism and NADPH depletion can be examined. Another advantage of using whole cells is that the drug metabolism system consists of both cytoplasmic and microsomal components. To study the effect of a regulator on total BP metabolism a complete drug metabolizing system should be

present. Two cell systems were used in this work. The first was the H4IIE rat hepatoma cell line. This cell line was derived from a Reuber hepatoma H-35 in 1961 (Reuber, 1961). The basal activity of cytochrome P-450 is very low and can be induced in vitro by adding an inducer to the medium. The second cell system used in this study was intact hepatocytes isolated from normal rat liver. The procedure used for the isolation of the cells was an in situ perfusion of the cell matrix disrupting enzymes, collagenase and hyaluronidase (Berry, 1974).

The second major area of work was that with a purified mixed function oxidase system. Cytochrome P-450 and NADPH-dependant cytochrome P-450 reductase were purified separately, then reconstituted to form a solubilized drug oxidation system. Using a purified drug metabolizing system, the direct effects of the K vitamins on the Phase I enzymes could be determined.

The data presented demonstrate the important balance between conjugation and functionalization in the mediation of toxification or detoxification. The K series of vitamins are shown here to have an important and previously unrecognized effect on the metabolism of xenobiotics by the microsomal mixed function oxidase system.

# MATERIALS AND METHODS

## H4IIE RAT HEPATOMA CELL LINE

### Background

The H4IIE cell line was derived from the Reuber H-35 hepatoma which was induced in a rat fed N-2-fluorenyl-diacetamide. The line has been maintained in cell culture since 1964 (Pitot et al, 1964). Whereas the H-35 hepatoma was a relatively well differentiated hepatoma, the H4IIE line is rather poorly differentiated. The line retains many of its original functions however, such as bile pigment secretion and drug metabolism. The growth rate has increased substantially upon prolonged culture. The doubling time of the H-35 line was reported as 220 hours in 1970 (Looney et al, 1970). Evans and Kovacs reported a doubling time of the H4IIE cell line in 1977 of 49 hours (Evans and Kovacs, 1977) in vivo as a solid tumor. The cells can be cultured in monolayer or in suspension, however growth in suspension is much slower. The basal level of aryl hydrocarbon hydroxylase activity is low. This activity can be induced in vitro by adding xenobiotics to the culture medium. A method for detecting polycyclic aromatic hydrocarbons has been developed using induction of P-450 enzyme activity in H4IIE culture (Bradlaw and Casterline, 1979).

Our H4IIE cell line was obtained from the American Type Culture Collection Cell Repository, Rockville, Maryland (ATCC CRL 1548) in August of 1982. Experiments with the hepatoma cells examined the effect of the K vitamins on BP metabolism in intact cells. A procedure for permeabilizing the membrane of the cells to allow for supplementation of polar molecules such as reduced dinucleotides is described.

Culture Conditions

The medium used for culture of the cell line was alpha minimal essential medium (MEM) plus 10% foetal bovine serum (FBS) supplemented with penicillin G (100 units/ml) and streptomycin sulfate (100 ug/ml). The cells were incubated in a 37°C humidity controlled incubator with a 5% $CO_2$ atmosphere. Cell suspensions were prepared using 0.2% Bacto trypsin in phosphate buffered saline (PBS). Routinely, plating density upon repassage of cells was 1 million cells per 10 cm diameter petri plate. The doubling time of the cell line was determined to be 13-15 hours, which is much shorter than the solid tumor form.

Induction of Aryl Hydrocarbon Hydroxylase and Assay of Enzyme Activity in Whole Cells

The procedure for induction and assay of enzyme activity was a modification of the procedure described by

Gielen and Nebert (1972). A trypsinized cell preparation was centrifuged and resuspended in MEM + 10% FBS and counted. The suspension was plated at a density of $10^6$ cells per 10 cm plate in 10 ml of medium. Growth was allowed to proceed for 24 hours at which point the plates were approximately 25% confluent. The medium was removed and replaced with MEM + 10% FBS + 0.25% DMSO + 1 uM 3-methylcholanthrene. The cells were again allowed to grow for 20-24 hours. The medium was removed at this point and replaced with 0.2% trypsin to harvest the cells. To obtain a single cell suspension the cells were trypsinized for 15-20 minutes at room temperature, mixing well with a pasteur pipette after the cells had detached from the surface of the plate. This treatment had little effect on the viability as measured by trypan blue exclusion (Phillips, 1973). Routinely viabilities of 98-99% were obtained providing the freshly trypsinized pelleted cells were resuspended in MEM + 10% FBS. To assay enzyme activity the cells were again pelleted and resuspended in MEM + 50mM Hepes buffer pH 7.4 at 37°C. 300 ul of the cell suspension was dispensed to 13x100 mm reaction tubes. A 10 mm stirring bar was added and the tubes preincubated for 5-15 minutes with gentle stirring at 37°C. Stirring the reaction prevented the cells from clumping and provided an even reaction suspension. BP was added in 6 ul acetone

to give a final concentration of 80 uM BP, thus starting the reaction. The reactions were allowed to proceed for 20 minutes whereupon they were stopped by the addition of 300 ul cold acetone. The tubes were immediately placed on ice. To determine the extent of BP metabolism, 3-OH BP was extracted from the reaction medium as described in the procedure for the spectrofluorimetric BP assay. To assay total BP metabolism a tritium labelled benzo(a)pyrene substrate was used with a specific activity of 12.5 uCi/umole. For this assay, the reaction was stopped with 300 ul of 0.5 M NaOH in 80% ethanol. Total BP metabolism was determined using the procedure described for the radioactive BP metabolism assay.

Permeabilized Cell Assay for AHH

The procedure for the permeabilization of the cell membrane was developed from a modification of an in vivo assay for ribonucleotide reductase (Dick and Wright, 1980; Hards and Wright; 1983; Wright et al, 1981). This technique involves the use of the non-ionic detergent Tween-80 to render the cell membrane permeable to large polar molecules such as reduced nicotinamide adenine dinucleotide phosphate (NADPH). Prolonged exposure of the cells to Tween-80 was found to be inhibitory to BP metabolism. This inhibition was probably mediated by a disruption of

the microsomal membrane or conversion of P-450 to the inactive form P-420 (Yamano and Ichikawa, 1978). Therefore, the standard assay for AHH in permeabilized cells allows for permeabilization of the outer membrane without interference with the endoplasmic reticulum. The trypsinized cell suspension in MEM + 10% FBS was pelleted and resuspended in permeabilizing buffer (0.25 M sucrose, 50 mM Hepes pH 7.4 and 1.0% Tween-80). The cells were mixed with a pasteur pipette to remove any clumps, and incubated at room temperature for 5-7 minutes. The cells were again pelleted and resuspended at a density of $5.26 \times 10^6$ cells/ml in 0.25 M sucrose, 50 mM Hepes pH 7.4. 200 ul of the permeabilized cells were added to 100 ul prewarmed reaction mixtures containing 240 uM BP (in 6ul acetone), 3mM NADPH and 9 mM $M9Cl_2$. The reactions were incubated with gentle stirring for 20 minutes at 37°C whereupon they were stopped by the addition of 300 ul cold acetone. The reaction tubes were immediately placed on ice. Extraction of 3-OH BP for assaying BP metabolism by the spectrofluorimetric assay is described in a later section dealing specifically with that assay procedure.

## NORMAL HEPATOCYTES

### Isolation

To facilitate the study of BP metabolism in normal

rat liver cells, morphologically intact isolated parenchymal cells were prepared using a liver perfusion technique (Berry, 1974). Male Sprague-Dawley rats weighing 150-250 grams were anesthetized with ether, placed on an operating board, and the abdomen opened completely. A ligature was loosely placed around the vena cava above the renal vein. A needle and catheter was placed into the portal vein, the needle was removed and the catheter secured by ligature. The inferior vena cava was immediately cut below the loose tie to allow the effluent to flow freely into the abdomen. Tubing from a buffer reservoir via a peristaltic pump was connected to the canula, the flow of buffer through the liver was started and gradually increased to a rate of 50-60 ml per minute. Prior to begining the operation the pump was run long enough to fill the tubing with buffer. This ensured that flow through the liver was begun as soon as possible. The perfusion buffer used was 150 ml Hanks Balanced Salt Solution (HBSS), buffered with 25 mM $NaHCO_3$ and carbon dioxide. The buffer was oxygenated throughout the procedure and maintained at 37°C in a water bath. The pH of the perfusate was kept as close as possible to 7.4, by regulating the flow of $CO_2$ to the reservoir. Once perfusion had begun, the lobes of the liver were rapidly cleared of erythrocytes resulting in a lightening of color. Immediately after flow was

established, the chest cavity was opened and a needle and canula inserted into the thoracic segment of the inferior vena cava. The needle was removed and the cannula secured by ligature. This effluent canula was connected to a tube which recirculated the buffer to the reservoir. The loose ligature on the inferior vena cava below the diaphragm was then tightened to force the effluent to recirculate. The collection reservoir was placed at a level below the animal to prevent hepatic swelling. About 50 ml of perfusion buffer was lost before the recirculating system was set up, leaving 100 ml. When flow through the liver was satisfactory, collagenase and hyaluronidase were added dissolved in 10 ml of buffer to give final concentrations of 0.05% and 0.1% respectively. The perfusion proceeded for 5-10 minutes at which time fluid began to leak from the surface of the liver, collecting in the abdomen. This was returned to the reservoir by syringe. When perfusion had progressed to the point where the liver was so digested that perfusion could not be maintained (25-45 minutes), the pump was stopped and the liver removed and transferred to a beaker. Using a spoon, the liver was broken apart completely. A successful perfusion resulted in a suspension with the consistency of a homogenate. 50 ml of perfusion buffer + 0.05% collagenase was added to the suspension and incubated for

10 minutes at 37°C with gentle shaking. It was found that oxygenation of the suspension at this point improved the viability of the cells. The suspension was then filtered through a cotton swab into siliconized conical centrifuge tubes, and cintrifuged for 2 minutes at 100g. The supernatant was decanted and the cells washed and resuspended in MEM + 50 mM Hepes buffer pH 7.4 at 37°C. A cell count in trypan blue (Phillips, 1973) was used to assay viability and total cell number. Viability in the experiments shown was typically 70-85%.

Assay of BP Metabolism

The BP metabolism assay in normal hepatocytes was a modification of the procedure described by Jones et al (1978) and is essentially the same as described for the H4IIE cells. 300 ul of a $3.5 \times 10^6$ cells/ml suspension was dispensed to 13x100 mm reaction tubes. A stirring bar was added and the cells preincubated for 10 minutes before adding the BP (80 uM in 6 ul acetone). The reactions were stirred gently so as not to disrupt the cellular integrity. The reactions were stopped with 300 ul acetone, or 300 ul 0.5 N NaOH in 80% ethanol if radioactive substrate was used. Extraction and assay of the products of BP metabolism were as described in the procedures for the radioactive and spectrofluorimetric AHH assays.

ROUTINE TECHNIQUES

Preparation of Microsomes From Rat Liver

Microsomes were used for the preparation of purified enzymes and solubilized carboxylase, or for assay of BP metabolism by microsomal P-450. The procedure for preparation was basically the same regardless of the final utilization. If induced microsomes were required, male Sprague-Dawley rats were given 2 intraperitoneal injections of 3-methylcholanthrene (3-MC) in corn oil on two consecutive days prior to sacrifice. The dose of the 3-MC was 40 mg/kg, dissolved at a concentration of 4 mg/ml. Induced as well as uninduced rats were fasted for 12-18 hours before sacrifice. The rats were killed by decapitation and the livers removed and placed in cold 0.25 M sucrose. Excess blood and tissue fragments were washed from the livers which were then dried and weighed. After mincing with scissors, the livers were homogenized in 3 volumes 0.25 M sucrose, using a cooled glass homogenizer with a teflon pestle. The homogenate was centrifuged at 3,000g for 10 minutes, and the resulting supernatant centrifuged again at 10,000g for 10 minutes to obtain a clean post-mitochondrial supernatant. To pellet the microsomes the supernatant was centrifuged at 105,000g for 60 minutes. The microsomal pellets were resuspended in 0.25 M sucrose to a concentration of 35 mg protein/ml, if

the preparation was to be used for microsomal BP metabolism. Protein concentrations were determined by the Lowry protein assay (Lowry et al, 1951). For preparation of purified enzymes, the pellets were resuspended by homogenizing in 1.15% KCl, 10 mM EDTA and recentrifuged to pellet. These washed microsomes were resuspended in 0.25 M sucrose to a protein concentration of 35 mg/ml and frozen at -80°C under a nitrogen atmosphere.

Microsomal Metabolism of Benzo(a)pyrene

Metabolism of BP by microsomal preparations was measured in a reaction containing 500 ug/ml microsomal protein, 3 mM $MgCl_2$, 1.0 mM NADP, 0.36 Units isocitrate dehydrogenase/ml, 6.0 mM isocitrate, 5 uM $MnCl_2$, 50 mM Tris-HCl pH 7.4 and 80 uM BP. The 300 ul reactions were preincubated for 5 minutes to generate NADPH before BP was added. The reactions were incubated at 37°C with vigorous stirring, and stopped by the addition of 300 ul acetone, or 300 ul 0.5 N NaOH in 80% ethanol if a radioactive substrate was used. The procedures for measuring the extent of BP metabolism were as described in the methods for the spectrofluorimetric and radioactive AHH assays.

Spectrofluorimetric Assay for BP Metabolism

The spectrofluorimetric assay measures production of

3-OH BP and was a modification of the procedure described by Nebert and Gelboin (1968). To extract 3-OH BP from the reaction solution, 975 ul n-hexane was added to the 600 ul stopped reaction mixture (300 ul acetone, 300 ul reaction). The tubes were stoppered to prevent the evaporation of hexane and vortexed vigorously. The two phases were allowed to separate at room temperature. 500 ul of the hexane layer was added to 1.5 ml 1.0 N NaOH. To reextract 3-OH BP into the aqueous layer the tubes were again vortexed vigorously. Once the phases had separated, a sample of the lower phase was read in the spectofluorometer (Aminco Bowman). The characteristic excitation and emission spectra of 3-OH BP is shown in Figure 7. For measuring 3-OH production the excitation wavelength was set at 396nm and the emission wavelength at 522nm. The concentration of 3-OH BP in the original reaction solution was extrapolated from a standard curve of the relative fluorescence vs. concentration of 3-OH (Figure 8). The fluorescence of 3-OH BP was linear with respect to concentration over a wide range. The maximum sensitivity of the assay was approximately 7.5 pmoles/ml. Fluorescence remained linear until at least 10 nmoles/ml. The standard curve was constructed using purified product obtained from IIT Research Institute, Chicago.

FIGURE 7: Fluorescence spectra of 3-OH BP in sodium hydroxide.

FIGURE 7

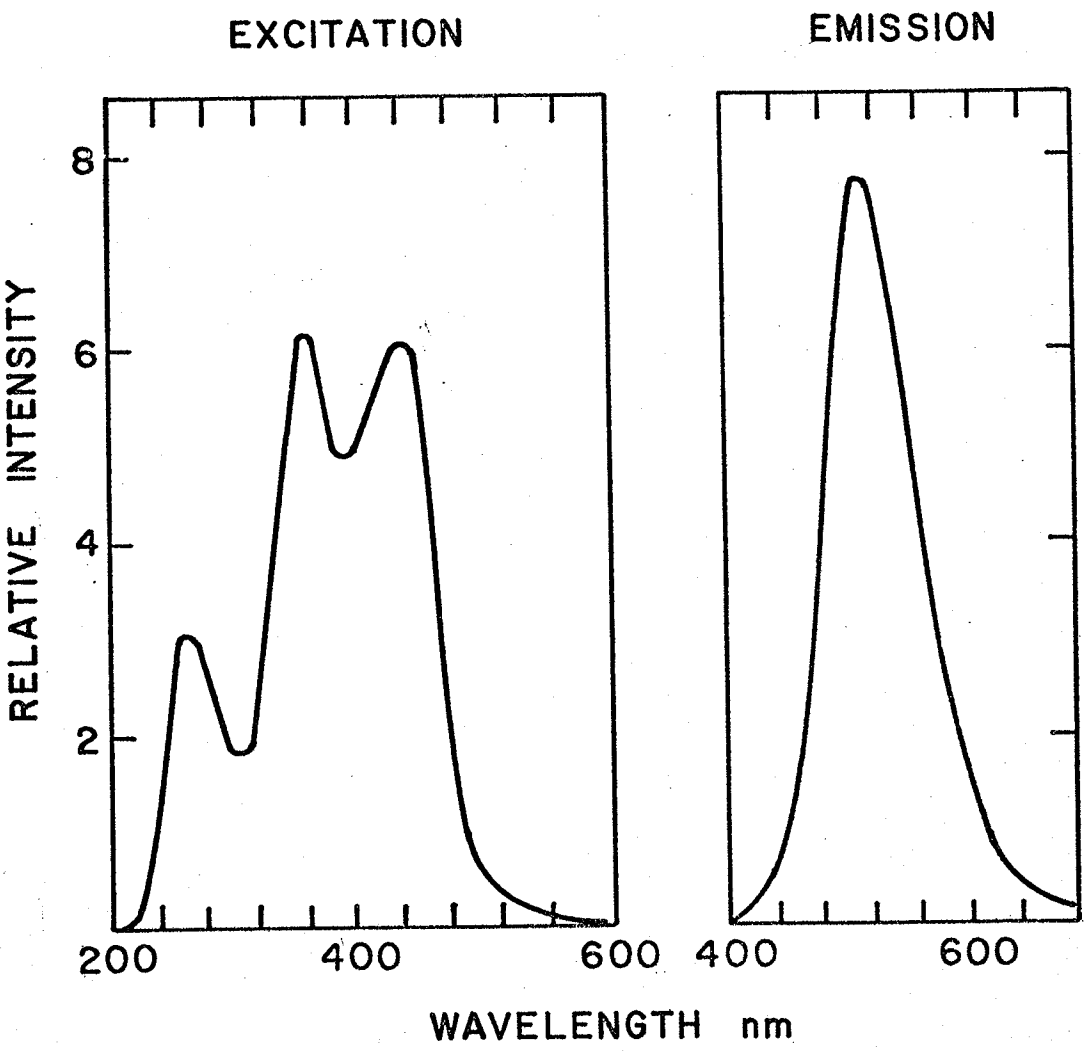
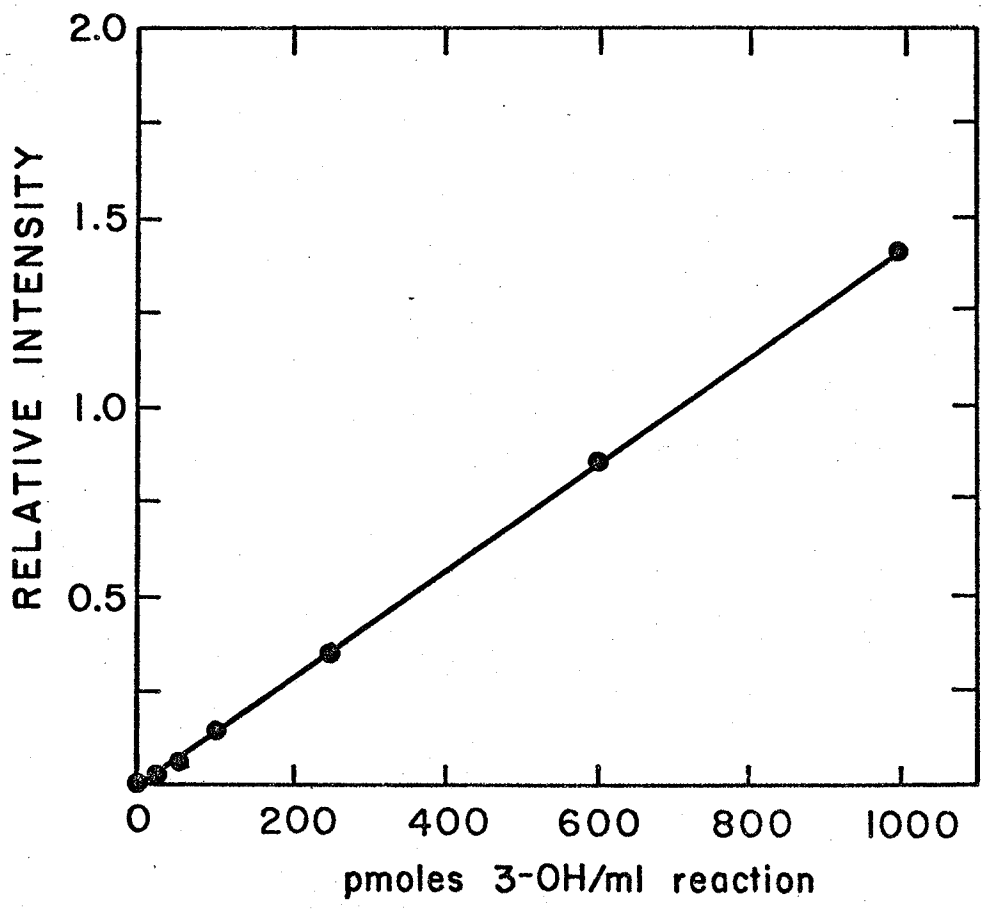

EXCITATION
EMISSION
RELATIVE INTENSITY
8
6
4
2
200
400
600
400
600
WAVELENGTH nm

FIGURE 8: Standard curve of relative fluorescent intensity vs. concentration of 3-OH BP. The 3-OH standard was diluted to the various concentrations in acetone. 6 ul of the standard was added to a stopped reaction containing 294 ul 50 mM Tris buffer pH 7.4 containing 500 ug/ml microsomes, and 300 ul acetone. The stopped reactions were extracted with hexane, and the organic layer reextracted with NaOH. The samples were read in a Aminco Bowman spectrofluorometer with excitation slits set at 1 nm and emmision slits at 3 nm. The excitation wavelength was 396 nm and emmision wavelength 522nm.

FIGURE 8


2.0
1.5
1.0
0.5
RELATIVE INTENSITY
0
200
400
600
800
1000
pmoles 3-OH/ml reaction

Radioactive Assay for BP Metabolism

To assay the total metabolism of BP, the radioactive assay was used to measure the proportion of BP in the aqueous layer after hexane extraction. The assay used was a modification of the procedure described by De Pierre et al (1975). To the 600 ul stopped reaction (300 ul reaction solution and 300 ul 0.5 N NaOH in 80% ethanol) 900 ul n-hexane was added. The tubes were stoppered to prevent the evaporation of hexane and vortexed vigorously for 2 minutes. To separate the layers, the tubes were spun at 1000g for 5 minutes. 300 ul samples of the lower phase and 25 ul samples of the organic phase were dispensed to scintillation vials. 10 ml aquasol was added and mixed thoroughly. The solutions were allowed to stand for 12 hours before counting to prevent interference of the NaOH in the lower phase samples with the aquasol. The proportion of counts was calculated for each phase, and the percentage of counts in the aqueous phase determined.

ASSAY OF VITAMIN K DEPENDANT CARBOXYLASE

Preparation of Solubilized Enzyme From Rat Liver

A representative assay of the activity of the Vitamin K cycle is the carboxylase reaction. The procedures used to prepare the enzyme, and its assay were those of Suttie et al (1980). Microsomes were prepared using the standard

procedure from uninduced or induced rat liver. Following the 105,000g spin, the surface of the pellet was washed with Buffer B (0.25 M sucrose, 25 mM Hepes Buffer pH 7.2, 0.5 M KCl, 1.0 mM dithiothreitol (DTT)). The microsomes were then suspended and solubilized in Buffer B + 1.5% Triton X-100, and diluted to a protein concentration of 10 mg/ml. After another spin at 105,000g to remove insoluble material, the solubilized microsomes were used directly in the carboxylase assay.

Preparation of Solubilized Enzyme from H4IIE Cells

To prepare enough solubilized microsomes to assay carboxylase activity, 70-100 plates of uninduced or induced H4IIE cells were harvested. The medium was removed and replaced with 2 ml MEM + 50 mM Hepes pH 7.4. The cells were scraped from the surfaces of the plates using a rubber spatula. Suspensions of the cells were placed in 50 ml conical centrifuge tubes and spun at 1500g for 10 minutes to pellet. The pellets were resuspended in a small volume of MEM + 50 mM Hepes, pooled and pelleted again. The pooled pellet was weighed and homogenized in 3 volumes of 0.25 M sucrose. Typically 2.5-3 grams of cell material could be obtained from 100 plates of cells. The cells were very difficult to break open, therefore a 30 second sonication at 75% output and tune setting #4

(Biosonic) was performed to lyse the remaining intact cells. From this point, the preparation of microsomes was as described; a 3,000g and 10,000g spin to obtain a post-mitochondrial supernatant, and a 105,000g spin to pellet the microsomes. The solubilization procedure for the H4IIE mocrosomes was the same as for rat liver.

Preparation of Vitamin K1 Hydroquinone

The procedure for preparation of reduced Vitamin K1 was that of Sadowski et al (1976). Vitamin K1 was dissolved in 10 ml diethyl-ether. The solution was mixed vigorously with 25 ml 5% sodium dithionite for 5 minutes in a separatory funnel. The ether layer was separated from the aqueous layer and dried under a constant stream of nitrogen. The remaining white residue was redissolved in ethanol to give a final concentration of 5 mg/ml. The reduced Vitamin was stored in the dark at -20°C under a nitrogen atmosphere.

Assay of Carboxylase Activity

To assay Vitamin K dependant carboxylase activity, the incorporation of $^{14}C$ labelled $CO_2$ into a non-TCA precipitable amino acid chain was measured. The carboxylase assay consisted of 200 ul undiluted solubilized microsomes, 50 ul of the artificial prothrombin precursor phe-leu-glu-glu-leu (5 mM), 5 ul of 1mCi/ml $NaH^{14}CO_2$ and

5 ul of 5 mg/ml hydroquinone. The peptide substrate is identical to a portion of the prothrombin molecule which becomes carboxylated. The reaction was started by the addition of reduced K1 and incubated at 17°C with stirring. K1 was always added within 15 seconds of adding the labelled $NaHCO_2$. To stop the reaction 1 ml of 10% Trichloroacetic Acid (TCA) was added, and the tubes placed on ice. After 10 minutes on ice, the tubes were centrifuged at 1500g for 10 minutes to pellet the precipitated protein. A 900 ul aliquot of the supernatant was gassed for 4 minutes with $CO_2$. 800 ul of the gassed supernatant was dispensed to scintillation vials and 10 ml aquasol added. The vials were mixed well before counting in a Beckman LS 7800 liquid scintillation counter.

## PURIFICATION OF CYTOCHROME P-448 AND NADPH-DEPENDANT CYTOCHROME P-450 REDUCTASE

### Cytochrome P-448 Concentration Determination

Cytochrome P-450 binds carbon monoxide when in the reduced state to generate a large absorbance peak at 450nm, or 448nm in the case of the 3-methylcholanthrene inducible form. The procedure for assaying the relative concentrations of P-448 was that of Omura and Sato (1964a). The protein to be analyzed was diluted to a concentration no greater than 1.0 mg/ml. The purer the preparation, the

less protein was required for accurate P-450 assay. A small amount of sodium dithionite (approximately 0.1 mg/ml) was added and the solution mixed well. 1 ml of the reduced preparation was dispensed to each of 2 matched cuvettes. The difference spectra of the cells were read to generate a baseline from 400nm to 500nm. The sample cuvette was gassed for 2 minutes with oxygen depleted carbon monoxide. The difference spectra was again recorded. The concentration of the cytochrome was calculated using an extinction coefficient of 91 $mM^{-1}$ $cm^{-1}$ between 448nm and 490nm. Cytochrome P-450 concentrations are expressed in nmoles per mg protein. All spectral determinations in these procedures were performed with a Beckman DU-8 spectrophotometer.

Assay of NADPH Dependant Cytochrome c Reductase

Cytochrome P-450 reductase activity can be measured by its ability to reduce the artificial electron acceptor cytochrome c. The procedure for assay of this activity was that of Strobel and Dignam (1978). The appearance of reduced cytochrome c was determined spectrophotometrically by measuring the increase in absorbance at 550nm. The standard assay consisted of 40 nmole cytochrome c, 300 umole potassium phosphate buffer pH 7.7, enzyme, and 0.1 umole NADPH per ml reaction. The reaction was initiated by

the addition of NADPH and allowed to proceed at 30°C while monitoring the change in absorbance at 550nm. An extinction coefficient of 21 $mM^{-1}$ $cm^{-1}$ at 550 nm was used to calculate the rate of reduction. One unit of NADPH-cytochrome P-450 reductase is defined as that amount of catalytic activity reducing 1 umole of cytochrome c per minute in the standard assay.

Purification of Proteins

For the purification of cytochrome P-448 and NADPH-dependant cytochrome P-450 reductase, a modification of various procedures was used to facilitate the resolution of the two proteins from the same microsomes. The methods for P-448 purification were derived from those of Levin et al (Lu and Levin, 1972; Levin et al, 1972, 1974; Ryan et al, 1978). Cytochrome c reductase was purified using modifications of the procedures described by Dignam and Strobel (1977; Strobel and Dignam, 1978).

Microsomes for purifications were prepared from 3-methylcholanthrene induced rats. Normally 2-3 grams of microsomal protein were required for a preparation. The microsomes were frozen for no longer than 3 weeks after sacrifice of the rats. All procedures were performed at 4°C. After thawing, they were diluted to 10 mg/ml in 0.2 M Tris-HCl pH 7.7 at 4°C, 30% glycerol, 1 mM EDTA, 0.1 mM

DTT. The non-ionic detergent Emulgen 913 was added as a 10% solution to a final concentration of 1.5%. The suspension was stirred for 20-30 minutes at 4°C to solubilize the microsomal membranes. 1.5% protamine sulfate was added to bring the concentration to .03% w/v. This suspension was stirred for 15 minutes at 4°C and centrifuged at 105,000g for 1 hour to remove insoluble material.

The cleared supernatant was loaded at 150 ml/hour onto a DEAE Sephadex A-25 column (26 mm x 100 cm) previously equilibrated with 0.1 M Tris-HCl pH 7.7 at 4°C, 1.0 mM EDTA, 0.1 mM DTT, 20% glycerol and 0.15% Emulgen 913. The column was then washed with 200 ml equilibration buffer at a rate of 200 ml/hour. A linear 2 litre gradient of 0-0.3 M KCl was applied after the wash to elute the reductase. Cytochrome P-450, cytochrome b5 and cytochrome b5 reductase are eluted in the flow through. Reductase elutes at a KCl concentration of approximately 0.1 M.

Fractions from the reductase peak were pooled and mixed with calcium phosphate (0.3 mg gel per mg protein). The suspension was stirred for 20 minutes to absorb the protein, then centrifuged at 10,000g for 20 minutes to pellet. The supernatant was drawn off and the pellet resuspended in 0.3 M potassium phosphate pH 7.7, 0.1 mM

DTT, 0.15% Emulgen 913 and 20% glycerol to elute the reductase from the gel. The suspension was stirred for 20 minutes, then centrifuged to pellet again. The supernatants were pooled and dialyzed overnight against 0.1 M Tris-HCl pH 7.7, 20% glycerol, 1 mM EDTA and 0.15% Emulgen 913. The dialyzed supernatant was frozen at -80°C and was stable for at least 3 months.

P-450 fractions eluting from the A-25 column flow through were pooled and concentrated on calcium phosphate gel (3 mg gel/mg protein). The gel was extracted once with 0.1 M potassium phosphate pH 7.7, then with 0.25 M potassium phosphate to elute the protein. The pooled extracted P-450 was concentrated to approximately 8 mg protein per ml using a Diaflo XM-100A ultramembrane filter, and dialyzed overnight against 5 mM potassium phosphate pH 7.7, 20% glycerol, 0.1 mM DTT, 0.1 mM EDTA, 0.05% cholate. The dialyzed sample was brought to 42% saturation with solid ammonium sulfate. This suspension was stirred for 20 minutes, then centrifuged at 10,000g for 20 minutes to pellet precipitated protein. The supernatant was then brought to 50% ammonium sulfate saturation, stirred for 20 minutes, then centrifuged to pellet. The supernatant was decanted and the pellet resuspended in 5 mM potassium phosphate, 20% glycerol, 0.1 mM DTT, 0.1 mM EDTA, 0.05% cholate. The redissolved pellet was dialyzed overnight

against the same buffer. The dialyzed preparation was frozen at -80°C under nitrogen and was stable for at least 3 months under these conditions.

Polyacrylamide Gel Electrophoresis

The method for SDS polyacrylamide gel electrophoresis was that of Laemmli (1970). The 10x15 cm slab gels containing 10% acrylamide were prepared from a 30% w/v acrylamide solution, and 0.8% w/v N-N'-bis-methyleneacrylamide. The stacking gels contained 3% acrylamide. Polymerization of the gel was induced by the addition of 0.025% tetramethylethylendiamine and ammonium persulfate. Final concentrations of reagents in the gel were 0.375 M Tris-HCl pH 8.8 and 0.1% SDS. The electrode buffers contained 25 mM Tris-HCl pH 8.3, 0.1% SDS and 0.192 M glycine. The samples were added in sample buffer containing 62.5 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 5% mercaptoethanol and 0.001% bromophenol blue. The proteins were denatured by immersing in a boiling water bath for 1.5 min before loading onto the gel. Electrophoresis was run until the dye marker reached the bottom of the vertical gel. Following electrophoresis, the gel was soaked in 50% methanol, 10% acetic acid overnight, and stained with 0.3% Coomassie blue in 50% methanol, 10% acetic acid for 1 hour at 37°C. Gels were destained in 40% methanol, 10% acetic acid.

Reconstitution of Mixed Function Oxidase Activity

When reconstituting the mixed function oxidase system for benzo(a)pyrene metabolism, the order of addition of the various components is important for maximum activity. For proper complex formation the order of addition was reductase, then the lipid component phosphatidyl choline, then cytochrome P-450. The complex was thoroughly mixed before adding any other reagents (Lu and Levin, 1974). The phosphatidyl choline solution prepared in 50 mM Hepes at a concentration of 900 ug/ml was sonicated before use for 30 seconds at 85% output with a tune setting at #4 (Biosonic). The standard reactions contained the following; 0.3 nmoles/ml cytochrome P-450, 0.04 Units/ml cytochrome c reductase, 30 ug/ml dilaurylphosphatidyl choline, 1 mM $MgCl_2$, 0.1 mM EDTA, 01% deoxycholate, 6 mM isocitrate, 1 mM NADP, 0.36 Units/ml isocitrate dehydrogenase, 5 uM $MnCl_2$ and 50 mM Hepes buffer pH 7.4 (Coon, 1978). The 300 ul reactions were incubated for 5 minutes at 37°C to allow for NADPH generation, before BP was added (80 uM in 6 ul acetone) to start the reaction. The reaction was stopped by the addition of 300 ul cold acetone. To measure BP metabolism, the spectrofluorometric 3-OH BP assay was used.

Reduction of artificial electron acceptors such as menadione by NADPH-cytochrome c reductase was measured by

monitoring the oxidation of NADPH at 340nm. The reactions contained 0.04 Units/ml reductase, 1 mM $MgCl_2$, 0.1 mM EDTA, 0.01% deoxycholate, 0.2 mM NADPH and 50 mM Hepes buffer pH 7.4. The mixture was preincubated at 37°C before adding the electron acceptor to start the reaction. The rate of NADPH oxidation was calculated using an extinction coefficient of 6.2 $mM^{-1}$ $cm^{-1}$. The reduction of the artificial electron acceptor dichlorophenolindophenol (DCIP) was monitored at 600nm using an extinction coefficient of 21 $mM^{-1}$ $cm^{-1}$.

SOURCE OF MATERIALS

Biochemicals were purchased from Sigma Chemical Co., St. Louis. All other materials were obtained from the sources listed in Table 1.

TABLE 1: Source of Materials



| MATERIAL | SOURCE |
|---|---|
| minimal essential medium | Flow Laboratories, Rockville, MD |
| foetal bovine serum | Gibco, Grand Island, NY |
| Bacto trypsin | Difco, Detroit, MI |
| petri plates | Miles Scientific, Naperville, MI |
| 3-methylcholanthrene | Aldrich, Milwaukee, WI |
| trypan blue | Gibco, Grand Island, NY |
| Tween-80 | J.T. Baker, Phillipsburg, NJ |
| benzo(a)pyrene | Aldrich, Milwaukee, WI |
| $^{3}H$ benzo(a)pyrene | Amersham, Arlington Heights, IL |
| Hanks balanced salt solution | Gibco, Grand Island, NY |
| phe-leu-glu-glu-leu | Vega Biochemicals, Tucson, AZ |
| $NaH^{14}CO_3$ | New England Nuclear, Boston, MA |
| Emulgen 913 | Kao-Atlas, Tokyo, Japan |
| DEAE Sephadex A-25 | Pharmacia, Uppsala, Sweden |
| Diaflo XM-100A filters | Amicon, Danvers, MA |
| tetramethylethylendiamine | Eastman, Rochester, NY |
| ammonium persulfate | J.T. Baker, Phillipsburg, NJ |
| sodium dodecylsulfate | Bio Rad Laboratories, Richmond, CA |
| mercaptoethanol | Eastman, Rochester, NY |

# RESULTS

## 1. H4IIE Rat Hepatoma Cell Line

### a) Growth and Induction of Cytochrome P-450 Activity

H4IIE cells seeded at a density of $10^6$ cells per 10 cm plate were allowed to grow for 24 hours before adding the inducer. 3-methylcholanthrene is very hydrophobic, therefore to dissolve it in the aqueous media DMSO was used as a solvent. Figures 9 and 10 show the effect of 1 uM 3-methylcholanthrene on the cells growing in monolayer. The basal level of Aryl Hydrocarbon Hydroxylase activity in uninduced H4IIE cells is low, but this activity could be measured using either the spectrofluorimetric (Figure 9) or radioactive BP (Figure 10) assays. Addition of 3-methylcholanthrene in 0.25% DMSO to the growth media of the cells caused an increase in the activity of BP metabolism detectable at 4 hours after addition (Figures 9 and 10). The activity increases for 12-16 hours after initiation of induction, whereupon it levels off. Replacing the medium at 24 hours with fresh MEM + 10% FBS + .25% DMSO + 1uM 3MC did not further increase enzyme activity as measured by 3-OH BP production (Figure 9). Total BP metabolism may increase after replenishment of the inducer at 24 hours as shown in Figure 10. 0.25% DMSO alone did not cause an induction of enzyme activity. Cells used for experiments were routinely induced for 18-

FIGURE 9: Effect of 3-methylcholanthrene induction on 3-OH BP production in whole unpermeabilized H4IIE cells. Cells were seeded at a density of $10^6$ cells/10 cm plate and allowed to grow for 24 hours, at which time the plates were divided into three groups. A control group received only a medium change of fresh MEM + 10% FBS ( ■ ), the DMSO group received a medium change to MEM + 10% FBS + 0.25% DMSO ( ▲ ). The induced groups medium was changed to MEM + 10% FBS + 0.25% DMSO + 1 uM 3-methylcholanthrene ( ● ). The cells were allowed to grow for the indicated times, harvested and assayed for BP hydroxylase activity. At 24 hours post induction all of the plates received medium changes with their corresponding additions. AHH activity is expressed in pmoles 3-OH BP/min/$10^6$ cells as measured by the spectrofuorometric assay. Data shown is an average of 2 experiments, the mean standard error was +/- 6.8%.

FIGURE 9

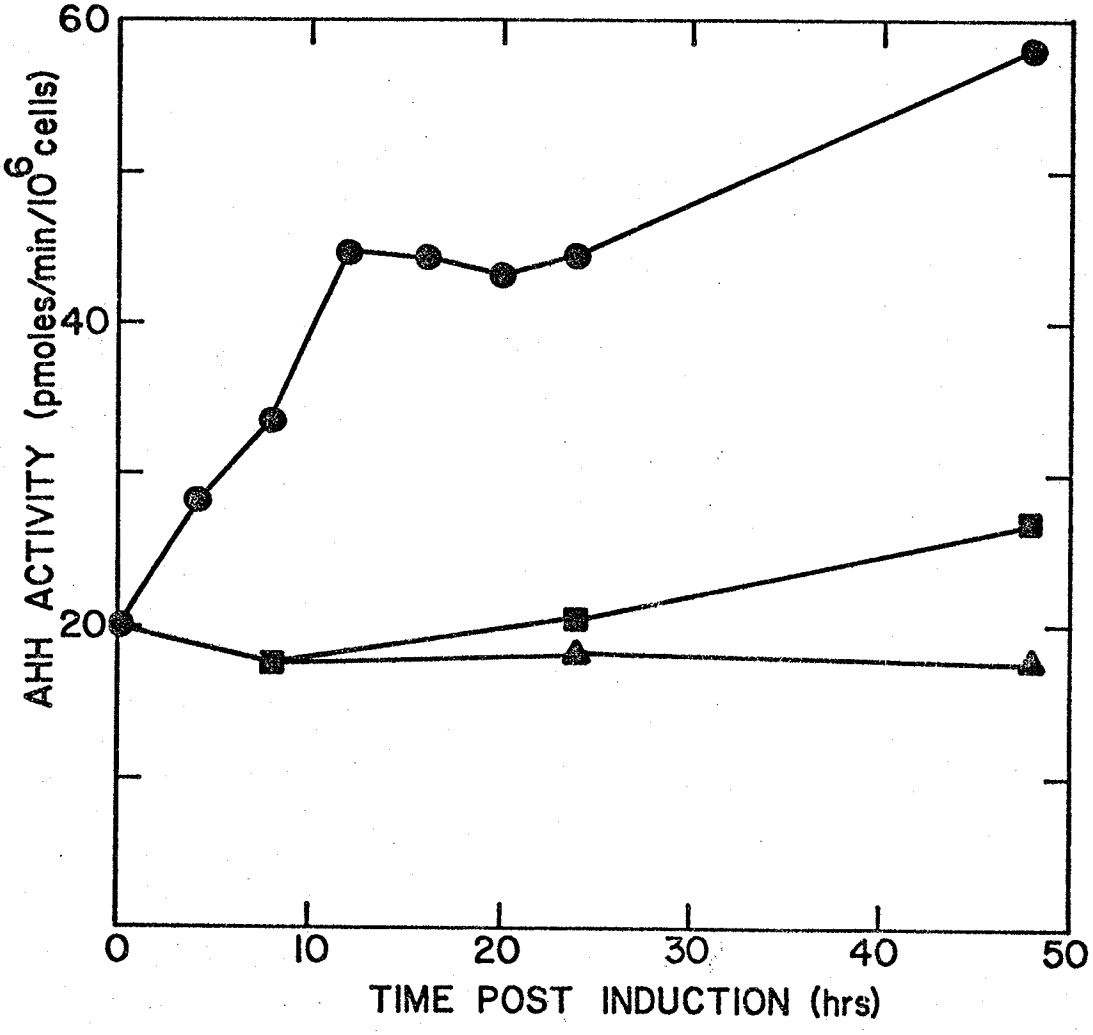
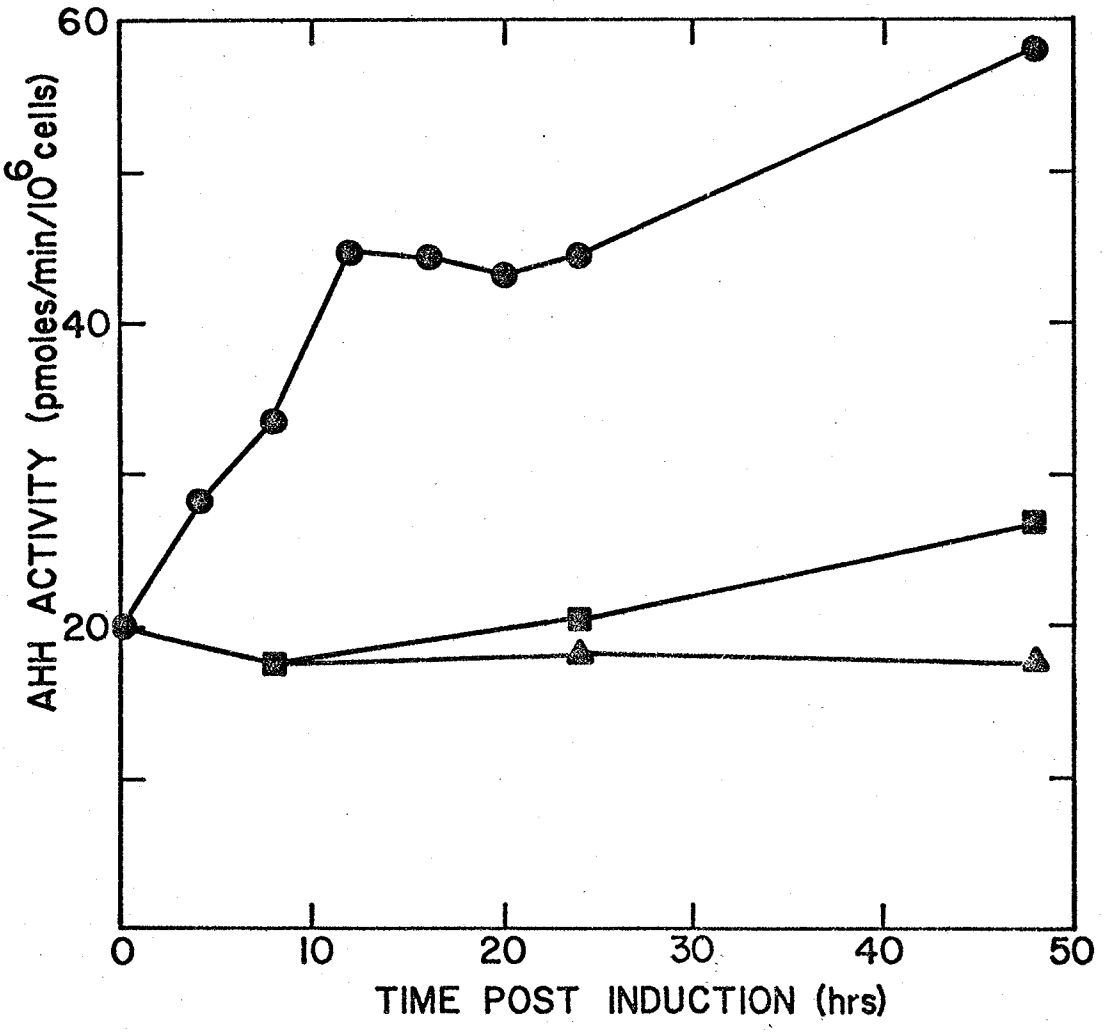

60
40
20
AHH ACTIVITY (pmoles/min/10^6 cells)
0
10
20
30
40
50
TIME POST INDUCTION (hrs)

FIGURE 10: Effect of 3-methylcholanthrene induction on total BP metabolism in unpermeabilized H4IIE cells. Cells were treated as described in the legend to Figure 9. Groups were; control ( ■ ), MEM + 10% FBS + 0.25% DMSO ( ▲ ), and MEM + 10% FBS + 0.25% DMSO + 1 uM 3-methylcholanthrene ( ● ). At the time specified the cells were harvested and assayed for total BP metabolism as determined by the radioactive assay. At 24 hours post induction all of the plates received medium changes with the appropriate additions. AHH activity is expressed in pmoles BP/min/$10^6$ cells. Data shown is an average of 2 experiments, the mean standard error was +/- 6.7%.

FIGURE 10


60
40
20
AHH ACTIVITY (pmoles/min/10^6 cells)
0
10
20
30
40
50
TIME POST INDUCTION (hrs)

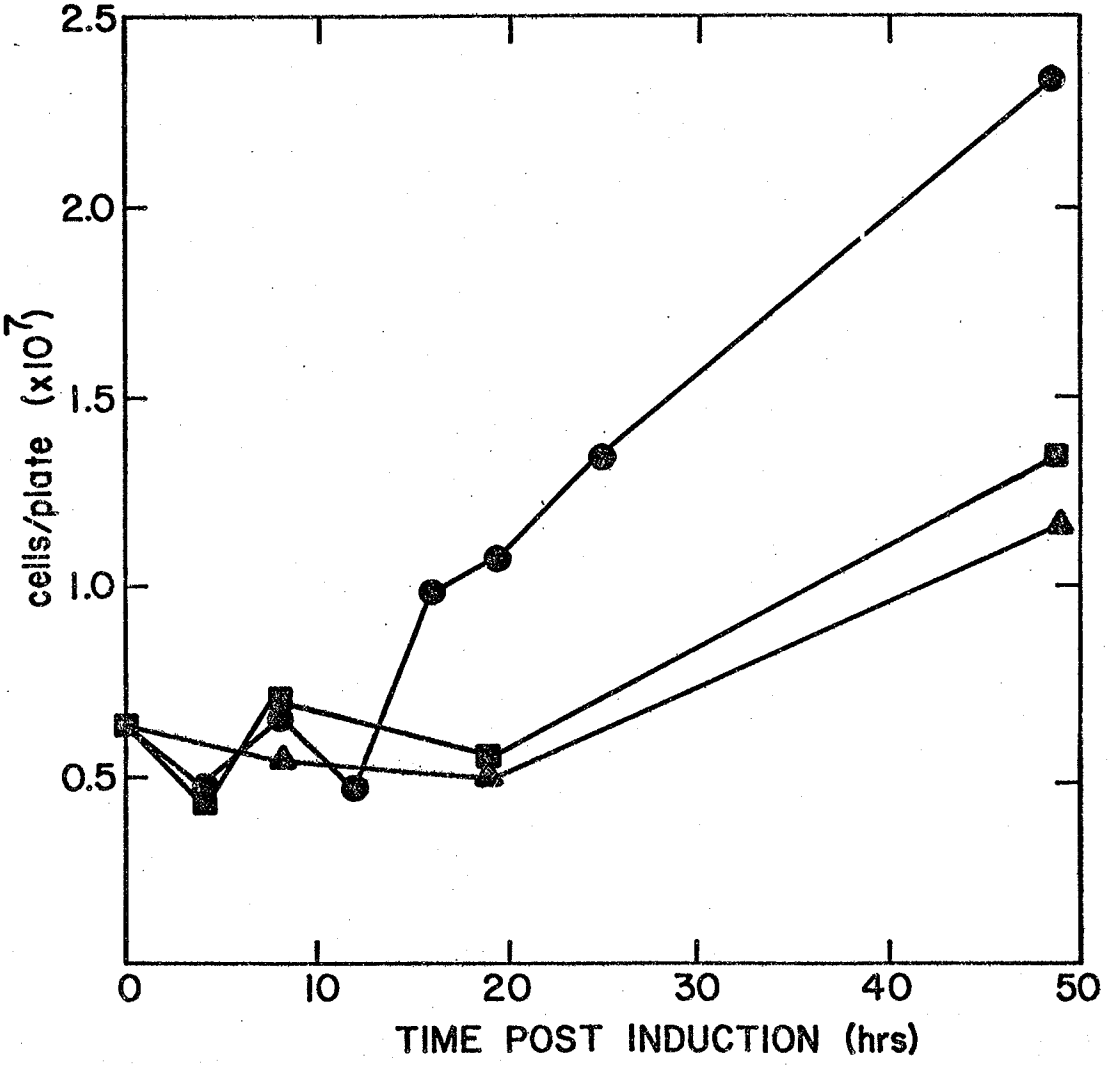
22 hours. The doubling time of cells grown in MEM + 10% FBS was determined to be 13.9 +/- 1.9 hours. Addition of 0.25% DMSO to the medium resulted in a slower doubling time of 15.1 +/- 1.7 hours. An interesting observation was that the growth rate of 3-methylcholanthrene treated cells was consistently faster than control cells, with a doubling time of 11.5 +/- 0.5 hours. The growth curves of the cells following induction are shown in Figure 11.

b) Permeabilized Cell Assay for AHH and Effect of Exogenous NADPH

To study the effect of NADPH on the metabolism of BP in intact H4IIE cells, a permeabilized cell assay was developed. This system provided valuable information on the role of NADPH as a rate limiting factor in BP hydroxylation. The technique for producing permeabilized H4IIE cells was derived from the assay for in vivo activity of ribonucleotide reductase ( R.Rase) (Wright et al, 1981). This R.Rase assay assay calls for a permeabilizing treatment of the cells in buffer consisting of 0.25 M sucrose, 50 mM Hepes buffer and 1% Tween-80. The permeabilization proceeds for 30 minutes at which time the cells are pelleted and resuspended in fresh permeabilizing buffer. The resuspended cells are then added directly to a reaction mixture. Figure 12 shows the effect of incubation in

FIGURE 11: Growth of H4IIE cells during induction with 3-methylcholanthrene. Cells were treated as described in the legends to figures 9 and 10. Groups were; control ( ■ ), DMSO treated ( ▲ ), and DMSO plus 1 uM 3-methyl-cholanthrene treated ( ● ). Data shown is an average of 2 experiments, the mean standard error was +/- 5.7%.

FIGURE 11


2.5
2.0
1.5
1.0
0.5
cells/plate (x10^7)
0
10
20
30
40
50
TIME POST INDUCTION (hrs)

permeabilizing buffer on the AHH activity in H4IIE cells. Exposure of the cells to the non-ionic detergent Tween-80 resulted in an increase in activity within the first five minutes of permeabilization, then a decrease in activity at longer permeabilizing times. An immediate drop in activity occurs after five minutes in the Tween buffer. Various nonionic and ionic detergents have been shown to be inhibitory to cytochrome P-450 (Yamano and Ichikawa, 1978). This inhibition may be mediated by a conversion of the hemoprotein to the inactive form P-420. This mechanism accompanied by a solubilization of the microsomal membrane is probably responsible for the inhibitory effect of Tween-80 on AHH.

To minimize the inhibitory of Tween-80 on AHH activity two alterations were made to the protocol. The assay was found to work well using only a very short exposure of the cells to the permeabilizing buffer (3-5 minutes). Secondly the cells were pelleted after permeabilization and resuspended in 0.25M sucrose, 50 mM Hepes rather than the complete permeabilization buffer. Figure 13 demonstrates the effect of such treatment on the cells (bar C). Resuspension of the cells in 50 mM Hepes alone resulted in a lower activity (bar B). Unpermeabilized cells (bar A) show a much slower rate of AHH activity, indicating that the short permeabilization is sufficient to allow

FIGURE 12: Effect of permeabilizing buffer on AHH activity in H4IIE cells. Induced cells were harvested by trypsinization washed in medium and resuspended in permeabilizing buffer. The cells were incubated in the buffer for the times indicated and centrifuged to pellet. The pellet was resuspended in 50mM Tris buffer pH 7.4 to a density of $5.25 \times 10^6$ cells/ml. To determine enzyme activity 200 ul of this suspension was added to 100 ul prewarmed reaction mixtures, to give a final concentration of $3.5 \times 10^6$ cells/ml, 1 mM NADPH, 3 mM $MgCl_2$ and 80 uM BP, as described in materials and methods. The reactions were run for 30 minutes at 37°C with stirring. Data shown is an average of 2 experiments, the mean standard error was +/- 8.4%.

FIGURE 12

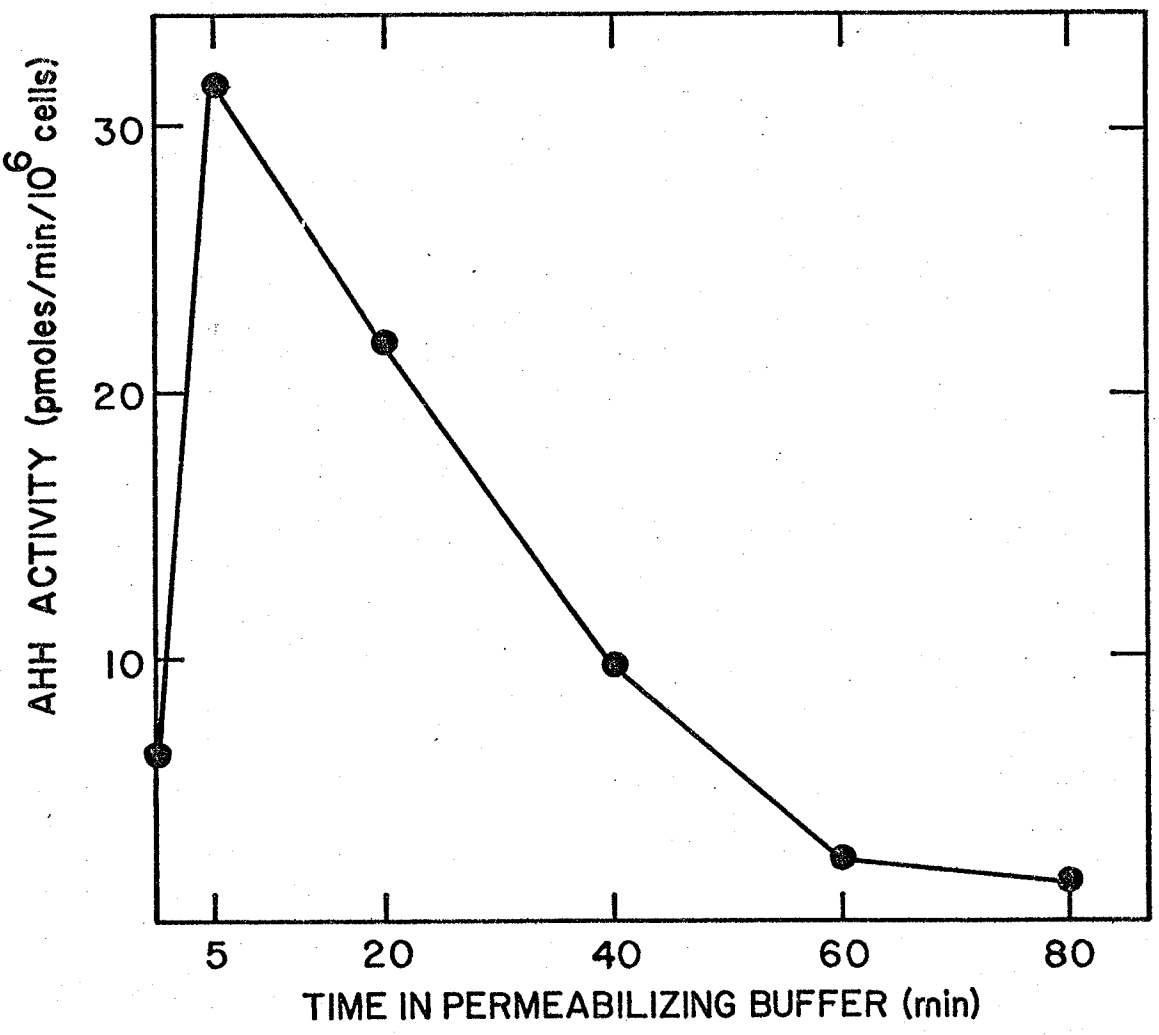
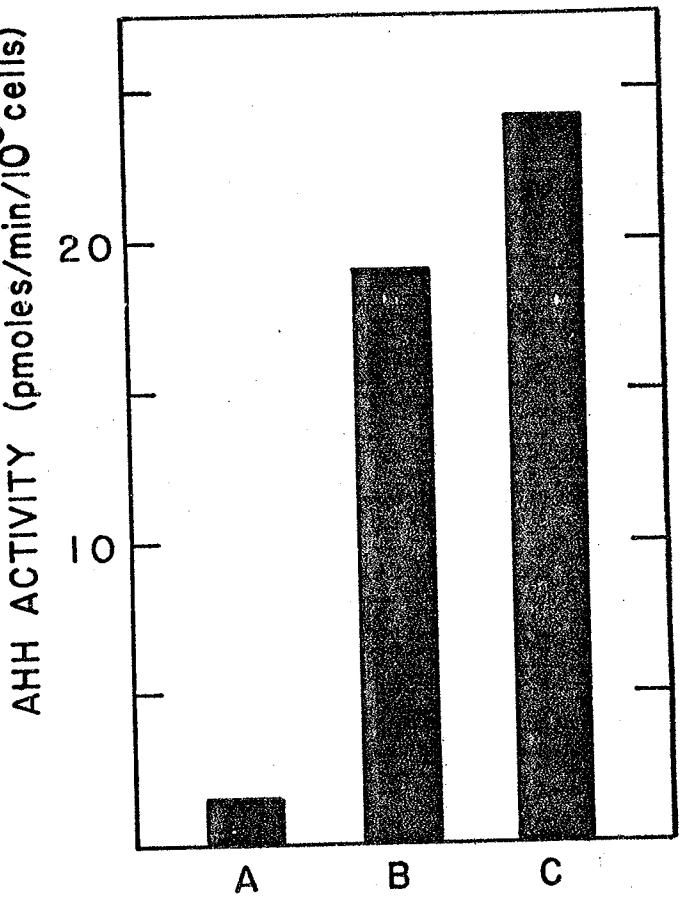

AHH ACTIVITY (pmoles/min/10^6 cells)
30
20
10
5
20
40
60
80
TIME IN PERMEABILIZING BUFFER (min)

FIGURE 13: Effect of permeabilization on AHH activity in H4IIE cells. Induced cells were harvested by trypsinization and washed in medium. Group A cells were pelleted and resuspended in 50 mM Hepes, 0.25 M sucrose and groups B and C resuspended in permeabilizing buffer. The cells were incubated for 5 minutes with mixing to remove clumps and pelleted again. Group A was again resuspended in 50 mM Hepes, 0.25 M sucrose, group B in 50 mM Hepes and group C in 50 mM Hepes plus 0.25 M sucrose. 200 ul samples of the cell suspensions were added to 100 ul reaction mixtures, as described in materials and methods. The reactions were allowed to proceed for 30 minutes. Data shown is an average of 2 experiments, the mean standard error was +/- 6.8%.

FIGURE 13



NADPH and benzo(a)pyrene to enter the cell. The sucrose in the reaction buffer aids in maintaining the structural integrity of the cell which seems to be required for maximum acitvity. The activity vs. cell concentration curve in Figure 14 indicates that the reaction was linear with respect to cell number. Stirring the reaction with the microbars aided in eliminating clumps which could cause a deviation from linearity. A similar observation was made in assaying R.Rase activity in permeabilized human diploid fibroblasts (Dick and Wright, 1980).

Figure 15 shows that unpermeabilized cells have a much slower rate of BP metabolism than do cells that had been treated with permeabilizing buffer. Adding 1mM NADPH to the unpermeabilized cells had no effect on the rate of BP hydroxylation. 1 mM NADPH increased the initial reaction rate in the permeabilized cells, however. With exogenous NADPH the hydroxylation of BP remained linear for at least 35 minutes. Without NADPH the reaction deviated from linearity after 20-25 minutes. The cells used for the experiment shown in Figure 15 were grown for 10 weeks in our laboratory. After prolonged culture of the hepatoma cells, a change in the effect of exogenous NADPH on the initial reaction velocity was observed. Figure 16 shows the metabolism of BP by cells that had been maintained for 40 weeks in our laboratory. The reaction rate in the

FIGURE 14: Effect of cell density on AHH activity in permeabilized H4IIE cells. Induced cells were harvested, washed and permeabilized for 4 minutes at room temperature. The permeabilized cells were resuspended in 0.25 M sucrose, 50 mM Hepes pH 7.4 and added at the cell densities indicated to reaction mixtures containing in final concentrations; 3 mM $MgCl_2$, 1 mM NADPH and 80 uM BP. The reactions were allowed to proceed for 20 minutes, stopped with cold acetone, extracted and the 3-OH production measured. AHH activity is expressed in pmoles 3-OH BP formed/minute. Data shown is an average of 3 experiments, the mean standard error was +/- 4.3%.

FIGURE 14

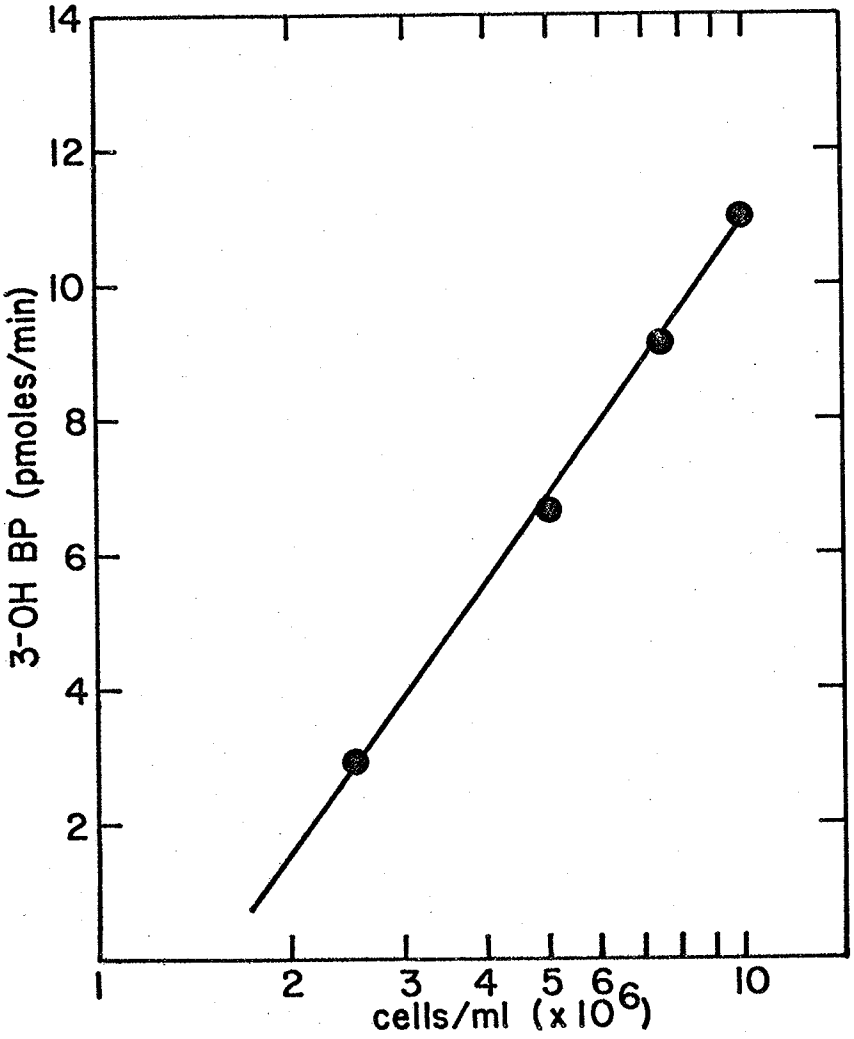


FIGURE 15: Effect of permeabilization and NADPH on the reaction velocity in H4IIE cells cultured for 10 weeks in our laboratory. Permeabilized cells were incubated in the presence of 1 mM NADPH ( ● ) and the absence of NADPH ( ○ ). Unpermeabilized cells were incubated with ( ■ ) and without ( □ ) NADPH. Activity is expressed in pmoles 3-OH BP formed/million cells. Data shown are an average of 3 experiments, the mean standard error was +/- 5.4%.

FIGURE 15

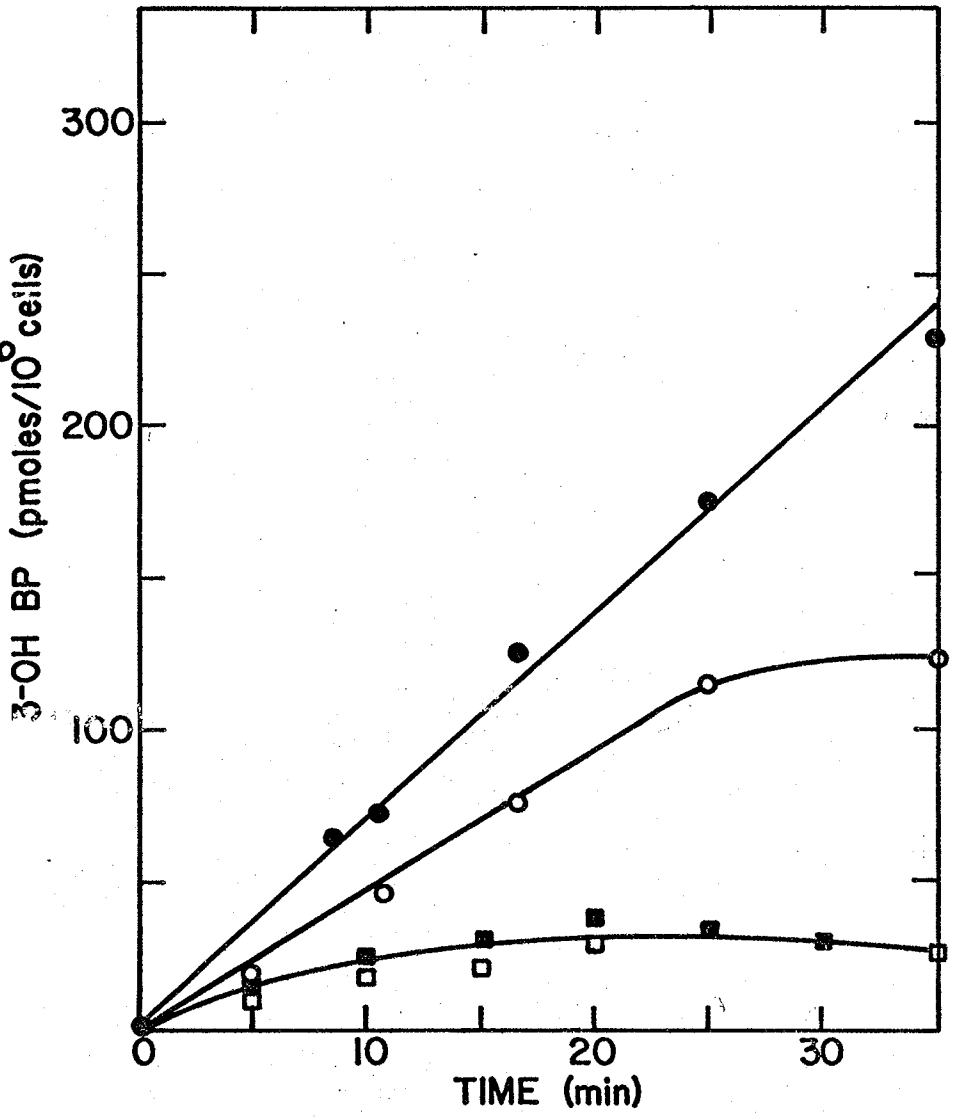
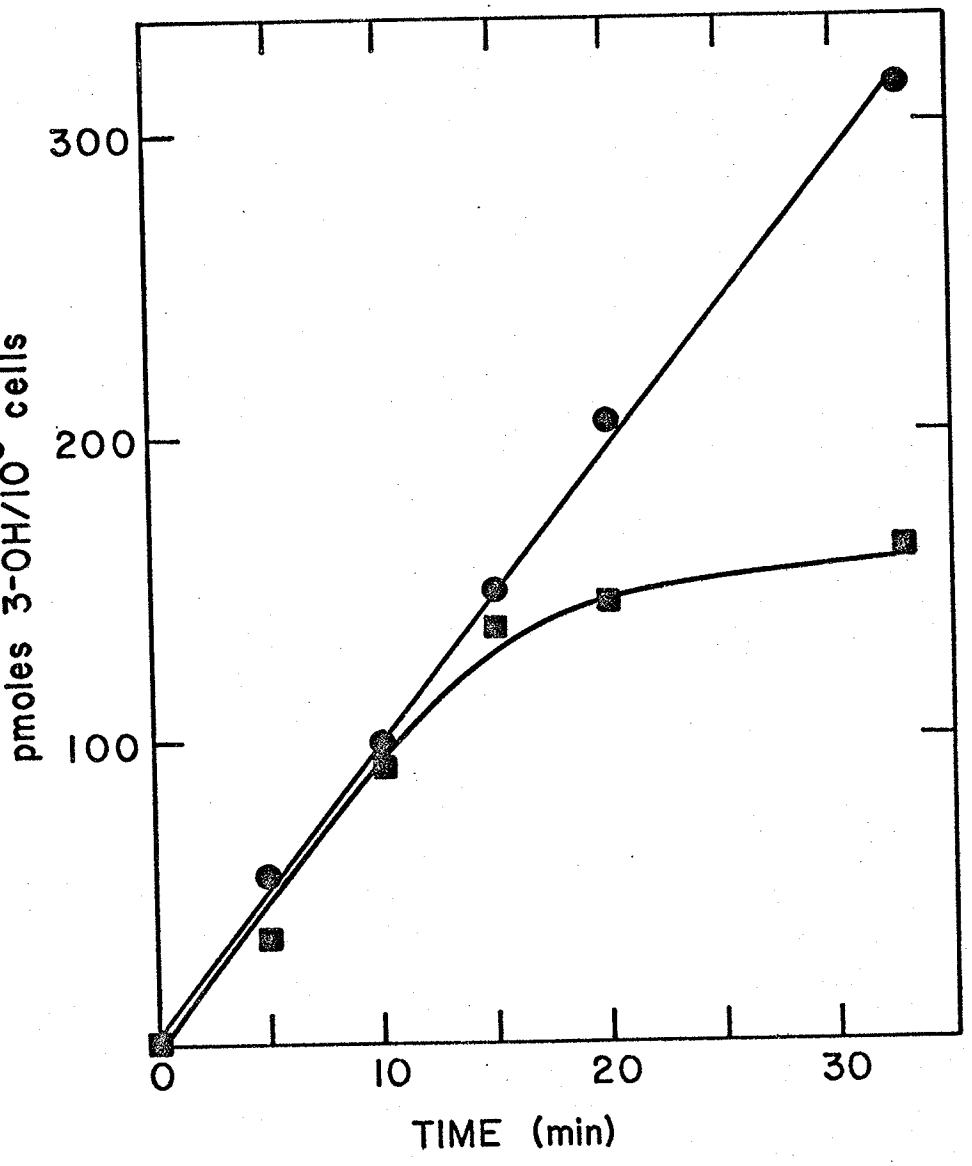


FIGURE 16: Effect of NADPH on permeabilized H4IIE cells that had been cultured for 40 weeks in our laboratory. Permeabilized cells were incubated in the presence ( ● ) and absence ( ■ ) of 1 mM NADPH. Activity is expressed in pmoles 3-OH BP formed/million cells. Data shown are an average of 2 experiments, the mean standard error was +/- 5.5%.

FIGURE 16



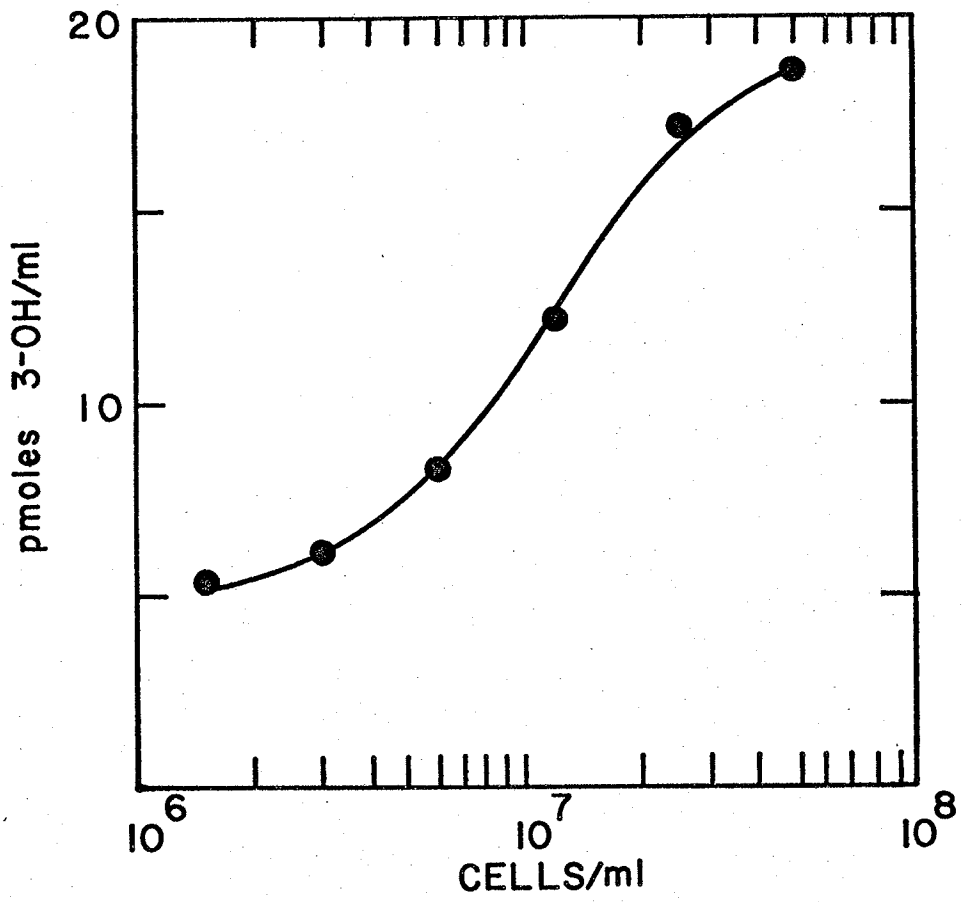
permeabilized cells without NADPH was linear for 15 minutes. 1 mM NADPH did not increase the initial rate. Without exogenous dinucleotide, however, this high rate of metabolism could not be maintained as the concentration of 3-OH BP in the reaction increased only very slowly after 15 minutes. In the presence of 1 mM NADPH, the reaction remained linear for at least 35 minutes. These studies indicate that NADPH is an essential rate limiting component in BP metabolism by intact cells, and the capacity of the hepatoma cells to generate NADPH was altered over the period the cells were cultured. A related observation was that cells of low passage number grew with a doubling time of approximately 35 hours. After prolonged culture in our laboratory the rate had increased to a doubling time of 14 hours.

c) Effect of Menadione and Phylloquinone on BP Metabolism in Whole Unpermeabilized Cells

To test the hypothesis that the two analogs of Vitamin K modulate MFO activity by altering the supply of available NADPH, whole intact unpermeabilized cells were used in order to utilize the limiting nature of reduced dinucleotide. Figure 17 indicates that the activity vs. cell concentration curve for unpermeabilized cells was linear for only a short range of cell densities between approxi-

FIGURE 17: Effect of cell density on AHH acitivity in unpermeabilized H4IIE cells. The cells were induced, harvested, washed, and resuspended in MEM + 50 mM Hepes buffer pH 7.4 to the cell densities indicated. 80 uM BP was added in 6 ul acetone to 300 ul of the cell suspensions and the reactions incubated for 20 minutes at 37°C, with stirring. The reactions were stopped, extracted, and measured for 3-OH BP production as described in materials and methods. Activity is expressed as pmoles 3-OH BP/min/ml reaction. Data shown are an average of 3 experiments, the mean standard error was +/- 5.0%.

FIGURE 17



mately 3 x $10^6$ and 1 x $10^7$ cells/ml. Unpermeabilized cells tended to clump more readily than did those which had been treated with Tween buffer.

Menadione is a very potent inhibitor of BP metabolism in the H4IIE cells as shown in Figure 18. 50% inhibition of activity was achieved at a K3 concentration of about 23 uM as determined by the spectrofluorometric assay. Production of 3-OH BP was competely inhibited at 50 uM. Total BP metabolism was slightly more resistant to menadione inhibition. 50% inhibition was reached at approximately 28 uM as determined by the radioactive BP metabolism assay. At 100 uM K3, 17% of the control activity remained.

The effect of K1 on mixed function oxidation is less clear. In Figure 19, K1 appears to induce an activation of mixed function oxidase acivity. Total BP metabolism as measured by the radioactive assay was increased by 25% at 5-10 uM. Only a slight increase in 3-OH BP production is evident at 25-50 uM. It must be noted that these results tended to vary from experiment to experiment. In 6 independant trials with the radioactive assay, 4 of these indicated that K1 was activating BP metabolism, the remaining two showed no effect. Hence the mean standard error for the radioactive assay data in Figure 19 is high at 14.1%. For the spectroflourometric assay

FIGURE 18: Effect of menadione on AHH acitvity in H4IIE cells. Induced cells were washed and resuspended in MEM + 50 mM Hepes pH 7.4 at a density of $3.5 \times 10^6$ cells/ml. Menadione was added in 3 ul acetone per 300 ul reaction. The control reactions contained an equivalent amount of acetone without menadione. The reactions were preincubated for 5 minutes with the menadione before adding BP in 6 ul acetone to give a final concentration of 80 uM. Reactions were incubated for 20 minutes, stopped with the appropriate solutions and measured for BP metabolism. Metabolism was measured by 3-OH BP production ( ● ) or total BP metabolism ( ■ ). Activity was measured in pmoles (3-OH) BP/min/million cells. Data shown is an average of 3 experiments, the mean standard error was +/- 3.2%.

FIGURE 18

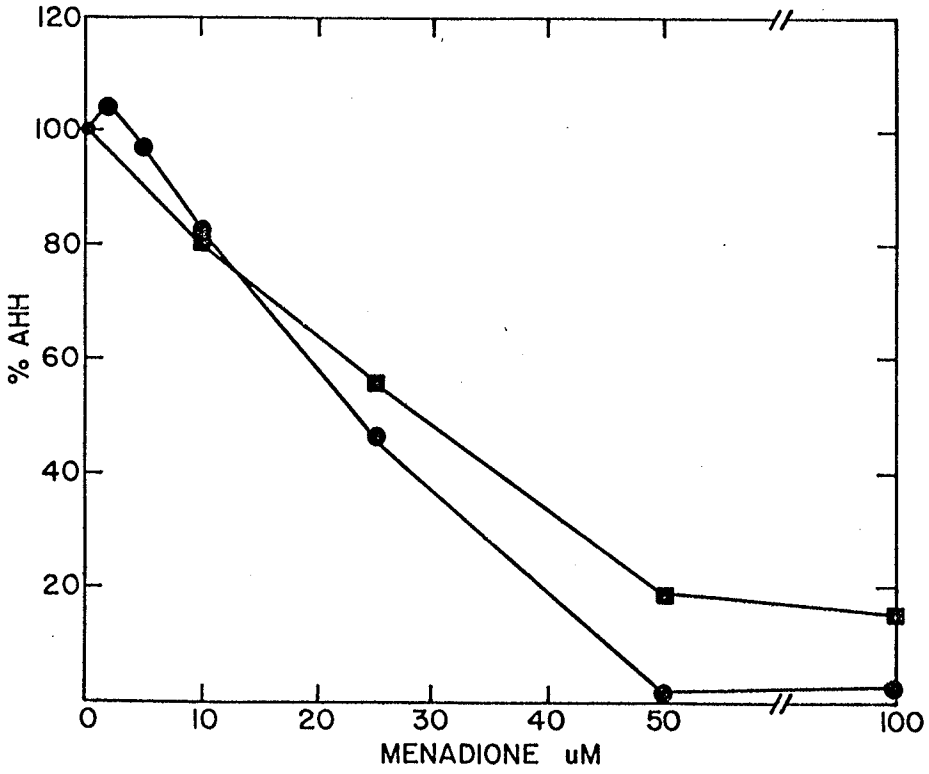
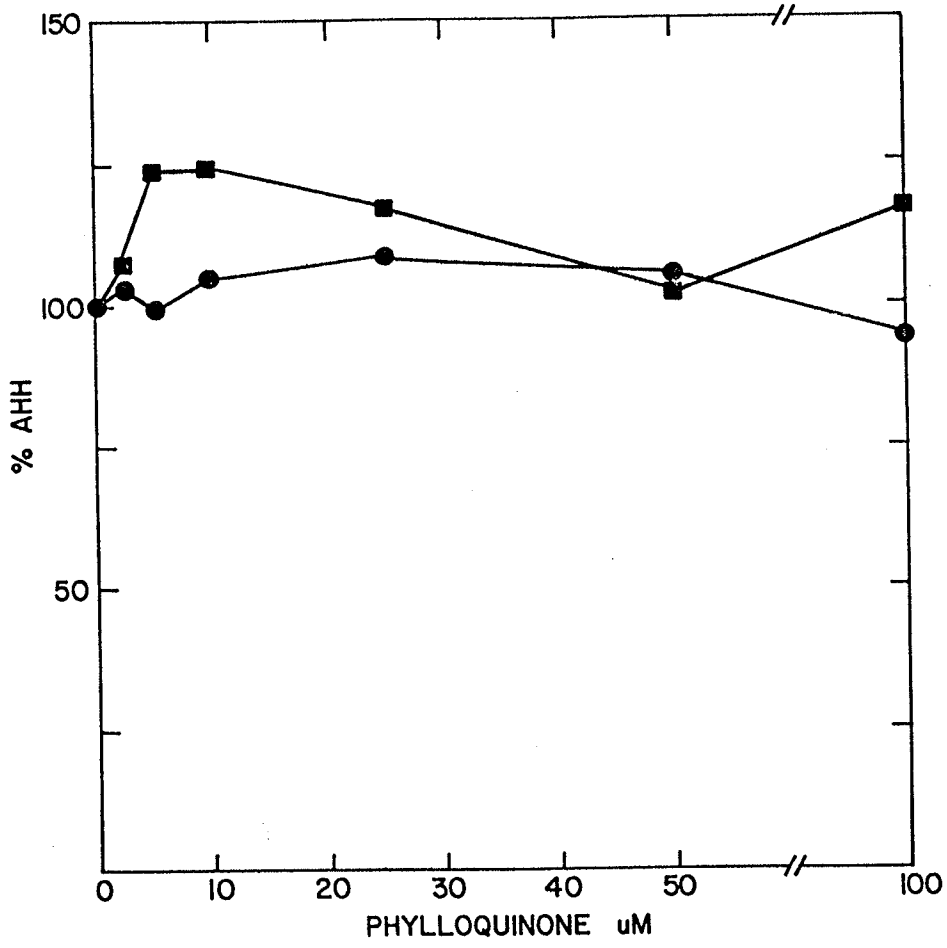


FIGURE 19: Effect of phylloquinone on AHH activity in H4IIE cells. Induced cells were washed and resuspended in MEM + 50 mM Hepes pH 7.4 at a density of 3.5 x $10^6$ cell/ml. Phylloquinone was added in 3 ul acetone per 300 ul reaction. The control reactions contained an equivalent amount of acetone. The reactions were pre-incubated for 5 minutes before adding the BP to start the reaction (80 uM in 6 ul acetone). The reactions were allowed to proceed for 20 minutes before stopping with the appropriate solutions, and measured for BP metabolism. Metabolism was measured by 3-OH BP production ( ● ) or total BP metabolism ( ■ ). Data shown for the radioactive assay is an average of 6 experiments, the mean standard error was 14.1%. Data for the spectrofluorimetric assay is an average of 9 experiments, the mean standard error was 6.7%.

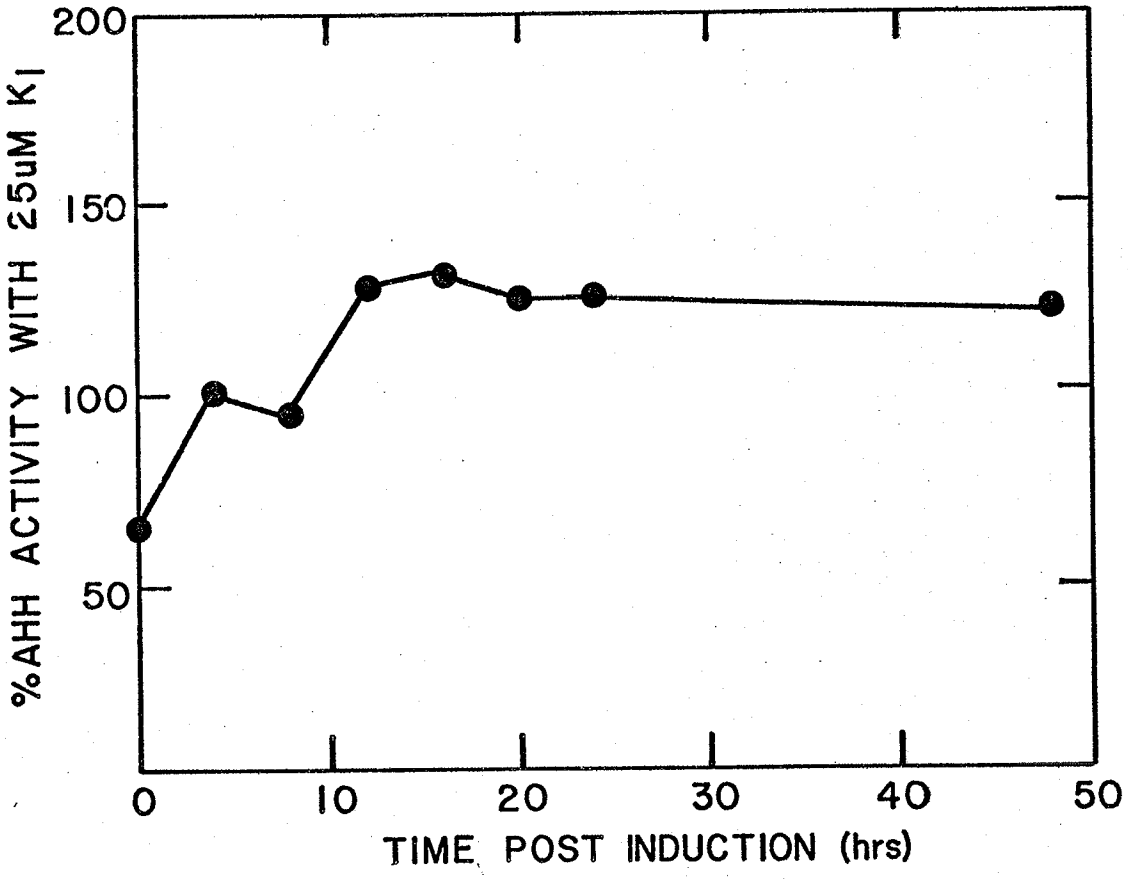
FIGURE 19



measuring 3-OH BP production only 3 out of 9 experiments indicated that K1 was increasing metabolism. The mean standard error for this data was lower at 6.7%. It became evident that there was an important factor responsible for activation by K1 that was varying from experiment to experiment. A great deal of effort was expended in trying to determine what this factor was. The effect of K1 could not be correlated with the length of time elapsed after harvesting the cells before they were used, the length of time the cells were preincubated with K1 before adding the BP, or the cell viability. Various reaction media such as MEM, HBSS, and PBS were used but none of these stabilized the effect of K1. A factor common to all experiments was that the cells were induced. The possibility was tested that the level of induction of the cells may have been responsible for the varying effect of phylloquinone. Figure 20 shows these results. BP metabolism was inhibited to 65% in uninduced cells with 25 uM K1. This inhibition disappeared as induction proceeded. At 12-25 hours post induction, K1 enhanced BP metabolism by 20%. The spectrofluorimetric assay was used for these experiments as the radioactive assay is not sensitive enough at low AHH activities. This data does not fully explain the variability of the K1 effect as the cells in subsequent experiments were all

FIGURE 20: Effect of induction on Kl activation of AHH activity in H4IIE cells. Cells were plated at a density of $10^6$ cells/10 cm plate and grown for 24 hours. At this point the media was changed to MEM + 10% FBS + 0.25% DMSO + 1 uM 3-methylcholanthrene. The cells were allowed to grow with the inducer for the times specified, whereupon they were harvested and washed. 20 minute reactions were run using whole unpermeabilized cells to determine the activity in the presence and absence of 25 uM Kl. The activity of the cells reacted with 25 uM Kl is expressed as a percentage of the control activity. Data shown is an average of 3 experiments, the mean standard error was 11.7%.

FIGURE 20


200
150
100
50
%AHH ACTIVITY WITH 25uM K1
0
10
20
30
40
50
TIME POST INDUCTION (hrs)

induced for the same length of time. Very slight alterations in inducible factors may be responsible for the variation in the K1 experiments.

2. Normal Hepatocytes

a) Effect of Menadione and Phylloquinone

To determine the effect of the K Vitamins on BP metabolism in normal rat liver cells, parenchymal cells were isolated using the perfusion technique of Berry (1974). Cell preparations with viabilities greater than 70% were used for the experiments shown. The rate of BP metabolism in the normal cells was much lower than that in the H4IIE cell line, therefore the radioactive assay could not be used with any degree of accuracy. Figure 21 shows the effect of K3 on AHH activity. Menadione inhibited at low concentrations, 50% inhibition being reached at approximately 20 uM. Metabolism was not completely inhibited at 100 uM as 35% of the control activity remained.

Phylloquinone again produced variable results, more so than in the H4IIE cells. The mean standard error for the data in Figure 22 was 23%. In an average of 5 independant experiments, K1 appears to cause an increase in BP hydroxylation activity 60% higher than the control at 25 uM (Figure 22). The activity of the cells varied from 20

FIGURE 21: Effect of menadione on AHH activity in normal hepatocytes. Normal hepatocytes were isolated from uninduced rat livers as described in materials and methods. The cells were washed and resuspended in MEM + 50 mM Hepes buffer pH 7.4 at 37°C, at a density of $3.5 \times 10^6$ cells/ml. Menadione was added to the cells in 3 ul of acetone, the control reactions contained an equivalent amount of acetone. The reactions were preincubated for 5 minutes before adding BP to start the reaction (80 uM in 6 ul acetone). Metabolism was measured by the spectrofluorometric assay. Activity was measured in pmoles 3-OH BP/min/$10^6$ cells. Data shown is an average of 3 experiments, the mean standard error was +/- 7.1%.

FIGURE 21

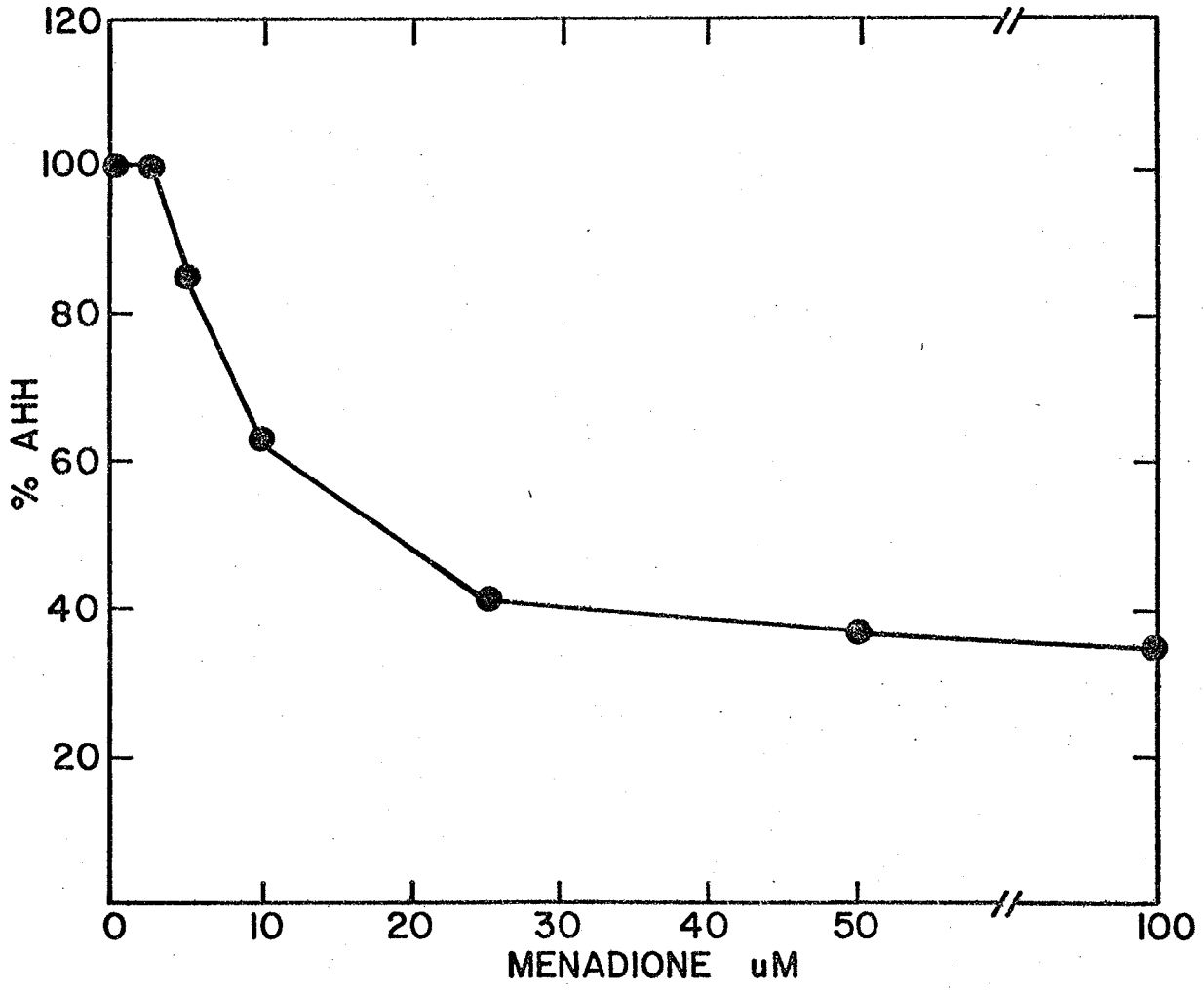
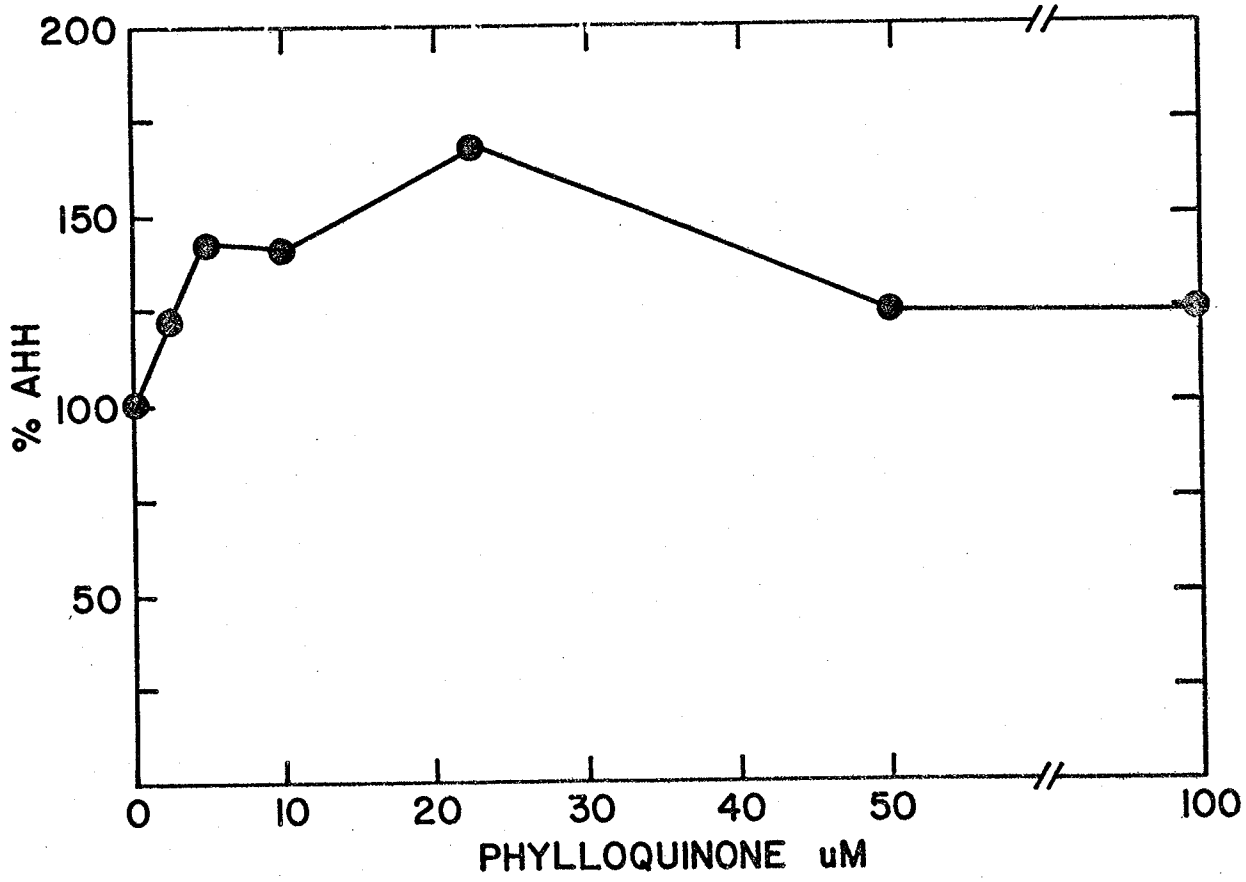


FIGURE 22: Effect of phylloquinone on AHH activity in normal hepatocytes. Normal hepatocytes were isolated from uninduced rat liver using the in situ perfusion method as described in materials and methods. The cells were washed and resuspended in MEM + 50 mM Hepes buffer pH 7.4 at 37°C, at a density of $3.5 \times 10^6$ cells/ml. Phylloquinone was added to the cells in 3 ul acetone per 300 ul reaction, the control reactions received an equivalent amount of acetone. The reactions were preincubated for 5 minutes before adding BP (80 uM in 6 ul acetone). Metabolism was measured by the spectrofluorometric assay. Activity was measured in pmoles 3-OH BP/min/$10^6$ cells. Data shown is an average of 5 experiments, the mean standard error was 23%.

FIGURE 22



pmoles 3-OH produced/min/$10^6$ cells to 90 pmoles/min/million cells. A correlation between the effect of K1 on BP metabolism and the control activity was observed in these isolated hepatocytes. Figure 23 shows that at low MFO activities K1 inhibited BP metabolism by 40%. This inhibition was absent in the cells that were more active. In cells with an activity of 85 pmoles/min/million cells K1 activated BP metabolism to 125% of the control. This correlation was observed only at a very low concentration of phylloquinone (2.5 uM). The correlation at this concentration was calculated to be equal to 0.923. The probability that there was no correlation was calculation to be less than 0.05%.

b) Assay of Carboxylase Activity and Comparison to the H4IIE Cells

A normal function of phylloquinone is the conversion of precursor proteins to active molecules required for coagulation. K1 modulates this conversion through a series of reactions known as the K-cycle (Figure 6). The carboxylase enzyme is a key step in this process and a representative assay for K cycling activity. The carboxylase activity was linear as a function of protein concentration (Figure 24), demonstrating that a single enzyme activity is involved in the carboxylation of the precursor peptide

FIGURE 23: Effect of AHH control activity on activation potential of Vitamin K1 in normal hepatocytes. The activity of the reactions incubated with 2.5 uM K1 expressed as a percentage of the control activity is plotted as a function of the control activity. Correlation coefficient (r) was calculated to be 0.9225. The probability that there was no correlation was calculated to be less than 0.05.

FIGURE 23

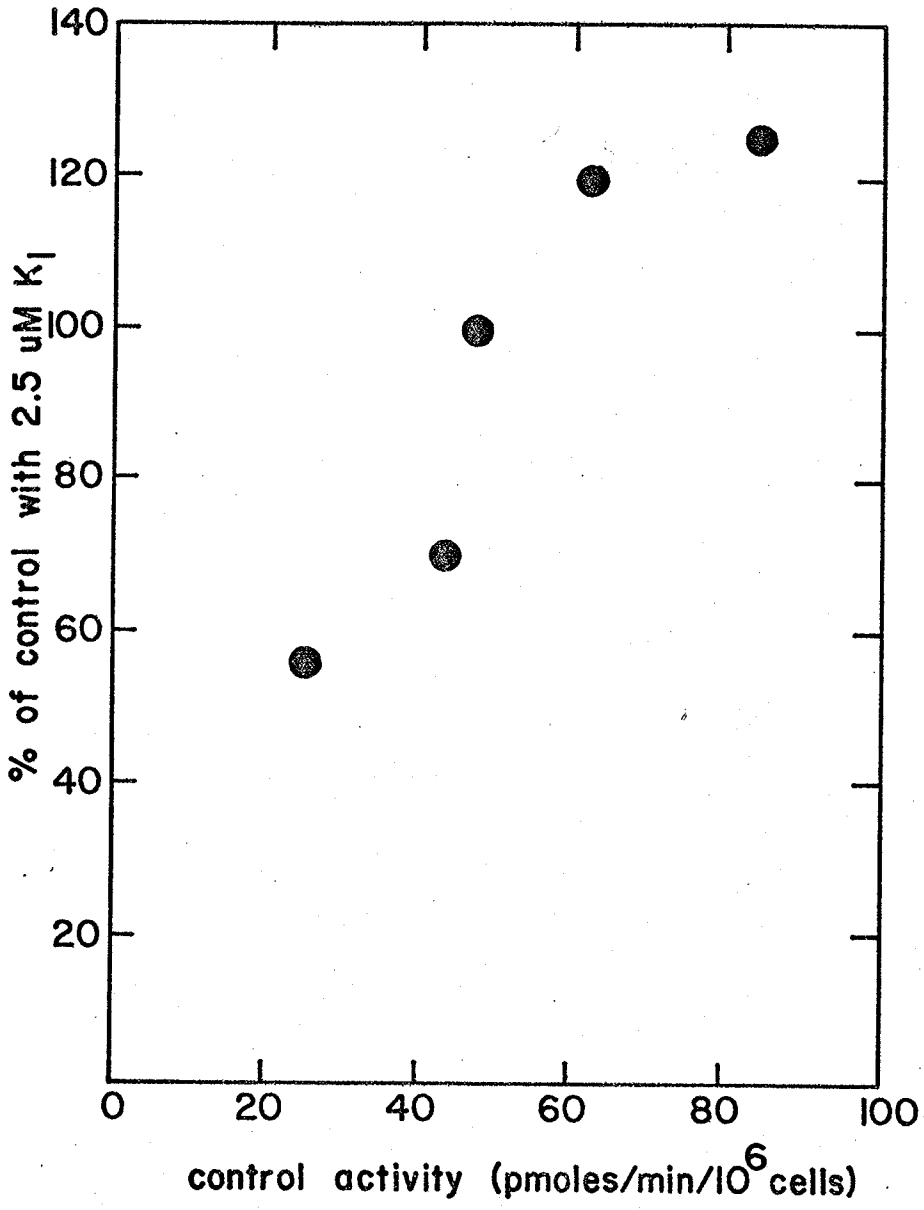
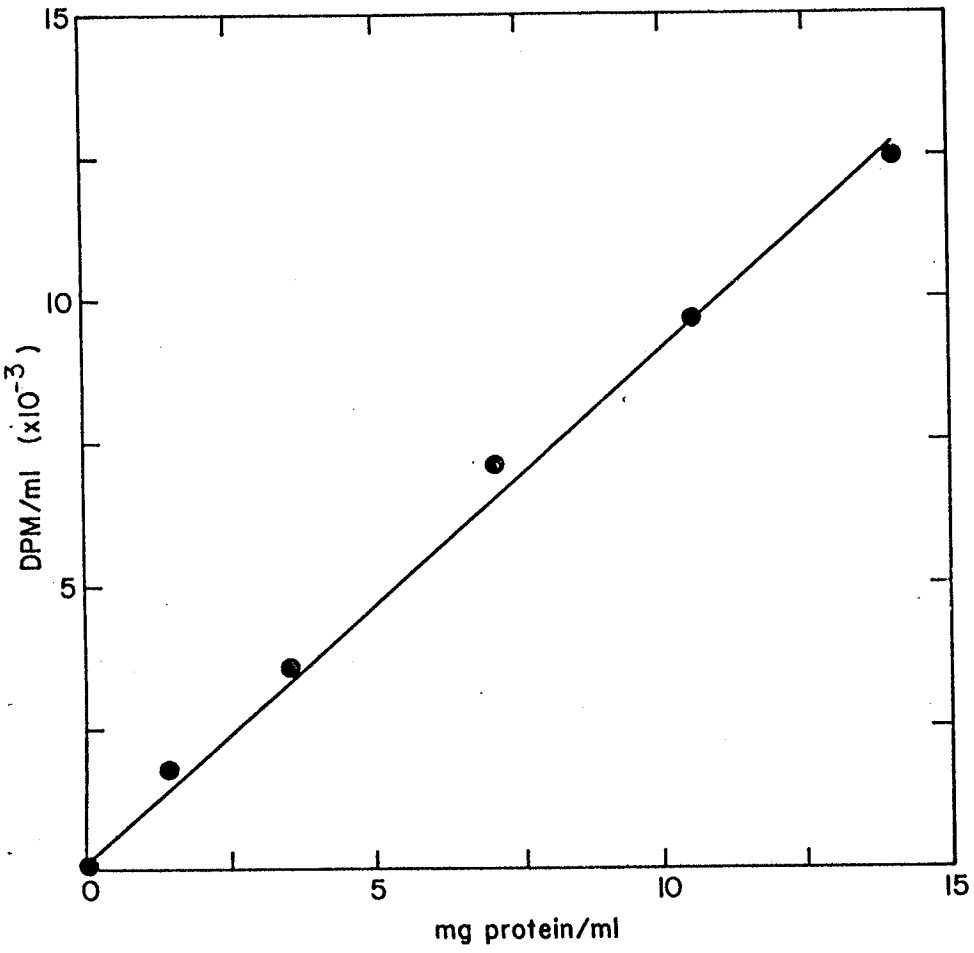

140
120
100
80
60
40
20
% of control with 2.5 uM $K_I$
0
20
40
60
80
100
control activity (pmoles/min/$10^6$ cells)

FIGURE 24: Effect of carboxylase protein preparation concentration on carboxylation activity. The carboxylase enzyme preparation from normal rat liver was diluted in buffer B + 1.5% Triton X-100, to give the protein concengrations indicated. The reactions were allowed to proceed for 20 minutes at 17°C. Activity was measured in counts incorporated into the non-TCA precipitable artificial prothrombin precursor peptide (DPM/ml). Data shown is an average of 2 experiments, the mean standard error was +/- 3.4%.

FIGURE 24



phe-leu-glu-glu-leu. Carboxylase activity was measured in preparations from both rat liver and microsomes prepared from H4IIE cells. Figure 25 shows that the activity was much higher in normal rat liver than in the hepatoma line. Induction with 3-methylcholanthrene did not alter the activities signifigantly. This data indicates that normal Vitamin K metabolism is altered in the hepatoma line, however a measurable amount of carboxylase activity is retained.

3. Effect of Vitamin K on Micorosomal Metabolism

a) Microsomal BP Metabolism

In the presence of an NADPH generating system, menadione inhibited microsomal BP metabolism as shown in Figure 26. 50% inhibition was achieved at approximately 6 uM menadione. At 50 uM K3 the AHH activity was almost completely inhibited. Phylloquinone again produced an enhancement of BP metabolism. The results, as with the whole cell experiments, were somewhat variable. Figure 27 shows a typical experiment in which K1 produced an activation of BP metabolism. 3-OH production was enhance to 128% of the control by 50 uM K1. The activation in this particular experiment was statistically significant ($P < 0.05$). Since microsomes produce the same type of variable results as do whole cells, the variations must

FIGURE 25: Carboxylase activity of preparations obtained from induced and uninduced rat liver and H4IIE microsomes. Solubilized carboxylase preparations were prepared from 3-methylcholanthrene induced ( O ) and uninduced ( ● ) rat liver, and from 3-methylcholanthrene induced ( □ ) and uninduced ( ■ ) H4IIE cells. Carboxylase activity was determined as described in materials and methods. The Figure shows the appearance of the carboxylated precursor peptide as a function of reaction time. Data shown is an average of 2 experiments, the mean standard error was +/- 2.4%.

FIGURE 25

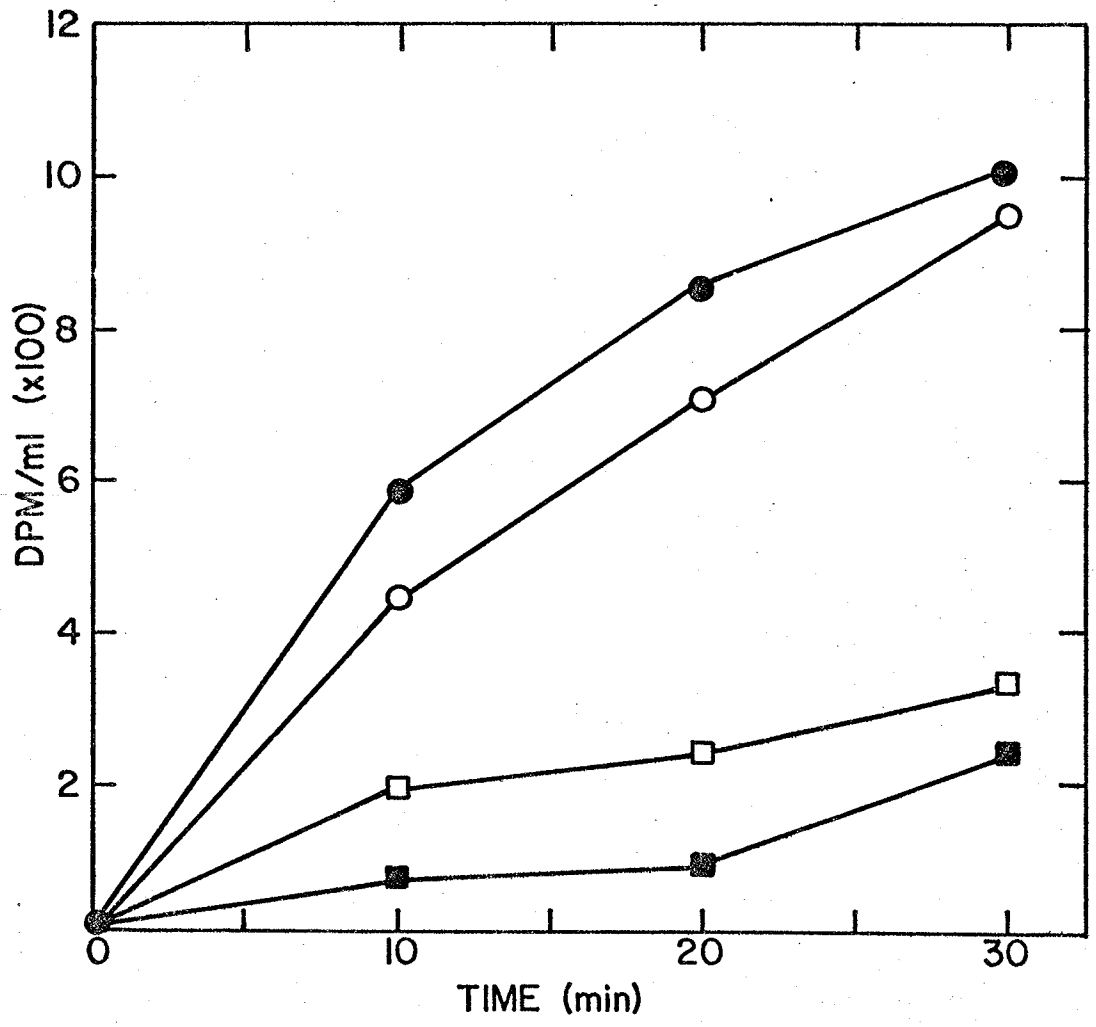


FIGURE 26: Effect of menadione on microsomal BP hydroxylation. Microsomal BP metabolism was measured as described in materials and methods. Menadione was added in 3 ul acetone per 300 ul reaction. The control reactions contained an equivalent amount of acetone. The reactions were preincubated for five minutes with K3 before adding the BP (80 uM in 6 ul acetone). The reactions were allowed to proceed for 10 minutes. Metabolism was measured in pmoles 3-OH BP formed/min/mg protein. Data shown is an average of four experiments, the mean standard error was +/- 3.1%.

FIGURE 26

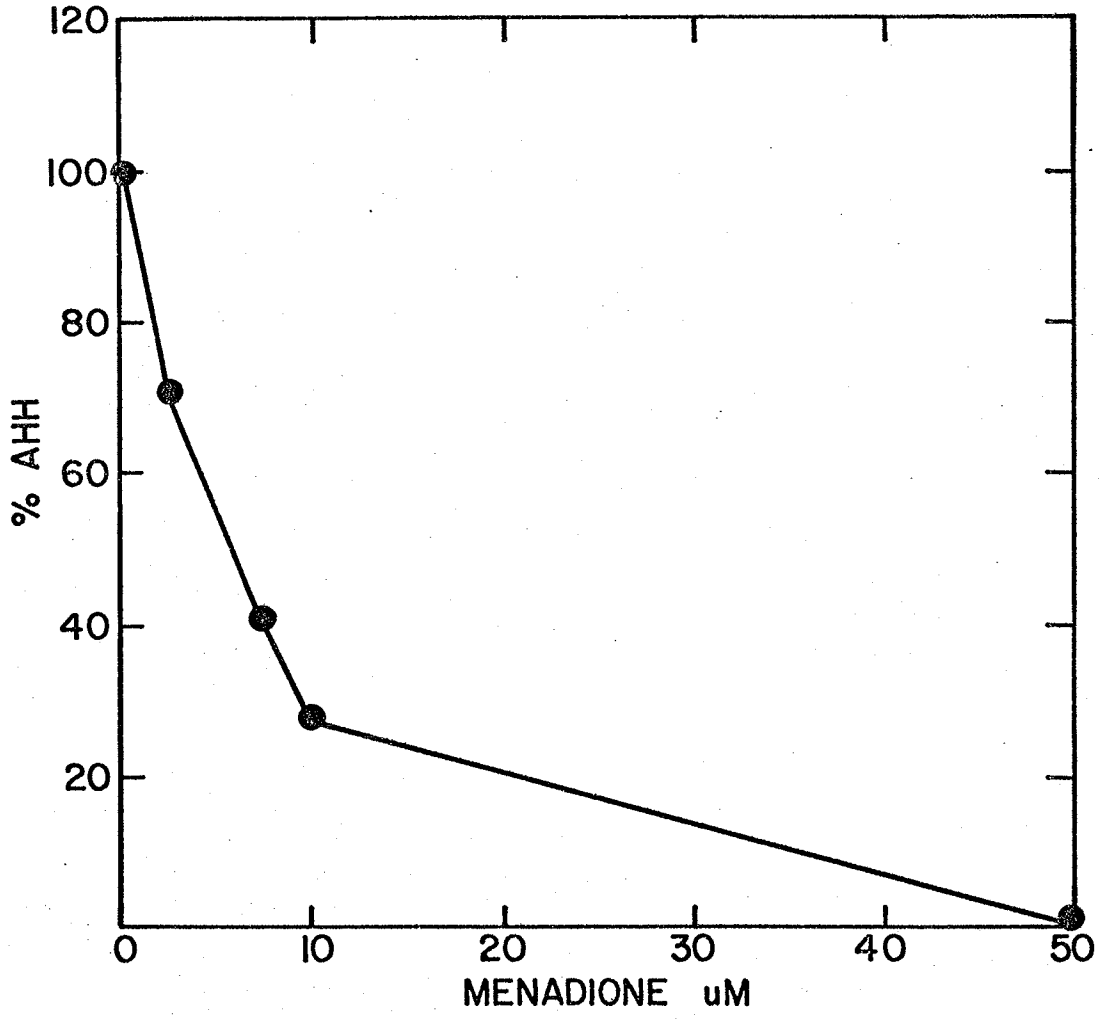
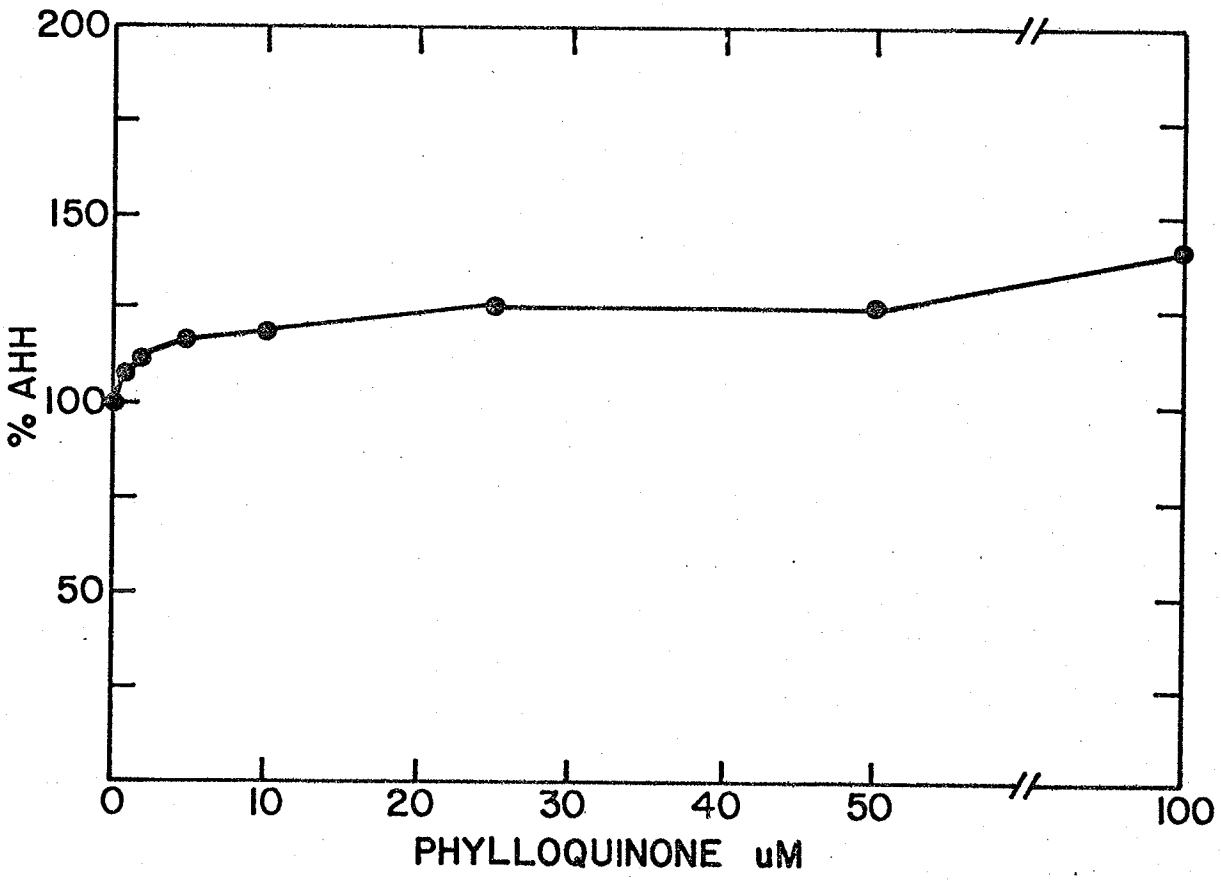


FIGURE 27: Effect of phylloquinone on microsomal BP hydroxylation. Microsomal BP metabolism was measured as described in materials and methods. Phylloquinone was added in 2 ul acetone per 300 ul reaction. The reactions were preincubated for five minutes before adding the BP (80 uM in 6 ul acetone). The reactions were allowed to proceed for 10 minutes. Metabolism was measured in pmoles 3-OH BP/min/mg protein. The mean standard error for this data was +/- 4.6%.

FIGURE 27



be due to a subcellular phenomenon.

b) Microsomal NADPH Oxidation

Microsomes incubated with reduced dinucleotide catalyze an oxidation of NADPH. Without an externally added electron acceptor, a background oxidation of NADPH could be measured (Figure 28). This background oxidation can be attributed to endogenous acceptors in the microsomal preparation. Adding 10 uM and 25 uM Menadione to the reaction increased the rate of oxidation proportionally. Menadione does not oxidize NADPH in the presence of heat treated microsomes suggesting that an enzyme is required for K3 reduction by NADPH. Several menadione reducing enzymes are present in microsomal preparations. These include NADPH-dependant cytochrome P-450 reductase, NADH-dependant cytochrome b5 reductase and microsomal DT-diaphorase. Phylloquinone did not accept electrons from any NADPH dependant enzyme in these microsomal preparations, as it did not enhance NADPH oxidation. On the contrary 10 and 25 uM K1 inhibited the background oxidation of NADPH (Figure 28). Warfarin has been shown to be a potent inhibitor of the vitamin K reducing enzyme DT-diaphorase (Jackson and Suttie, 1977). Warfarin did not inhibit NADPH oxidation by menadione as shown in Figure 29. At 100 uM warfarin the activity was still

FIGURE 28: Effect of K ivtamins on NADPH oxidation by microsomes. Microsomes were prepared from uninduced rats. The reactions contained the following; 50 mM Tris buffer pH 7.4, 1 mM NADPH, and 250 ug micorsomal protein per ml. Control reactions contained 10 ul acetone per ml reaction ( ● ). Menadione was added in acetone to concentrations of 10 uM ( □ ) and 25 uM ( △ ). Phylloquinone was added in acetone to concentrations of 10 uM ( ■ ) and 25 uM ( ▲ ). Heat treated microsomes (50°C for 10 minutes) were incubated with 1 mM NADPH and 25 uM menadione( ○ ). All reactions were started by the addition of NADPH. To measure NADPH oxidation, the absorbance at 340 nm was measured every 5 minutes (Beckman DU-8 spectrophotometer). All reactions were at 37°C. Data shown is an average of 2 experiments, the mean standard error was +/- 2.1%.

FIGURE 28

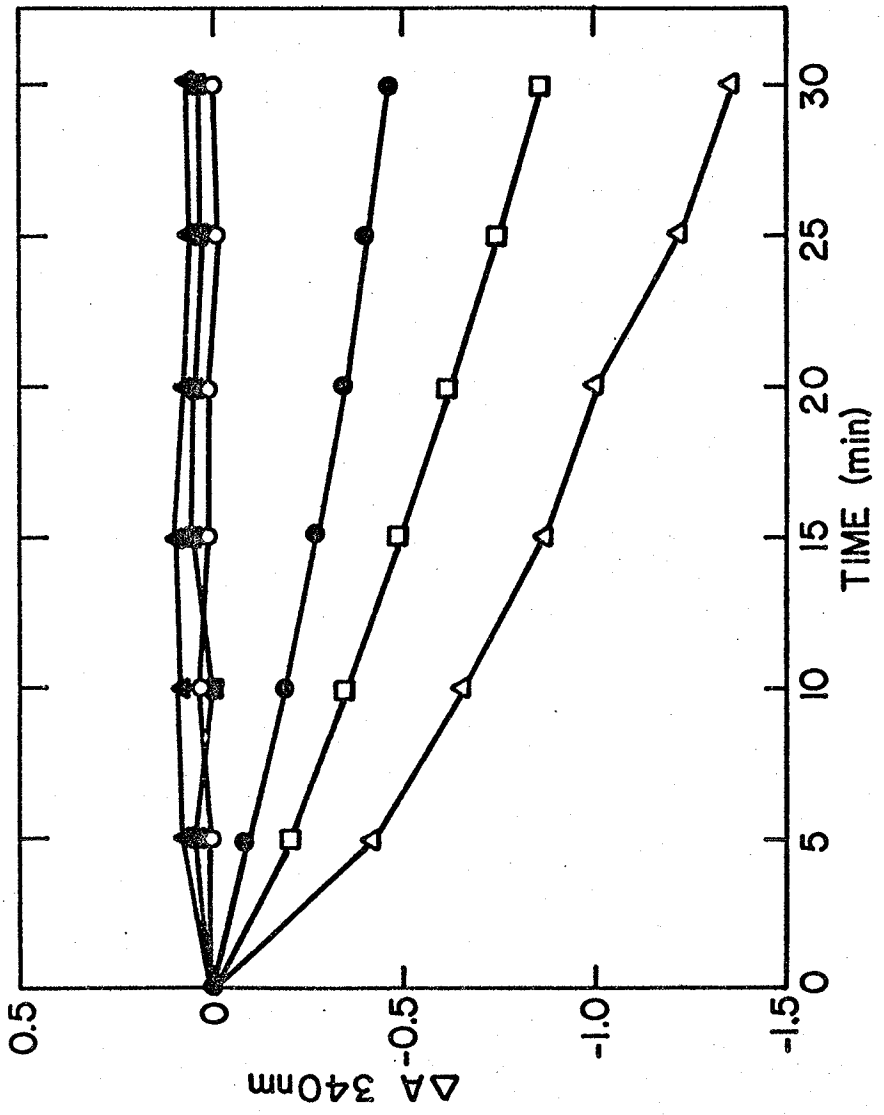
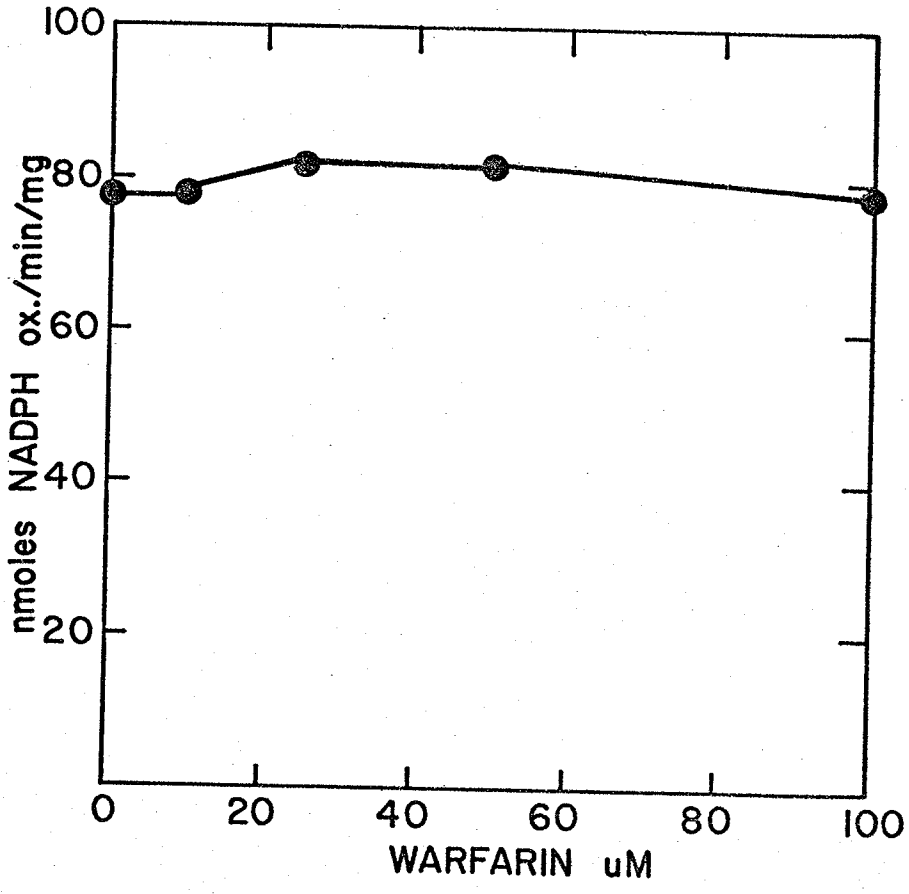


FIGURE 29: Effect of warfarin on menadione induced oxidation of NADPH by rat liver microsomes. Microsomes were prepared from uninduced rat liver. The reactions contained the following; 50 mM Tris buffer pH 7.4, 250 ug/ml microsomal protein, 1 mM NADPH, 25 uM menadione and the indicated concentrations of warfarin. Reactions were started by the addition of NADPH. The oxidation of NADPH was determined at 37°C by monitoring the change in absorbance at 340 nm. Data shown is an average of 2 experiments, the mean standard error was +/- 2.4%.

FIGURE 29


100
80
60
40
20
nmoles NADPH ox./min/mg
0
20
40
60
80
100
WARFARIN uM

near that of the control. Since DT-diaphorase is very sensitive to warfarin inhibition, the major NADPH-dependent enzyme reducing K3 has to be NADPH-dependent cytochrome P-450 reductase. The data in Figure 30 show that reduction of menadione by microsomal preparations follows Michaelis-Menton kinetic parameters. The apparent Km of the microsomal P-450 reductase for menadione was calculated to be 9.5 uM.

4. Purified Mixed Function Oxidase System

a) Purification of Cytochrome P-448 and NADPH-dependent Cytochrome P-450 Reductase

After solubilization of the 3-methylcholanthrene induced microsomes with the non-ionic detergent Emulgen 913 and treatment with protamine sulfate, the proteins of the mixed function oxidase system were resolved on a DEAE Sephadex A-25 column (Figure 31). Cytochrome P-448 elutes in the flow through, and represents the large protein peak eluting in fractions 0-45. P-448 binds carbon monoxide when in the reduced state to generate a large absorbtion peak at 448 nm. Figure 32 shows the characteristic difference spectra formed by the hemoprotein. Partial purification of cytochrome P-448 was done in four steps (Table 2), resulting in a 10 fold increase in specific activity.

FIGURE 30: Kinetics of menadione NADPH oxidation by uninduced microsomes. The reactions contained the following; 50 mM Tris pH 7.4, 1 mM NADPH, the indicated amounts of menadione added in 10 ul acetone per ml reaction and 50 ug/ml microsomal protein. Reactions were started by the addition of NADPH. NADPH oxidation was measured at 37°C by monitoring the change in absorbance at 340 nm. The velocity was measured in nmoles NADPH oxidized/min/mg protein. Data shown is an average of 3 experiments, the mean standard error was +/- 5.0%.

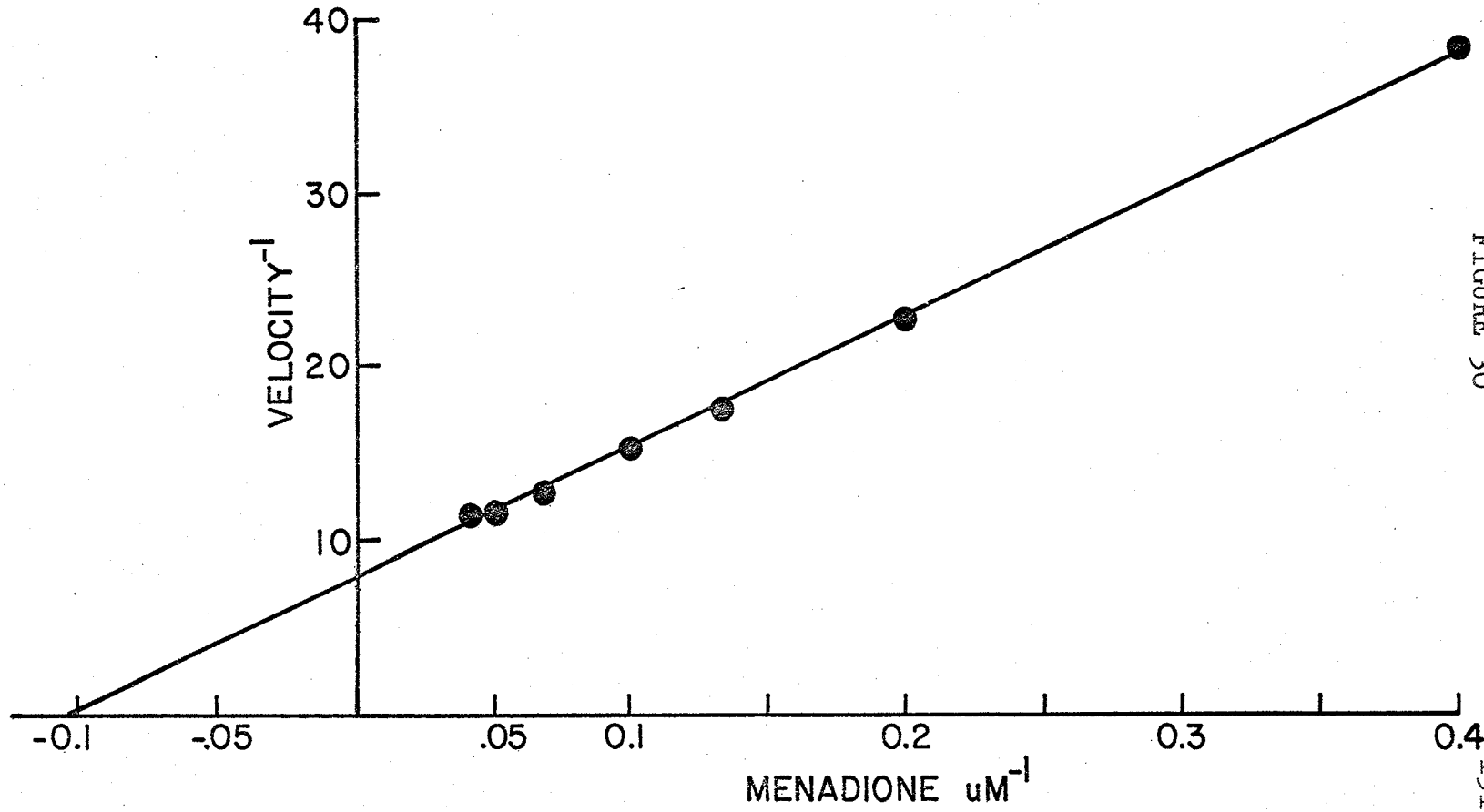



FIGURE 30

FIGURE 31: Chromatography of Emulgen 913 solubilized, protamine sulfate treated microsomes on a DEAE Sephadex A-25 column. See materials and methods for details.

FIGURE 31

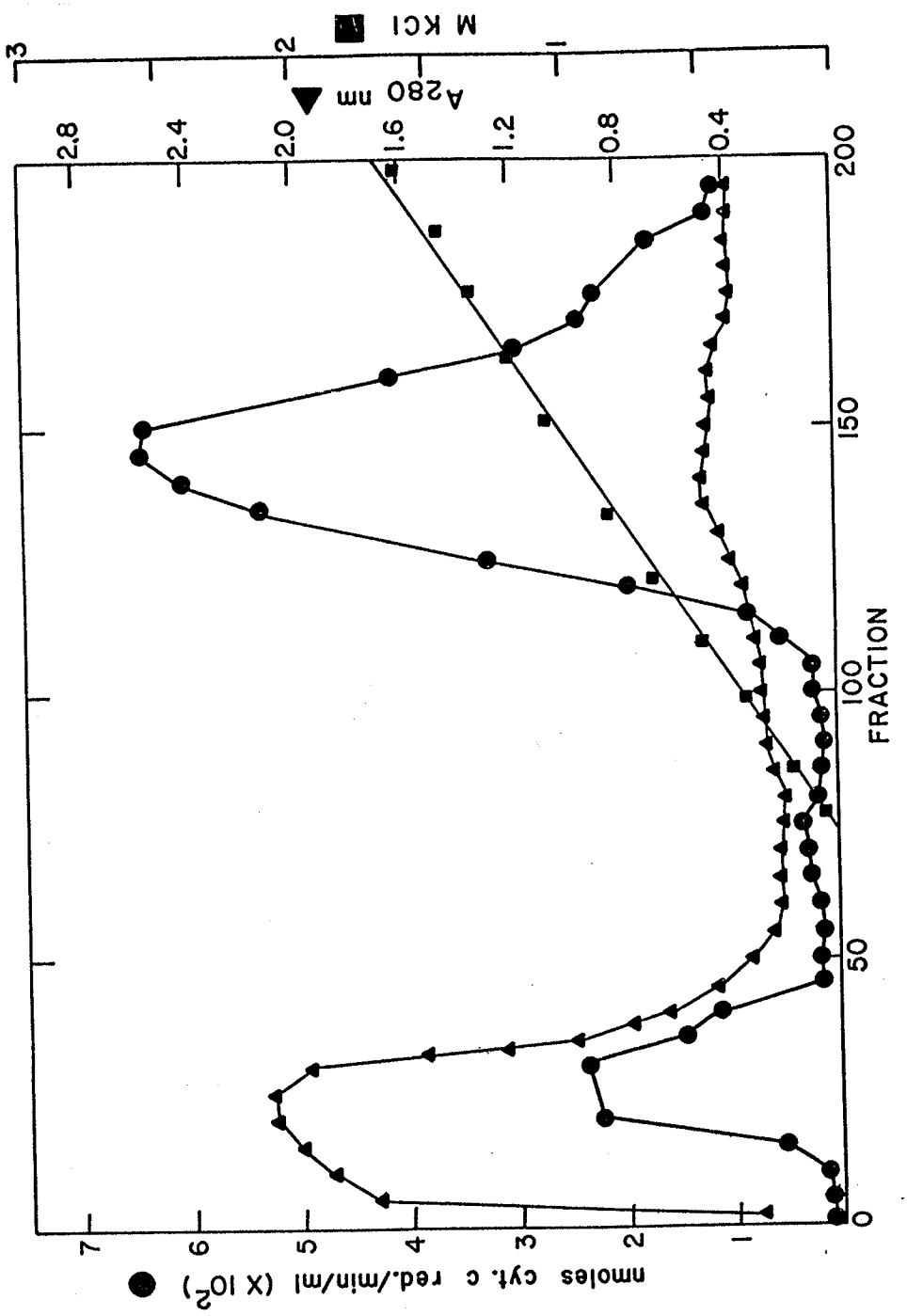

M KCl
$A_{280}$ nm
nmoles cyt. c red./min/ml (X $10^2$)
FRACTION
0
50
100
150
200
1
2
3
4
5
6
7
0.4
0.8
1.2
1.6
2.0
2.4
2.8

FIGURE 32: Difference spectra of partially purified Cytochrome P-448. The baseline represents the difference spectra of the sodium dithionite reduced sample against itself. The peak at 448 nm is formed following a short gassing of the sample with carbon monoxide.

FIGURE 32

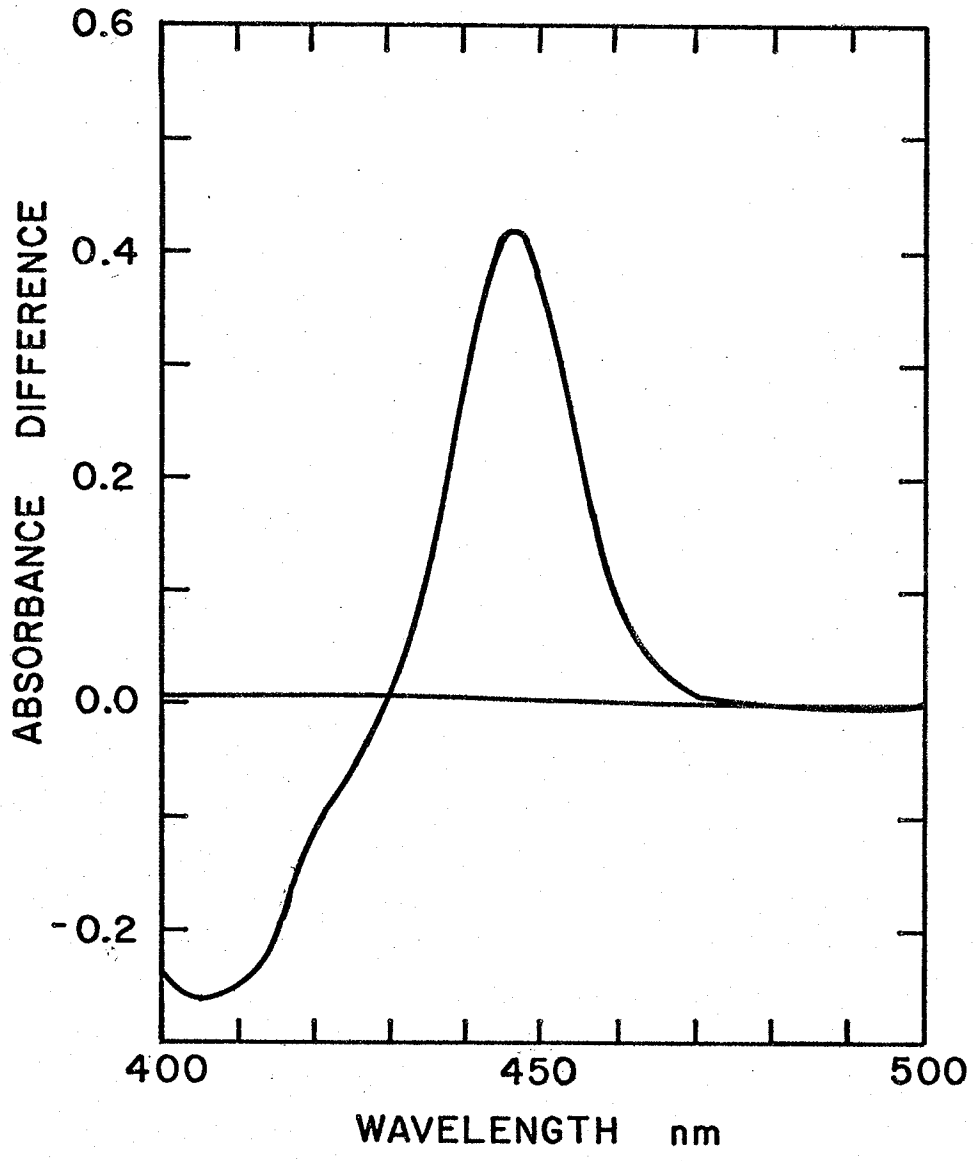

0.6
0.4
0.2
0.0
-0.2
ABSORBANCE DIFFERENCE
400
450
500
WAVELENGTH nm

TABLE 2

CYTOCHROME P-448 PURIFICATION

| STEP | VOLUME (ml) | PROTEIN (mg/ml) | SPECIFIC ACTIVITY [1] (nmoles/mg) | % YIELD |
|---|---|---|---|---|
| MICROSOMES | 52 | 45 | 0.423 | 100 |
| DEAE A-25 | 290 | 1.91 | 1.51 | 85 |
| CALCIUM PHOSPHATE | 9.8 | 1.54 | 2.85 | 43 |
| AMMONIUM SULFATE | 7.0 | 3.5 | 4.40 | 10.9 |

1. Specific Activity is measured in nmoles P-450/mg protein.

Cytochrome P-450 reductase elutes from the A-25 column at a KCl concentration of 0.1 M (Figure 31). A portion of the reductase activity eluted with the P-448 fraction and represents that amount of reductase in complex formation with the hemoprotein. Fractions containing reductase were pooled and concentrated on calcium phosphate. Total purification of the activity (Table 3) was 6 fold over the microsomal preparation.

SDS-polyacrylamide gel electrophoresis of the protein preparations revealed that the cytochrome P-450 preparation still retained a large number of other microsomal proteins (Figure 33). Molecular weights of the proteins in the preparations were estimated from a standard curve of the migration of the molecular weight markers through the gel (Figure 34). The five large bands migrating between the 47-55,000 mw region on the gel are isozymes of cytochrome P-450. These proteins were the major components of the preparation. The purified reductase had fewer contaminating proteins (Figure 33). Reductase is the band migrating at approximately 67,000 mw. A large amount of a 55,000 mw P-450 contaminant was present in the reductase preparation. This contaminant was present in all of three different reductase preparations. The level of contamination was determined to be 0.147 nmoles P-450/mg protein. To determine if the contaminating hemoprotein would affect

TABLE 3

CYTOCHROME P-450 REDUCTASE PURIFICATION

| STEP | VOLUME (ml) | PROTEIN (mg/ml) | SPECIFIC ACTIVITY[1] (umoles/mg) | % YIELD |
|---|---|---|---|---|
| MICROSOMES | 52 | 45 | 0.429 | 100 |
| PROTAMINE SULFATE | 260 | 6 | 0.547 | 85 |
| DEAE A-25 | 360 | 0.64 | 0.896 | 20.6 |
| CALCIUM PHOSPHATE | 34 | 0.84 | 2.58 | 7.3 |

1. Specific Activity is measured in umoles cytochrome c reduced/min/mg.

FIGURE 33: Polyacrylamide gel electrophoresis of partially purified preparations. Lane A) cytochrome P-448 preparation, lane B) cytochrome P-450 reductase preparation, and lane C) molecular weight markers. Markers used were bovine albumin (66,000), egg albumin (45,600), pepsin (34,700) and carbonic anhydrase (29,000). Migration was from top to bottom.

FIGURE 33

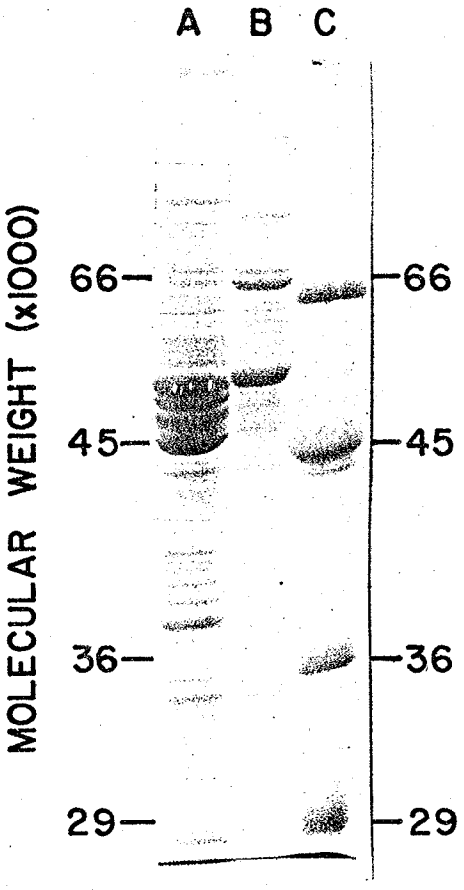
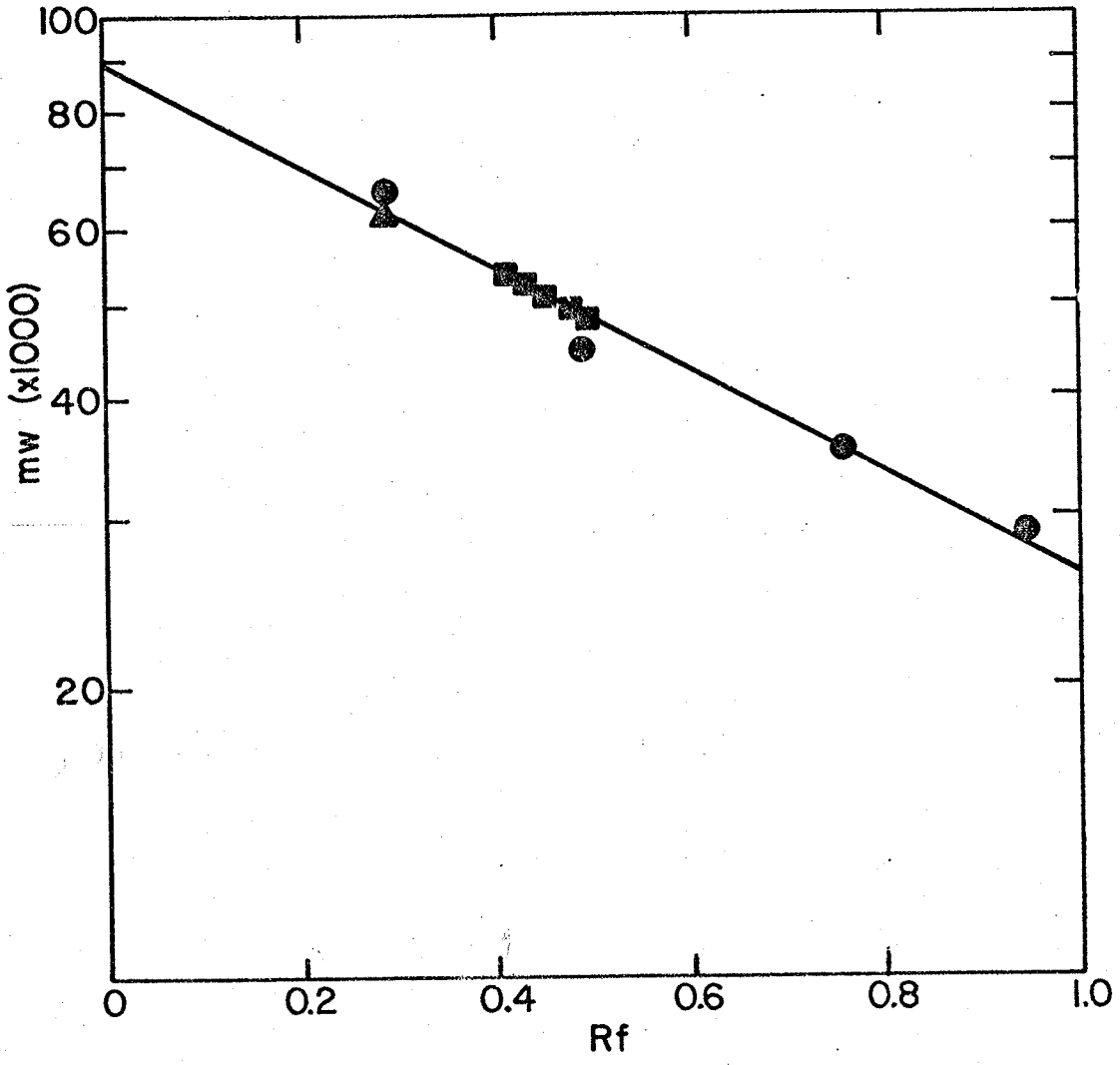


FIGURE 34: Calibration curve of molecular weight markers. The molecular weight standards were 66,000: bovine albumin, 45,600: egg albumin, 34,700: pepsin, and 29,000: carbonic anhydrase ( ● ). Rf values of reductase ( ▲ ) was 0.28 and for the five P-450 isozymes ( ■ ) between 0.4 and 0.48.

FIGURE 34



the results of the experiments undertaken, binding studies were done with benzo(a)pyrene. Binding of substrate to the catalytic site induces an alteration in the spin state of the porphryn prosthetic group on the P-450 molecule. A characteristic shift of the absolute absorption maximum from 420 to 385 will occur, in a difference spectra a decrease in absorption will occur at 420 nm and an increase at 385 nm (Backes and Canady, 1981). Benzo(a)-pyrene has three absorption maxima in the 360-420 nm region, these are at 368, 390 and 404 nm. Figure 35 shows that the contaminating P-450 did not bind BP, therefore it can be concluded that the isozyme in question could be an uninduced form incapable of catalyzing hydroxylation of BP.

The isozymes present in the P-448 preparation did bind BP, however, as shown in Figure 36. The decrease in absorbance at 420 nm is proportional to the amount on BP bound to the active site. A spectral dissociation constant (Ks) for BP can be calculated using these shifts in absorbance at 420. These data are shown in Figure 37. The apparent Ks was determined to be 17.2 uM BP.

b) Reconstitution of BP Hydroxylation Activity

In the presence of 0.04 units/ml reductase, 30 ug/ml dilaurylphosphatidylcholine and an NADPH generating system,

FIGURE 35: Difference spectra of P-450 reductase preparation in the presence of BP. The cytochrome P-450 reductase preparation was diluted to 1 mg protein/ml in reducatse dialysis buffer (0.1 M Tris pH 7.4, 20% glycerol, 1 mM EDTA and 0.15% Emulgen 913). The difference spectra was measured first against itself to generate the baseline. The difference spectra was then recorded in the presence of 5, 10 and 20 uM BP. The increase in absorbance at 368, 390, and 404 nm corresponds to the absorption maxima of BP.

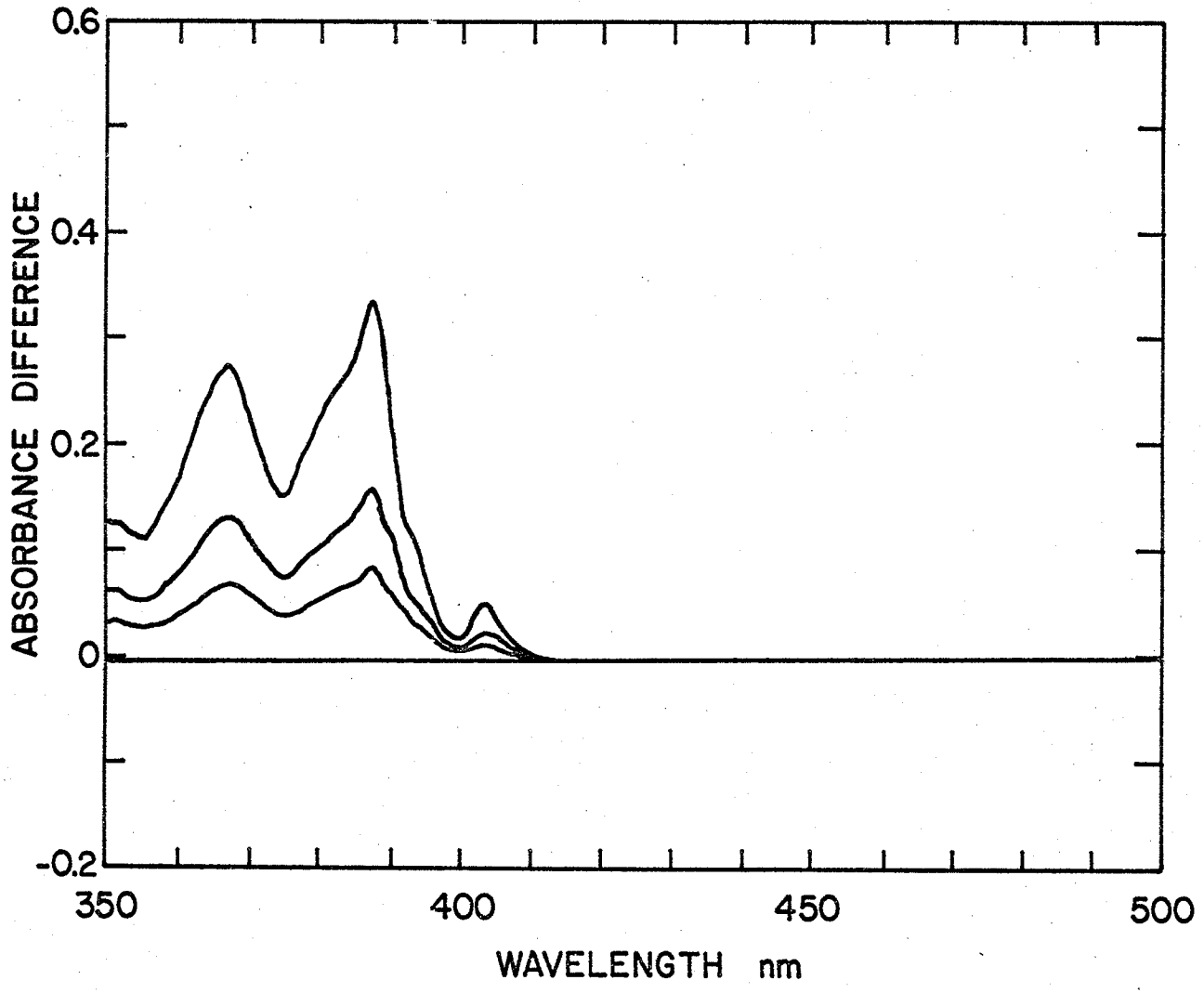

0.6
0.4
0.2
0
-0.2
ABSORBANCE DIFFERENCE
350
400
450
500
WAVELENGTH nm

FIGURE 35

FIGURE 36: Difference spectra of P-448 preparation in the presence of BP. The P-450 preparation was diluted to 1 mg protein/ml in P-450 dialysis buffer (5 mM potassium phosphate pH 7.7, 20% glycerol, 0.1 mM DTT, 0.1 mM EDTA and 0.05% cholate). The difference spectra of the preparation was first measured against itself to generate the baseline. The difference spectra was then recorded in the presence of 5, 10, 25, 50 and 100 uM BP. The spectra were measured at 30°C, all samples were prewarmed at this temperature for 5 minutes before reading. Increases in absorbance at 368, 390, and 404 nm correspond to the absorption maxima of BP. The decreases in absorbance at 420 nm correspond proportionally to the shift of the heme iron from the low spin state to the high spin state.

FIGURE 36

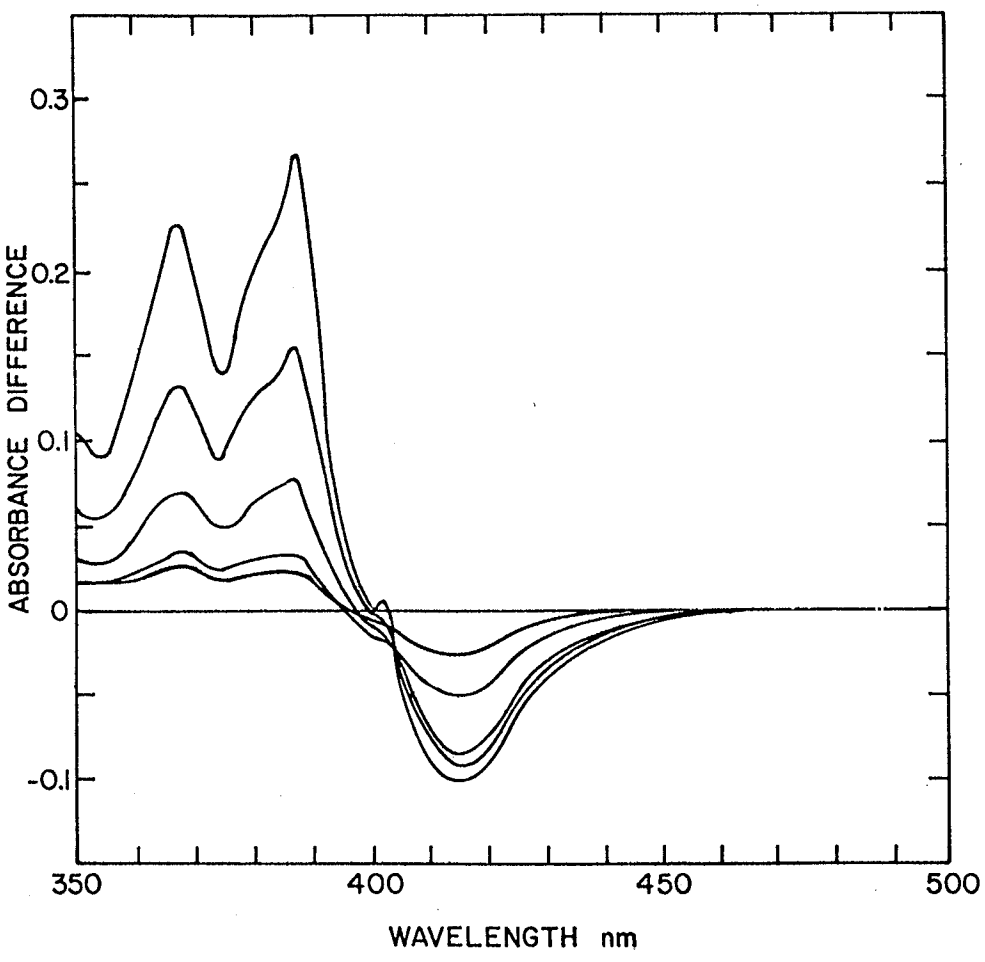
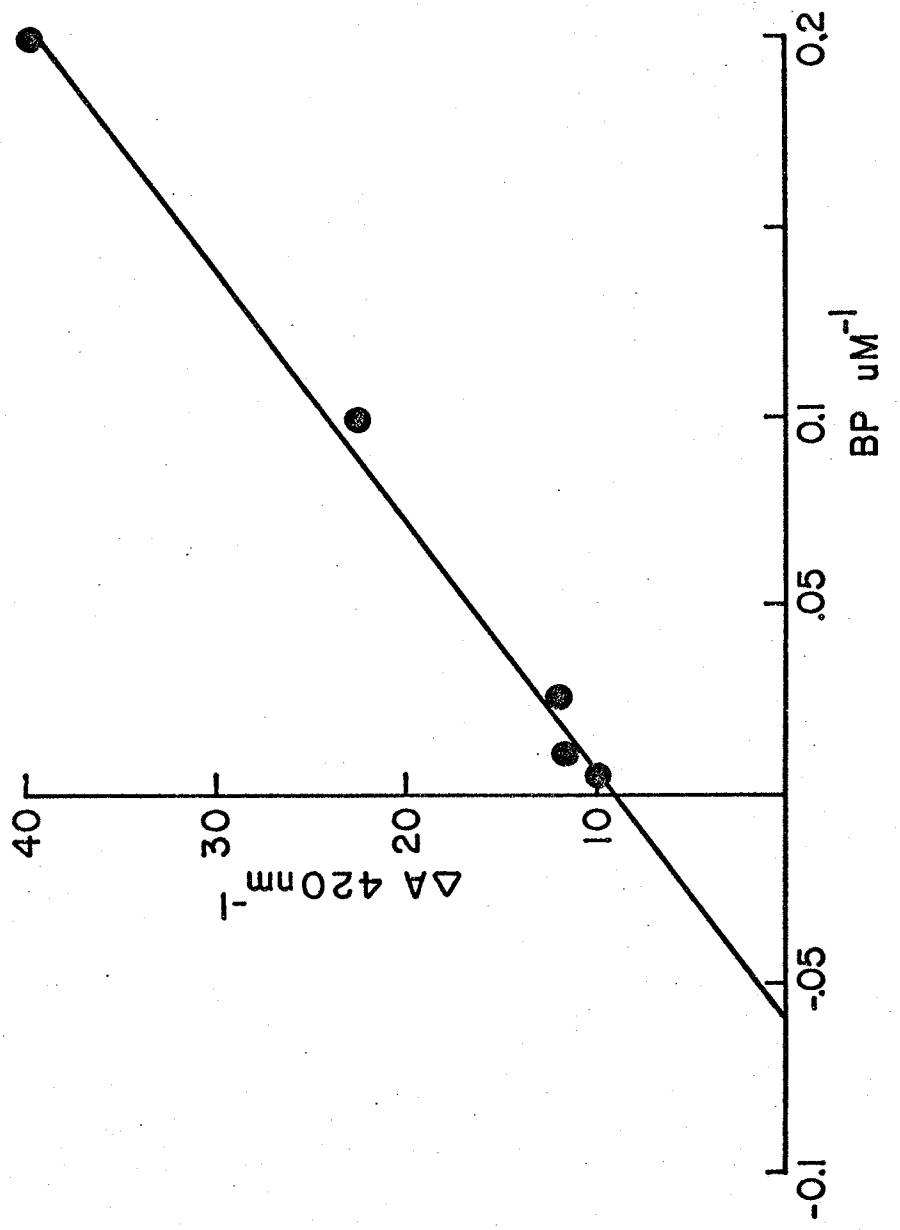

0.3
0.2
0.1
0
-0.1
ABSORBANCE DIFFERENCE
350
400
450
500
WAVELENGTH nm

FIGURE 37: Kinetics of spectral change induced by BP binding to cytochrome P-448. Replot of reciprocal of change in absorbance at 420 nm vs. reciprocal of BP concentration in uM. Data shown is an average of 2 experiments, the mean standard error was +/- 2.8%.

FIGURE 37



0.3 nmoles/ml of the cytochrome P-450 preparation hydroxylated benzo(a)pyrene. The reaction was linear for at least 15 minutes (Figure 38). 0.3 nmoles/ml cytochrome P-448 had a small amount of BP hydroxylating activity without adding purified reductase (Figure 39). This activity at 0 units reductase/ml is indicative of the level of contamination of the P-448 preparation by reductase. Increasing the concentration of reductase resulted in a linear increase in activity until 0.4 units reductase/ml at which point the hydroxylase activity was not enhanced further by adding excess reductase. The cytochrome P-450 reductase preparation did not hydroxylate BP, suggesting that it is free of contaminating P-448. This result corroborates the earlier binding studies with the reductase preparation. The dependance of the reconstituted MFO system on phosphatidylcholine is shown in Figure 40. By adding lipid, the activity could be increased to double the control activity. The activity at 0 ug/ml is indicative of the amount of lipid that is tightly associated with the P-448 molecule. Early preparations of the hemoprotein exhibited a smaller dependance on external lipid for maximal activity. For the reconstituted MFO experiments 30 ug/ml dilaurylphosphatidylcholine was routinely used.

FIGURE 38: Production of 3-OH BP by the reconstituted MFO system. Appearance of 3-OH BP as a function of time. Data shown is an average of 3 experiments, the mean standard error was +/- 2.5%.

FIGURE 38

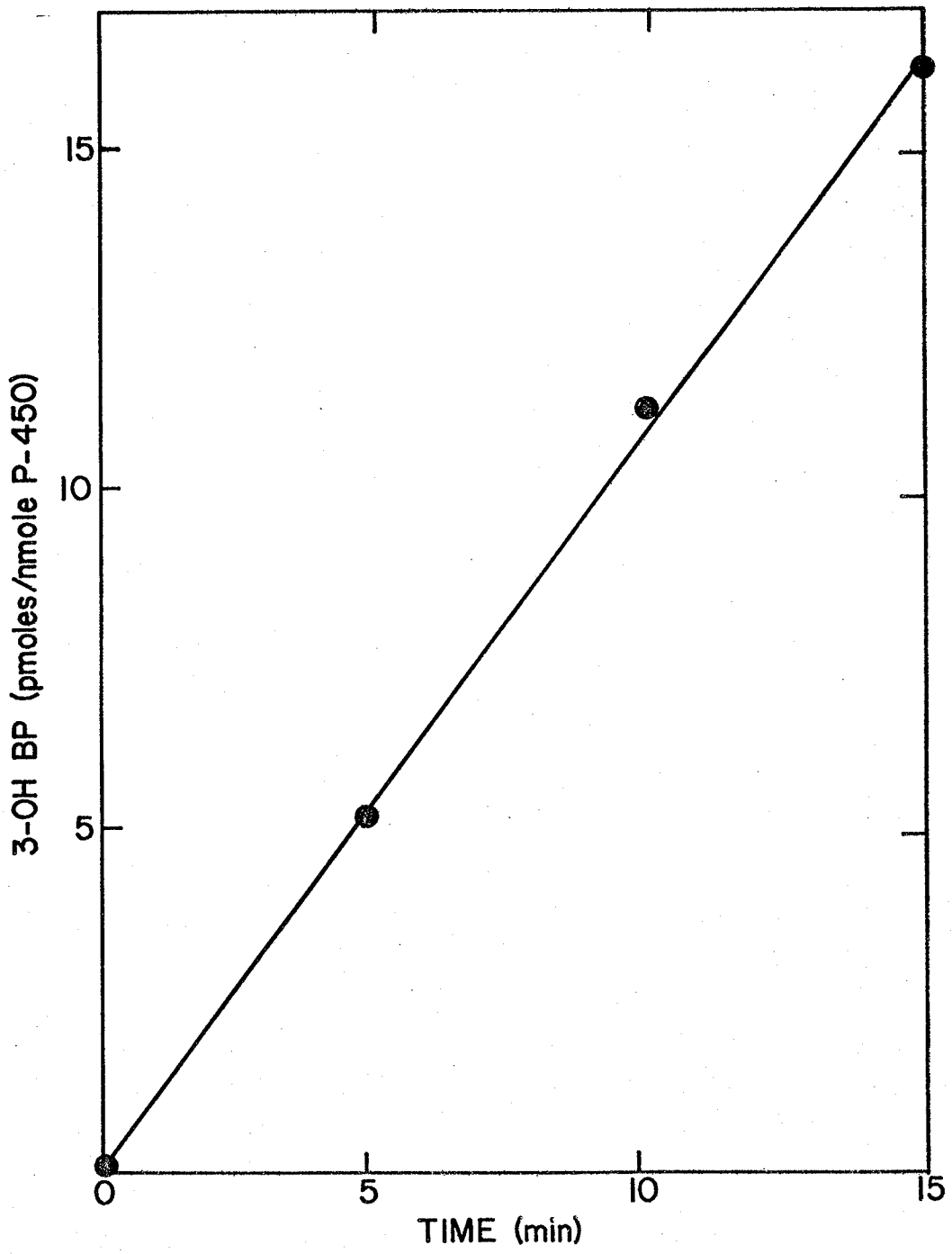


FIGURE 39: The effect of Cytochrome P-450 Reductase on BP hydroxylation activity of the reconstituted mixed function oxidase system. The concentration of P-448 was 0.3 nmoles/ml, phosphatidylcholine was used at 30 ug/ml. BP hydroxylation activity was measured in umoles 3-OH BP formed/min/nmole P-448. One unit of Cytochrome P-450 reductase is defined as that amount reducing 1 umole of cytochrome c/min in the standard cytochrome c reductase assay. Data shown is an average of 3 experiments, the mean standard error was +/- 9.1%.

FIGURE 39

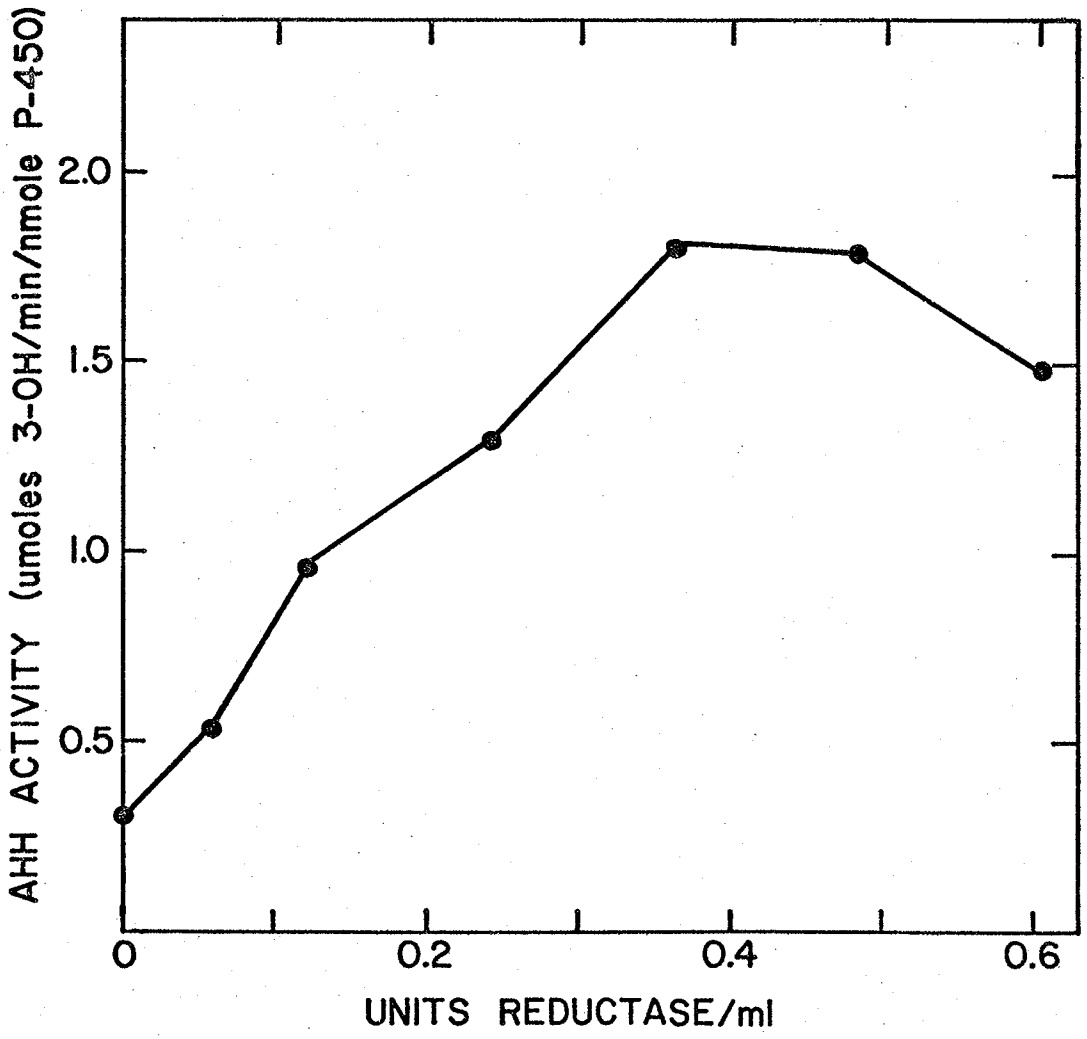
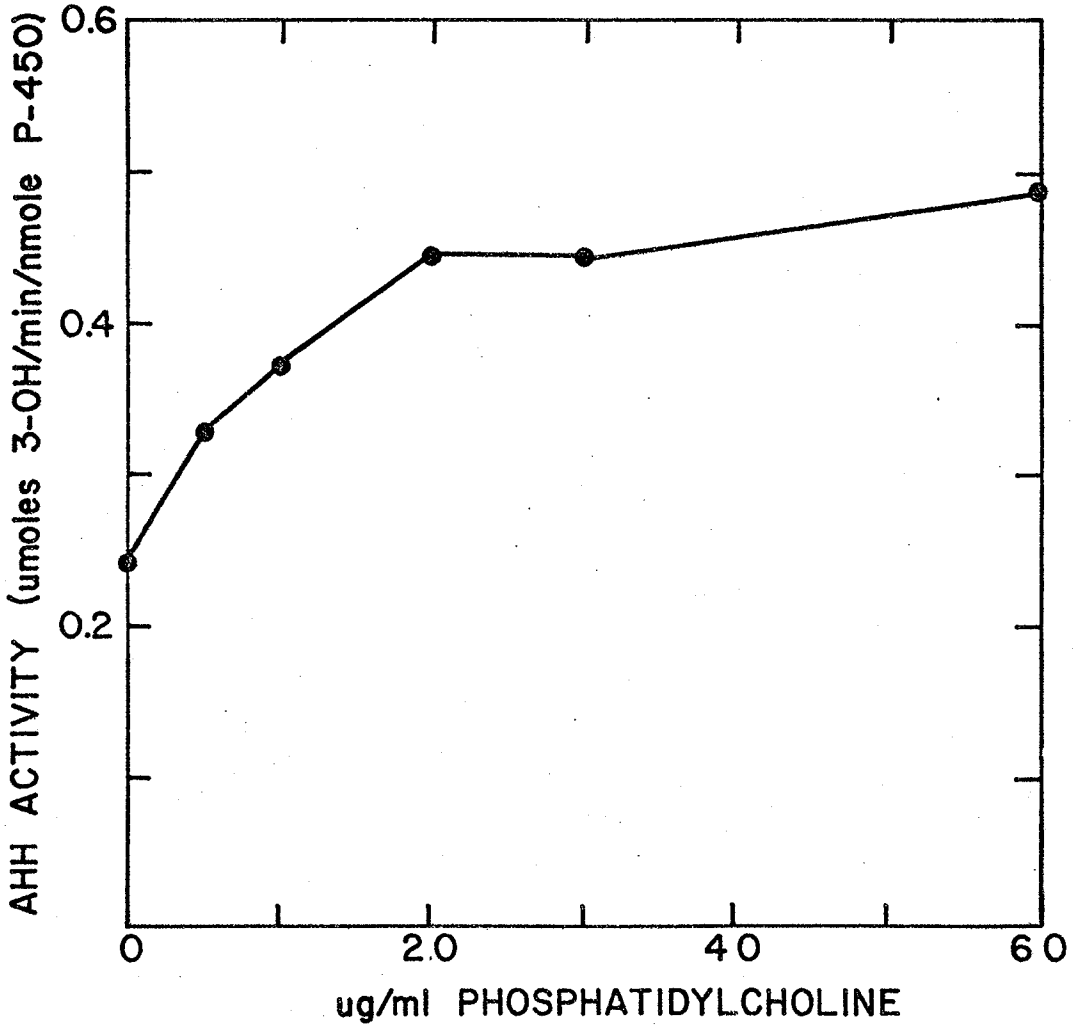

AHH ACTIVITY (umoles 3-OH/min/nmole P-450)
2.0
1.5
1.0
0.5
0
0.2
0.4
0.6
UNITS REDUCTASE/ml

FIGURE 40: The effect of dilaurylphosphatidylcholine on the BP hydroxylation activity of the reconstituted mixed function oxidase system. Data shown is an average of 3 experiments, the mean standard error was +/- 10.2%.

FIGURE 40


AHH ACTIVITY (umoles 3-OH/min/nmole P-450)
0.6
0.4
0.2
0
20
40
60
ug/ml PHOSPHATIDYLCHOLINE

c) Inhibition of Reconstituted MFO Activity by Menadione Analogs

Menadione and 1, 4-napthoquinone were very potent inhibitors of the reconstituted BP hydroxylating system (Figures 41 and 42). The concentration at which the activities were inhibited by 50% (estimated Ki) for menadione was calculated to be 3.63 uM, which is similar to the Ki for 1, 4-napthoquinone at 3.70 uM, The menadione bisulfite analog inhibited half maximally at 28.6 uM, which is almost 3 times higher than the other two analogs (Figure 43). Figure 4 shows the molecular structure of the three analogs used.

d) Reduction Kinetics of Menadione Analogs by Cytochrome P-450 Reductase and the Relationship to Inhibition of the MFO System

The Km for menadione reduction by cytochrome P-450 reductase was determined to be 3.75 uM as measured by NADPH oxidation (Figure 44). The double reciprocal plot was consistantly curvilinear as were the plots for 1, 4-napthoquinone (Figure 45). The Km for 1, 4-napthoquinone was determined to be 3.23 uM. Menadione bisulfite had a much higher Km at 29.4 uM (Figure 46). Velocity vs. enzyme concentration and NADPH kinetic experiments were done to test the possibility that the curvilinear Line-

FIGURE 41: Effect of Menadione on the reconstituted mixed function oxidase system; % activity of BP hydroxylation vs. concentration of menadione. Menadione was added dissolved in 6 ul acetone per 300 ul reaction. Data shown is an average of 3 experiments, the mean standard error was +/- 3.8%.

FIGURE 41

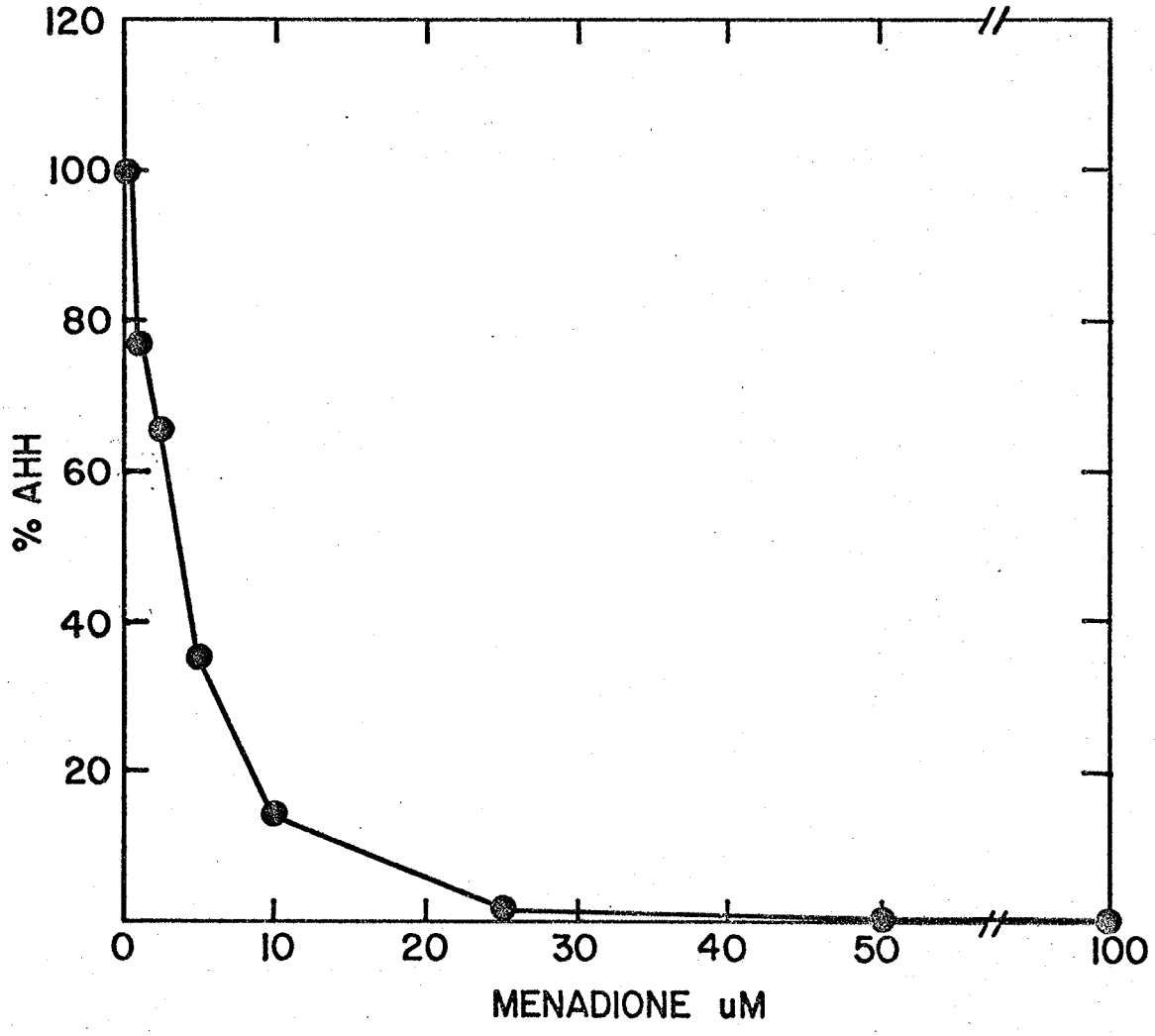


FIGURE 42: Effect of 1, 4-Napthoquinone on the reconstituted mixed function oxidase system; % activity of BP hydroxylation vs. concentration of napthoquinone. The quinone was added dissolved in 6 ul acetone per 300 ul reaction. Data shown is an average of 3 experiments, the mean standard error was +/- 6.9%.

FIGURE 42

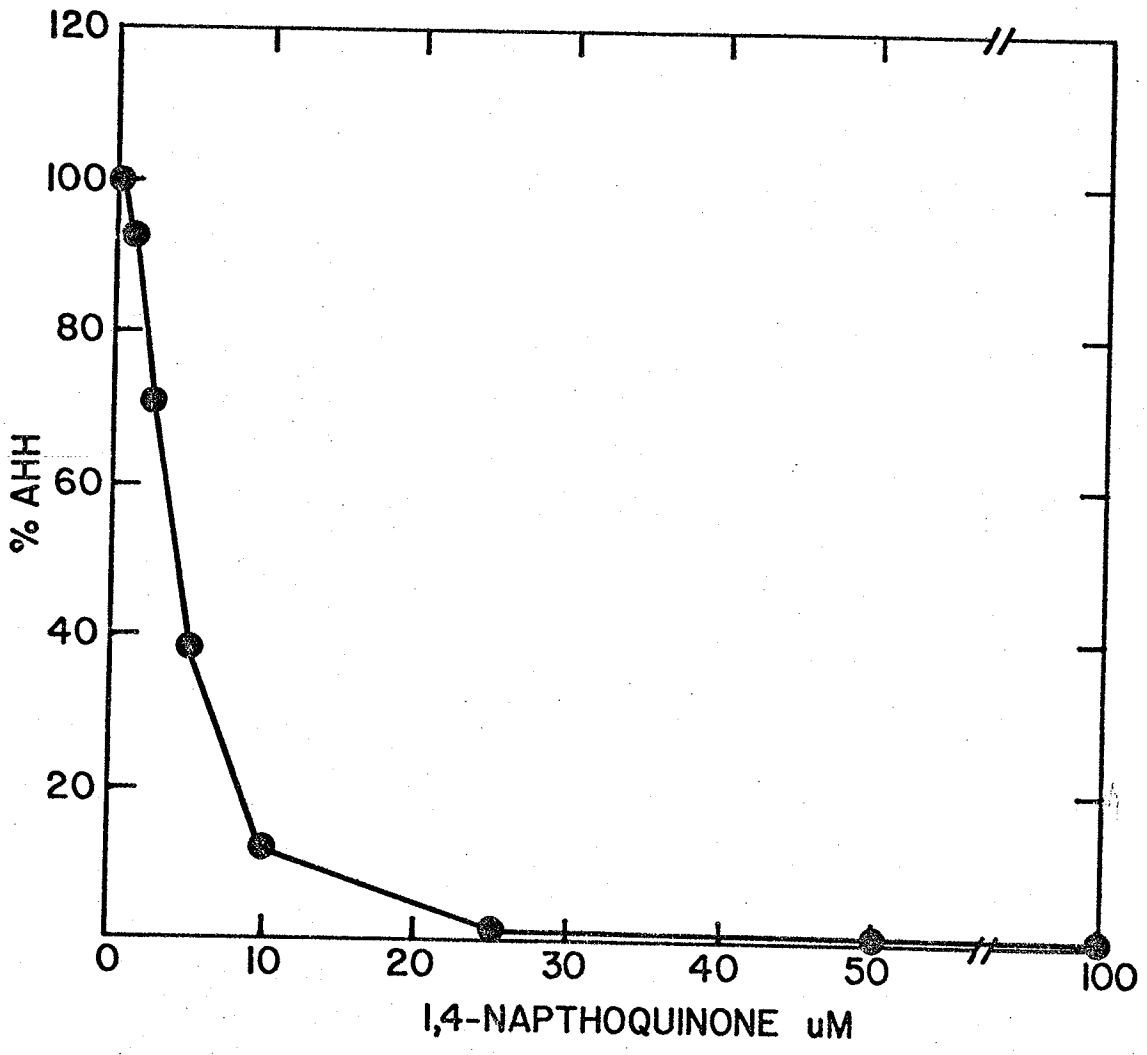


FIGURE 43: Effect of Menadione Bisulfite on the reconstituted mixed function oxidase system; % activity of BP hydroxylation vs. concentration of Bisulfite. Menadione Bisulfite was added in 6 ul water per 300 ul reaction. Data shown is an average of three experiments, the mean standard error was +/- 8.7%.

FIGURE 43

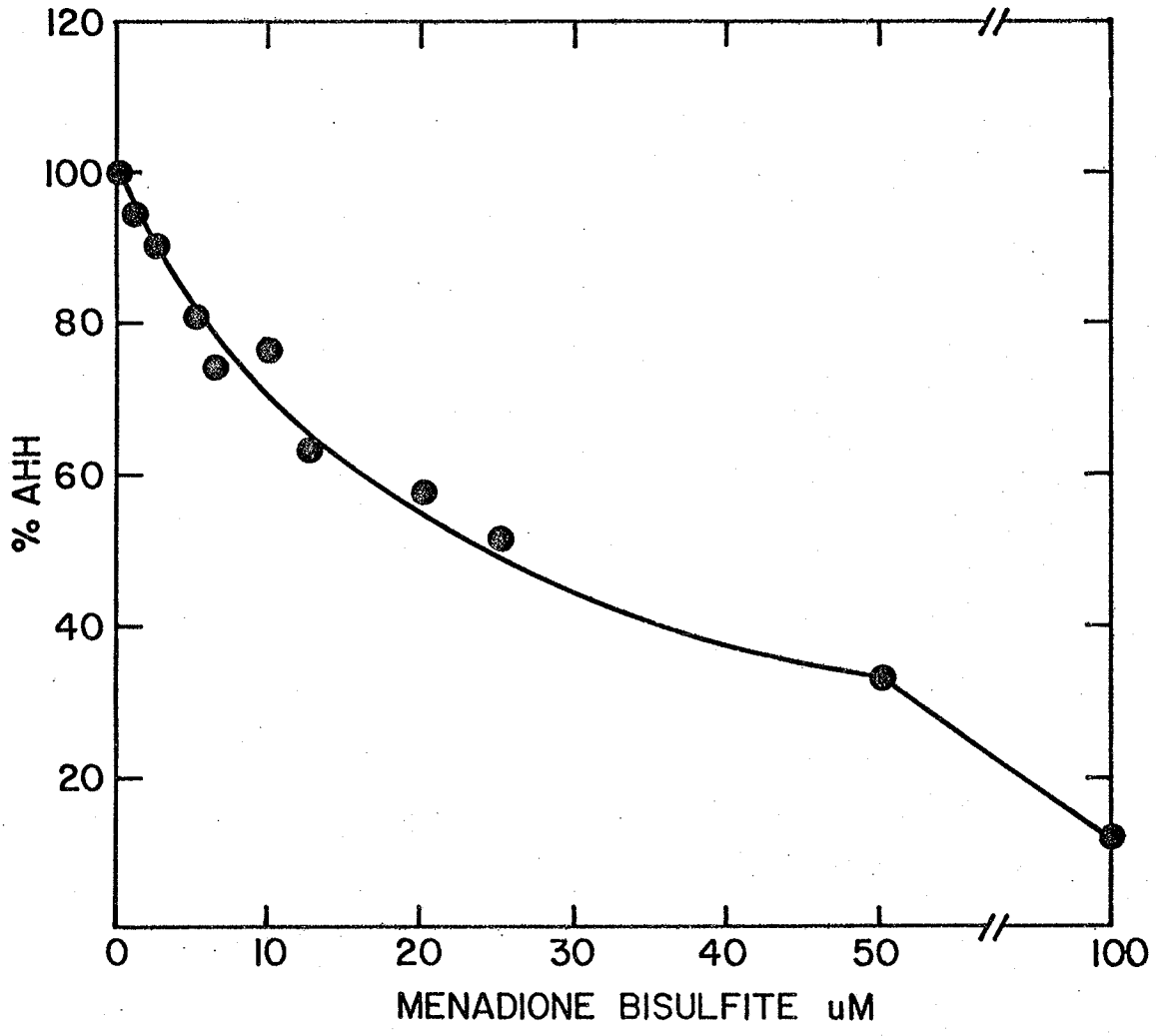

120
100
80
60
40
20
% AHH
0
10
20
30
40
50
100
MENADIONE BISULFITE uM

FIGURE 44: Lineweaver Burk plot of reduction of Menadione by P-450 Reductase as measured by NADPH oxidation. Inset shows the velocity vs. concentration data. Menadione was added dissolved in 6 ul acetone per 300 ul reaction. Velocity was measured in nmoles NADPH oxidized/min/unit reductase. Data shown is an average of 3 experiments, the mean standard error was +/- 5.9%.

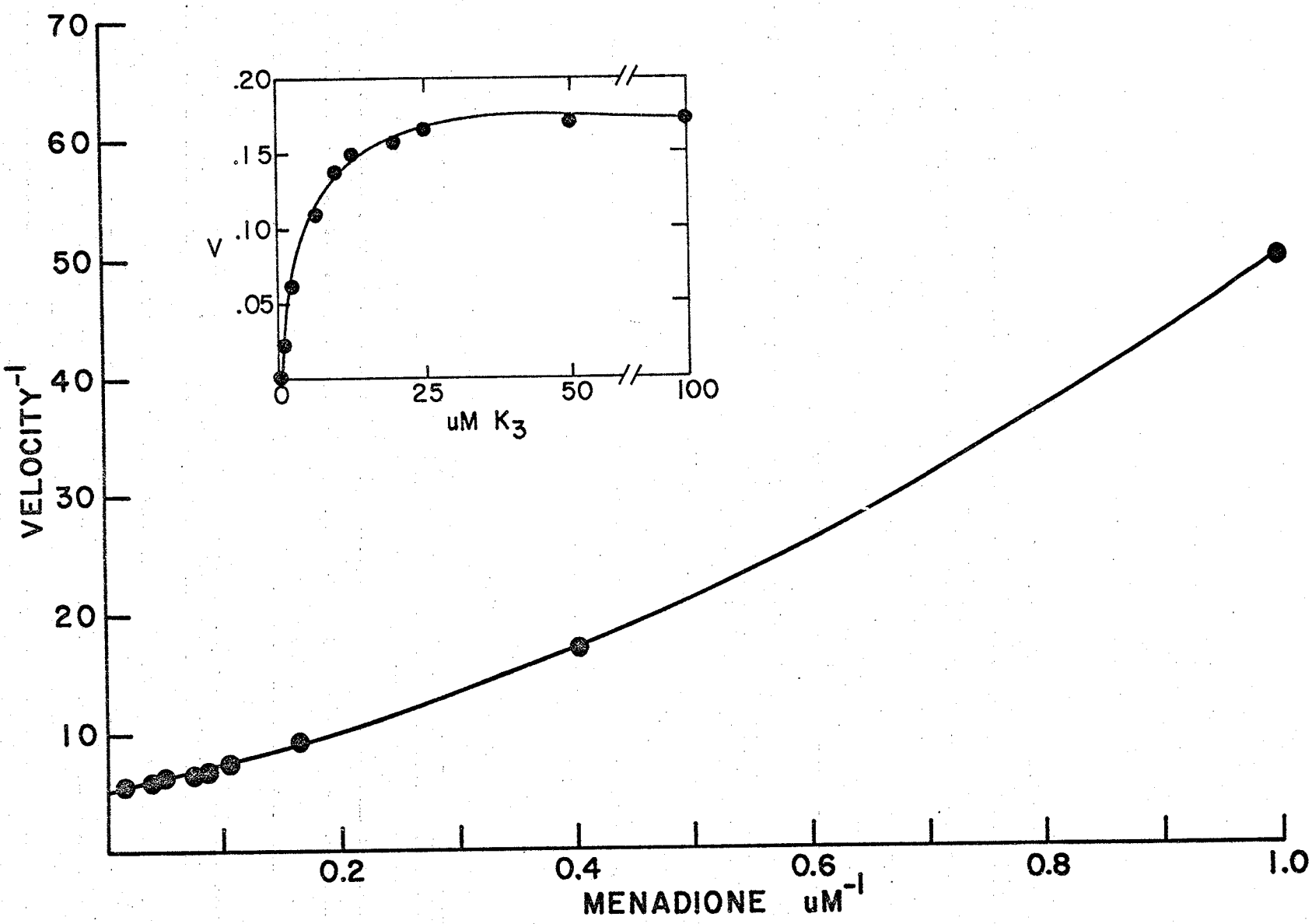

70
60
50
40
30
20
10
VELOCITY$^{-1}$
0.2
0.4
0.6
0.8
1.0
MENADIONE uM$^{-1}$
.20
.15
.10
.05
V
0
25
50
100
uM $K_3$

FIGURE 44

FIGURE 45: Lineweaver Burk plot of reduction of 1, 4-Napthoquinone by P-450 Reductase as measured by NADPH oxidation. Inset shows the velocity vs. concentration data. The quinone was added dissolved in 6 ul acetone per 300 ul reaction. Velocity was measured in nmoles NADPH oxidized/min/unit reductase. Data shown is an average of 3 experiments, the mean standard error was +/- 4.3%.

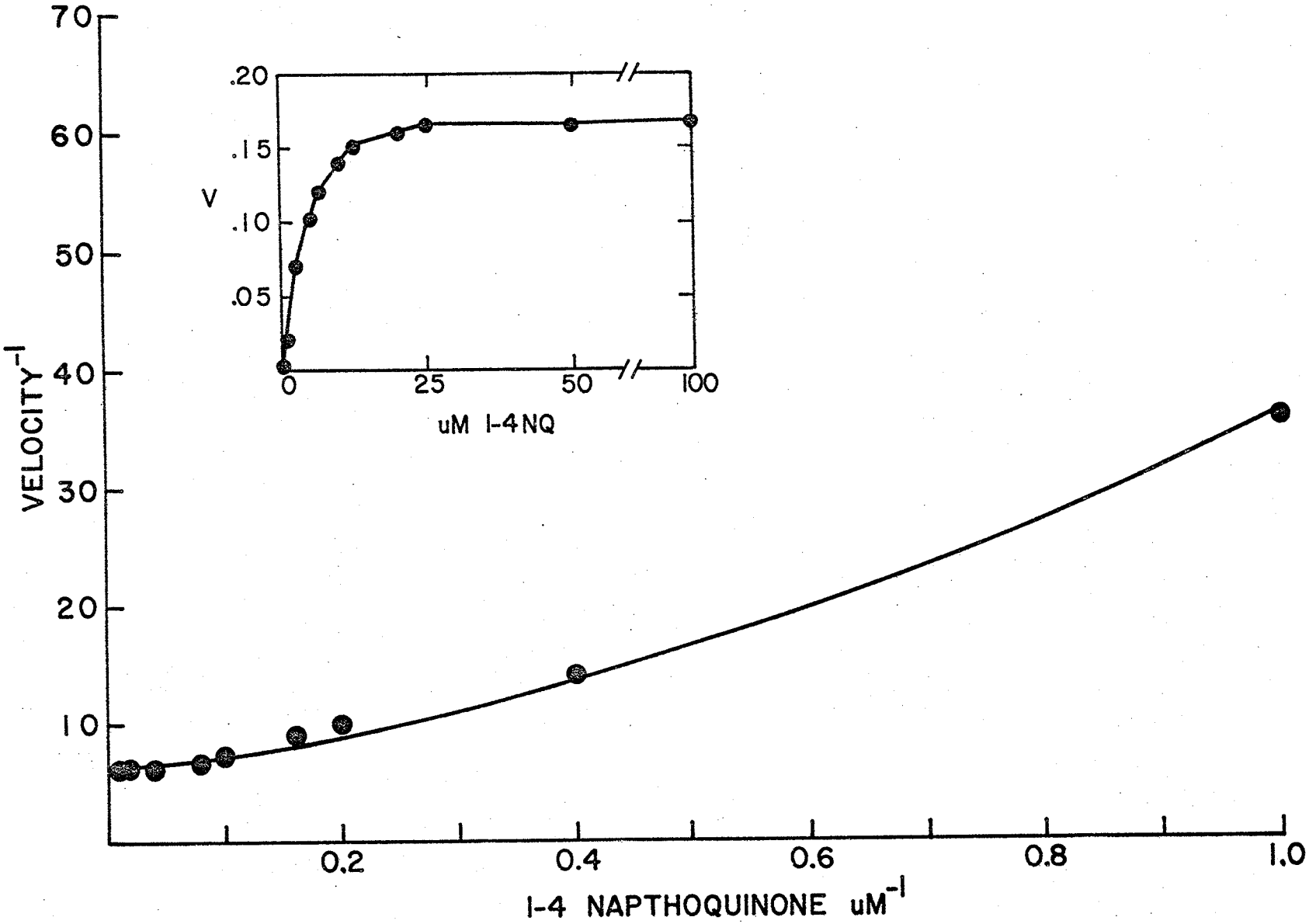
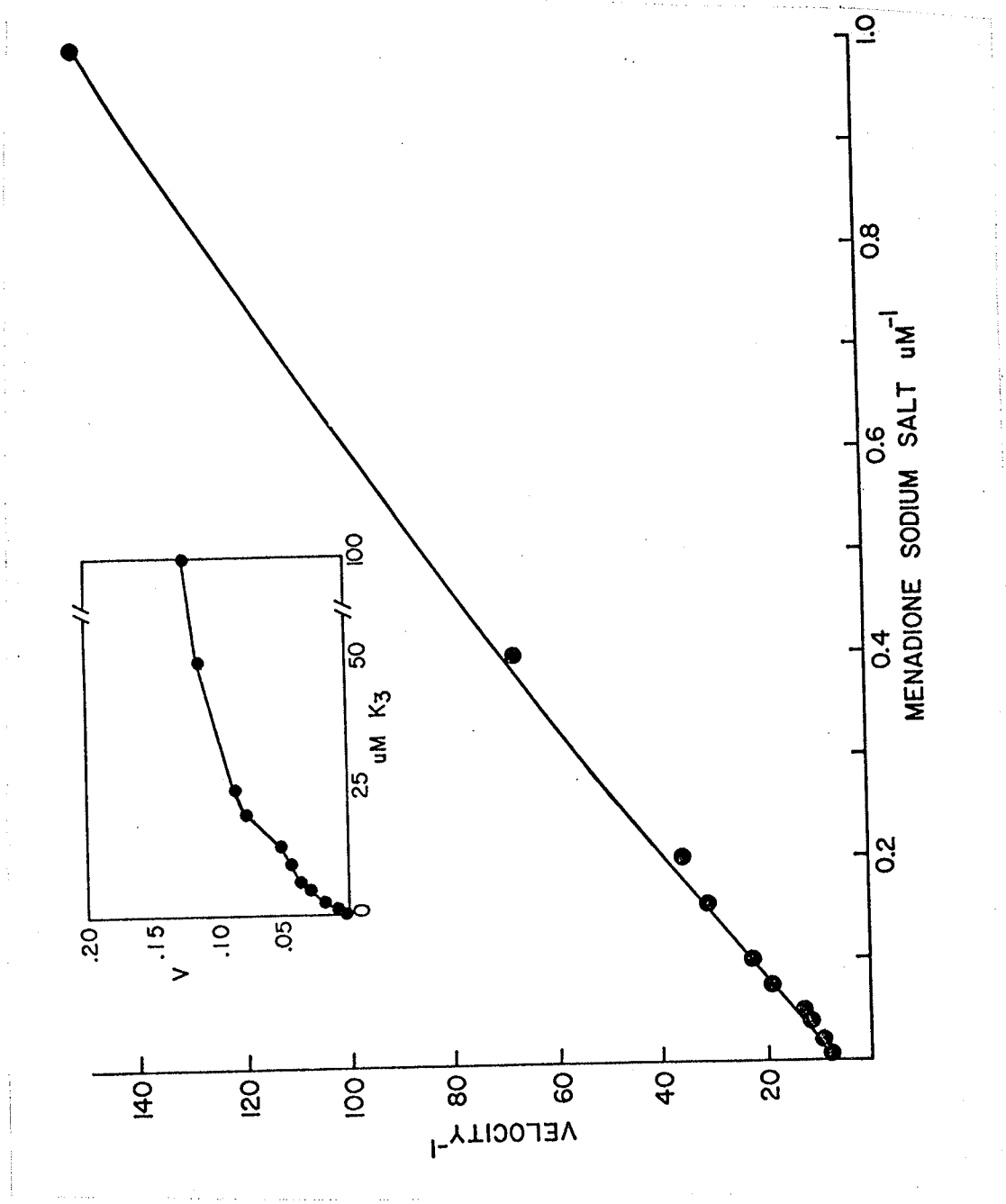

70
60
50
40
30
20
10
VELOCITY$^{-1}$
.20
.15
.10
.05
V
0
25
50
100
uM 1-4NQ
0.2
0.4
0.6
0.8
1.0
1-4 NAPTHOQUINONE uM$^{-1}$

FIGURE 45

FIGURE 46: Lineweaver Burk plot of reduction of Menadione Bisulfite by P-450 Reductase as measured by NADPH oxidation. Inset shows the velocity vs. concentration data. Menadione bisulfite was added dissolved in 6 ul water per 300 ul reaction. Velocity was measured in nmoles NADPH oxidized/-min/unit reductase. Data shown is an average of 4 experiments, the mean standard error was +/- 4.6%.

FIGURE 46


VELOCITY-1
140
120
100
80
60
40
20
MENADIONE SODIUM SALT uM-1
0.2
0.4
0.6
0.8
1.0
1/V
.20
.15
.10
.05
0
25
50
100
uM K3

weaver Burk plots were due to a contaminating NADPH-dependant reductase. Figure 47 demonstrates the activity vs. enzyme concentration for the reduction of the artificial electron acceptor 2, 6-dichlorophenolindophenol (DCIP). Reduction velocity vs. enzyme concentration yielded a linear plot as did the double reciprocal plot of velocity vs. NADPH concentration (Figure 48). Despite the partially purified state of the reductase enzyme preparation, the linear relationships in Figures 47 and 48 indicate that the activity was relatively pure. The Km of reductase for NADPH as determined from Figure 48 was approximately 10 uM. A Lineweaver Burk plot of velocity vs. DCIP concentration (Figure 49) indicated a Km for DCIP of 55 uM.

The reduction of menadione by the single electron donator P-450 reductase results in the formation of an unstable semiquinone. This reactive form of menadione rapidly reoxidizes while reducing oxygen to form a superoxide anion. The possibility was tested that the curvilinear reduction kinetics were due to an inhibition of the reductase at high K3 concentrations by superoxide. The reduction kinetics were repeated in the presence of superoxide dismutase. The results of these experiments are shown in Figure 50. Superoxide dismutase did not correct the non-linearity signifigantly, however, the Km for menadione was increased from 3.75 to 7.5 uM. These results

FIGURE 47: Velocity vs. enzyme concentration for 2,6-Dichlorophenolindophenol reduction by Cytochrome P-450 Reductase. Data shown is an average of 3 experiments, the mean standard error was +/- 1.3%.

FIGURE 47

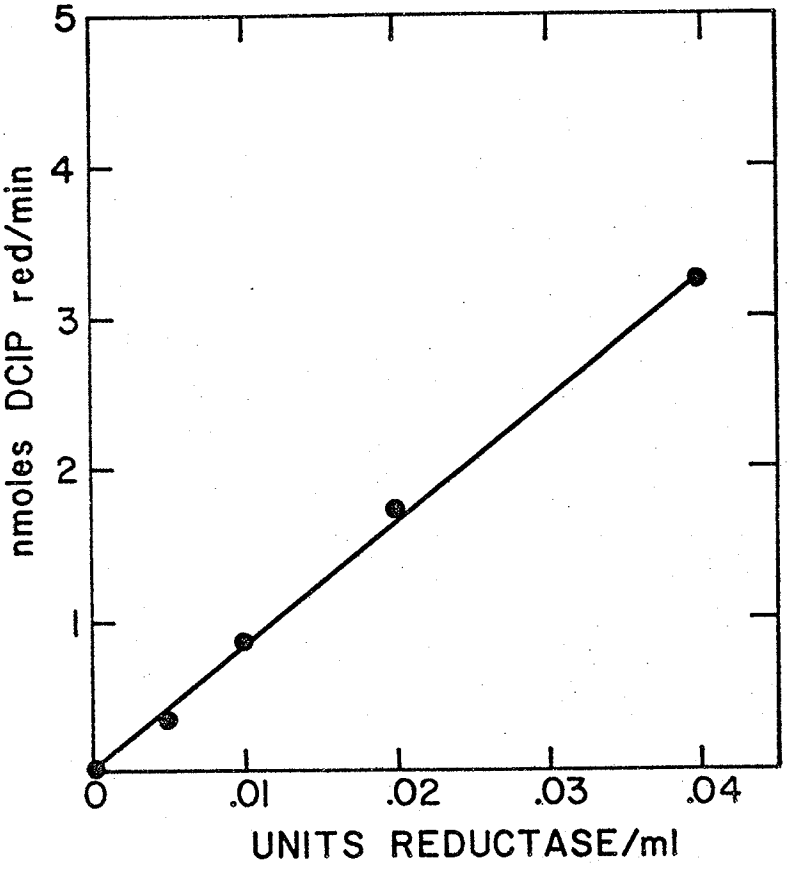


FIGURE 48: Lineweaver Burk plot of velocity vs. NADPH concentration for reduction of DCIP BY Cytochrome P-450 Reductase. Velocity was measured in nmoles DCIP reduced/-min/unit reductase. Data shown is an average of 3 experiments, the mean standard error was +/- 5.0%.

FIGURE 48

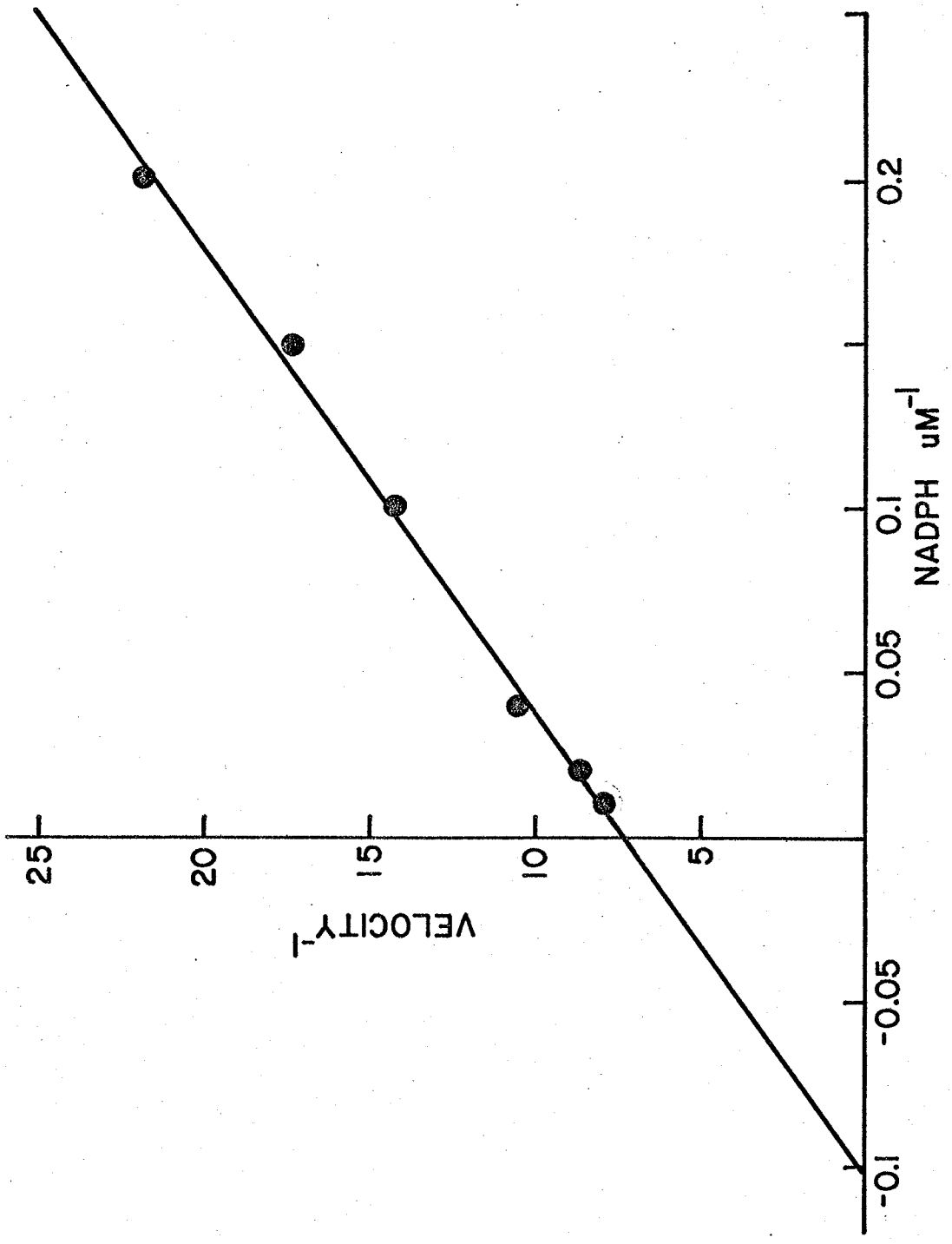


FIGURE 49: Lineweaver Burk plot of velocity vs concentration of DCIP for reduction by Cytochrome P-450. Velocity was measured in nmoles DCIP reduced/min/unit reductase. Data shown is an average of 3 experiments, the mean standard error was +/- 3.0%.

FIGURE 49

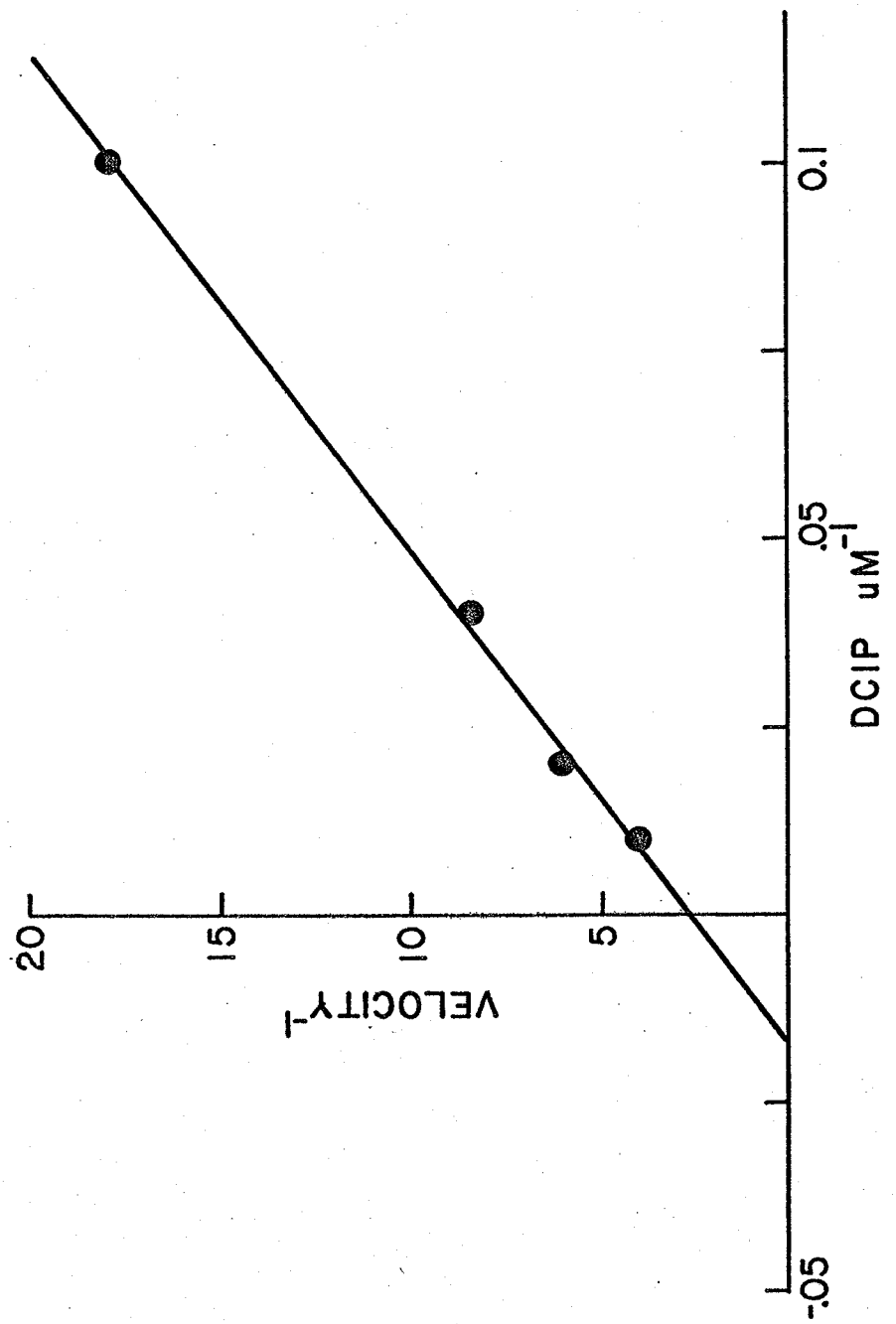


FIGURE 50: Effect of superoxide dismutase on reduction kinetics of menadione by P-450 reductase. Linweaver Burk plots of velocity vs. concentration for reduction of menadione by cytochrome P-450 reductase in the presence ( ■ ) and absence ( ● ) of 182 units/ml superoxide dismutase. Inset shows velocity vs. menadione concentration data. Velocity was measured in nmoles NADPH oxidized/min/unit reductase. Data shown is an average of 2 experiments, the mean standard error was +/- 2.7%.

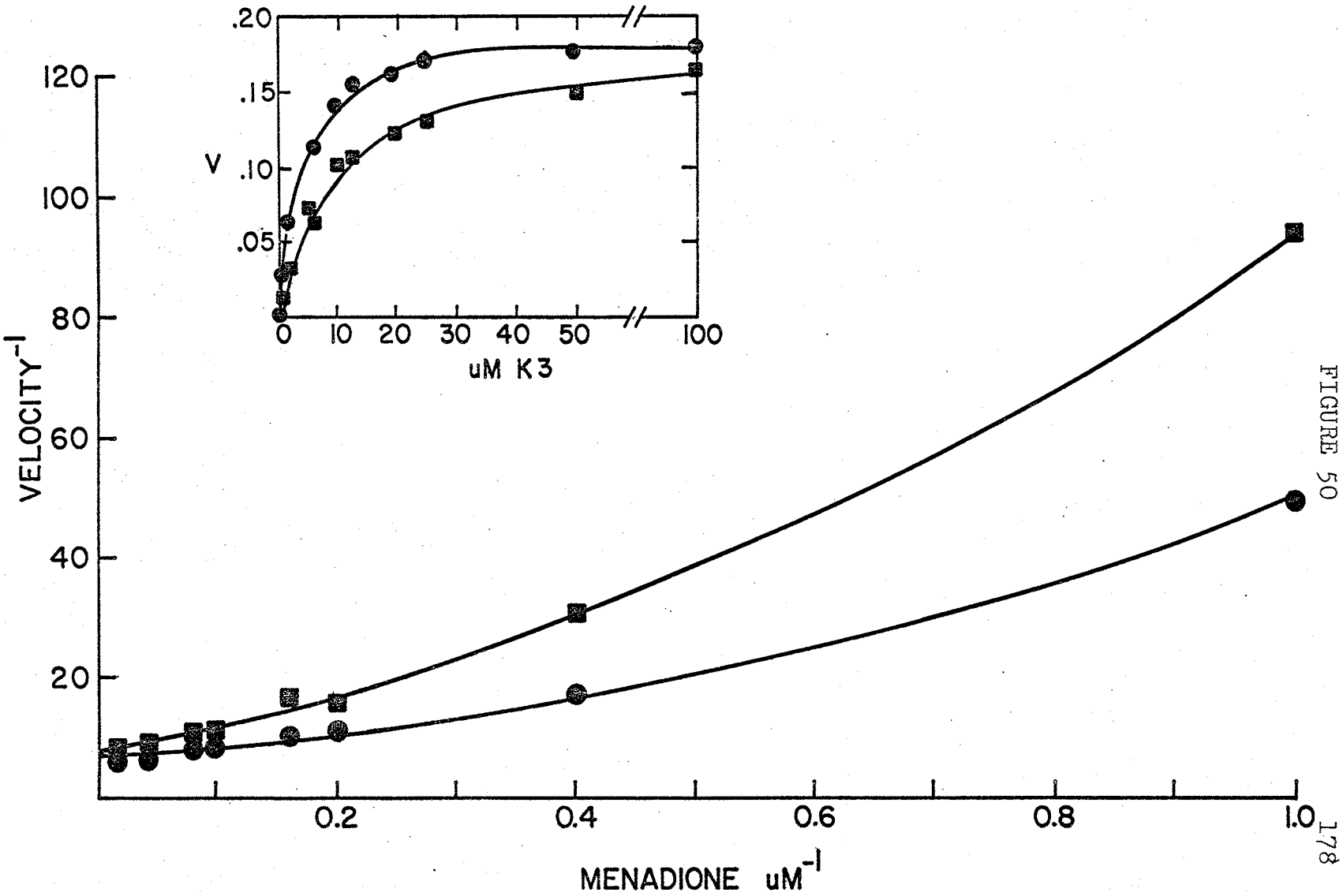

.20
.15
V .10
.05
0 10 20 30 40 50 100
uM K3
120
100
80
60
40
20
VELOCITY$^{-1}$
0.2
0.4
0.6
0.8
1.0
MENADIONE uM$^{-1}$

FIGURE 50

suggest that the reactive oxygen species was probably not responsible for the non-linear reduction kinetics. Quinone reduction by NADPH-dependant cytochrome P-450 reductase has been shown to be dependant upon ionic strength (Phillips and Langdon, 1962). A hypothesis was tested that the curvilinear lines generated by the Line-weaver Burk plots of quinone reduction in these experiments was due to low ionic strength of the reaction media. Figure 51 shows the effect of 0.3 M KCl on reduction kinetics of menadione. The double receiprocal plot at high ionic strength was linear, and the Km for menadione was higher (6.25) than at the low ionic strength.

Table 4 compares the Kms for reduction by P-450 reductase with the estimated Kis of the electron acceptors in the total reconstituted system. For all three menadione analogs, the Ki in the MFO system was very close to the Km for reduction by the reductase enzyme. The maximum reaction velocity for all three menadione analogs were similar at approximately 170 nmoles reduced/min/unit reductase. The maximum velocity of DCIP reduction was higher at 357 nmoles/min/unit.

e) Competitive Nature of Menadione Inhibition

Under saturating benzo(a)pyrene concentrations (100 uM) the ratio of P-448 to reductase was varied to determine the effect of menadione on the affinity of reductase for

FIGURE 51: Effect of ionic strength on reduction kinetics of Menadione by Cytochrome P-450 Reductase. Enzyme acitivity was measured by NADPH oxidation. Velocity was measured in nmoles NADPH oxidized/min/unit reductase. Data shown is an average of 2 experiments, the mean standard error was +/- 3.8%.

FIGURE 51

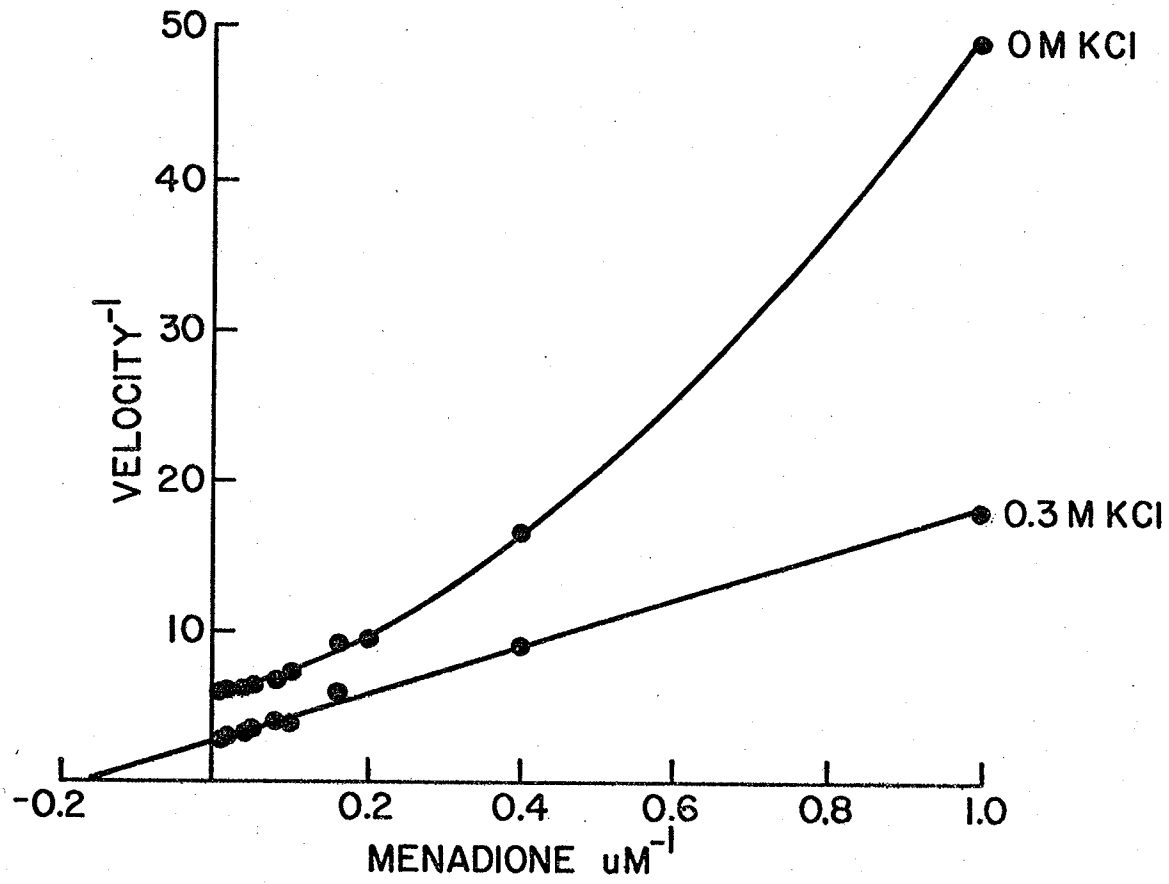

50
40
30
20
10
VELOCITY$^{-1}$
0 M KCl
0.3 M KCl
-0.2
0.2
0.4
0.6
0.8
1.0
MENADIONE uM$^{-1}$

TABLE 4: Kinetic parameters for reduction of various electron acceptors by cytochrome P-450 reductase, and estimated Kis of the quinones in the reconstituted mixed function oxidase system.

| ELECTRON ACCEPTOR | Km (uM) | Vmax [1] (nmoles/min/unit) | Ki in reconstituted MFO (uM) |
|---|---|---|---|
| 2-6 DICHLOROPHENOLINDOPHENOL | 55.2 | 357 | — |
| MENADIONE | 3.75 | 177 | 3.63 |
| 1-4 NAPTHOQUINONE | 3.23 | 166 | 3.70 |
| MENADIONE BISULFITE | 29.4 | 189 | 28.6 |

1. Maximum velocities are measured in nmoles NADPH oxidized/min/unit reductase.

cytochrome P-448. The Km for cytochrome P-448 was determined to be .096 +/- .009 nmoles/ml. Figure 52 demonstrates that menadione is a competitive inhibitor of P-448 reduction as measured by BP metabolism. The Ki for menadione inhibition from these experiments was determined to be 4.97 +/- 1.56 uM. This value is in accordance with the affinity of reductase for menadione (Figure 44) and the Ki as estimated by inhibition in the reconstituted system using a single concentration of P-448 (Figure 41).

f) Effect of Phylloquinone on the Reconstituted MFO System

K1 induced an activation of BP metabolism by the mixed function oxidase system. These results were more consistant than the whole cell data or microsomal data, but the level of activation varied from experiment to experiment. On average at 25 uM the MFO system was activated to approximately 130% of the control (Figure 53). This activation was statistically signifigant (P 0.05). Vitamin K1 did not accept electrons from NADPH-dependant cytochrome P-450 reductase under the conditions of our experiments. Phylloquinone was found to be an inhibitor of NADPH oxidation by reductase in the presence of an artificial electron acceptor. Figure 54 shows that at 100 uM K1, NADPH oxidation was inhibited to 83% and to 50% by 200 uM.

FIGURE 52: Inhibition of Cytochrome P-450 activity by Menadione. Concentrations of Menadione were; ( ● ) 0 uM, ( ▲ ) 2.5 uM, ( ■ ) 5 uM, and ( ◆ ) 10 uM. Cytochrome P-448 activity was measured in nmoles 3-OH formed/min. Data shown is an average of 3 experiments, the mean standard error was +/- 11.2%.

FIGURE 52

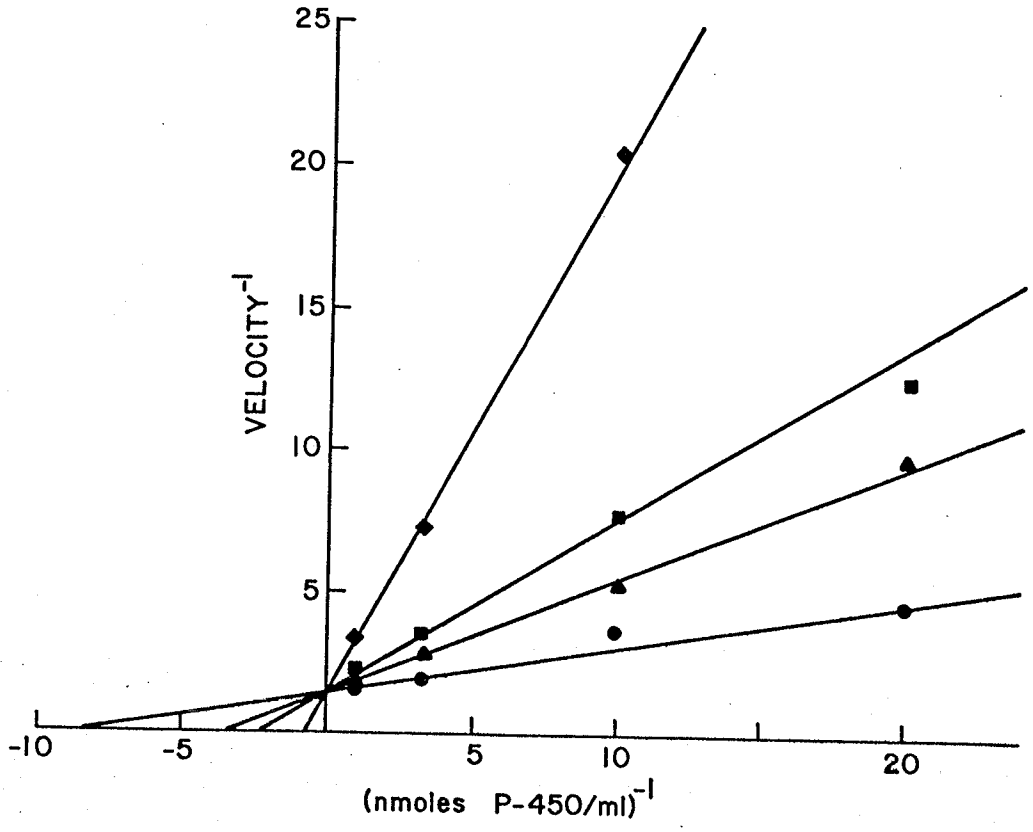


FIGURE 53: Effect of phylloquinone on the reconstituted mixed function oxidase system. Phylloquinone was added dissolved in acetone to the concentrations indicated. Control reactions contained an equivalent amount of acetone. The reactions were preincubated 5 minutes before adding BP. Reactions were allowed to proceed for 10 minutes before stopping, extracting, and measuring BP metabolism. Data shown is an average of 6 experiments. The mean standard error was +/- 10.3%. For all concentrations of K1 the probability that the activities were significantly higher than the control was greater than 90% ($P < 0.10$). At 25 uM K1 the probability that the activity was significantly higher than the control was greater than 95% ($P < 0.05$).

FIGURE 53

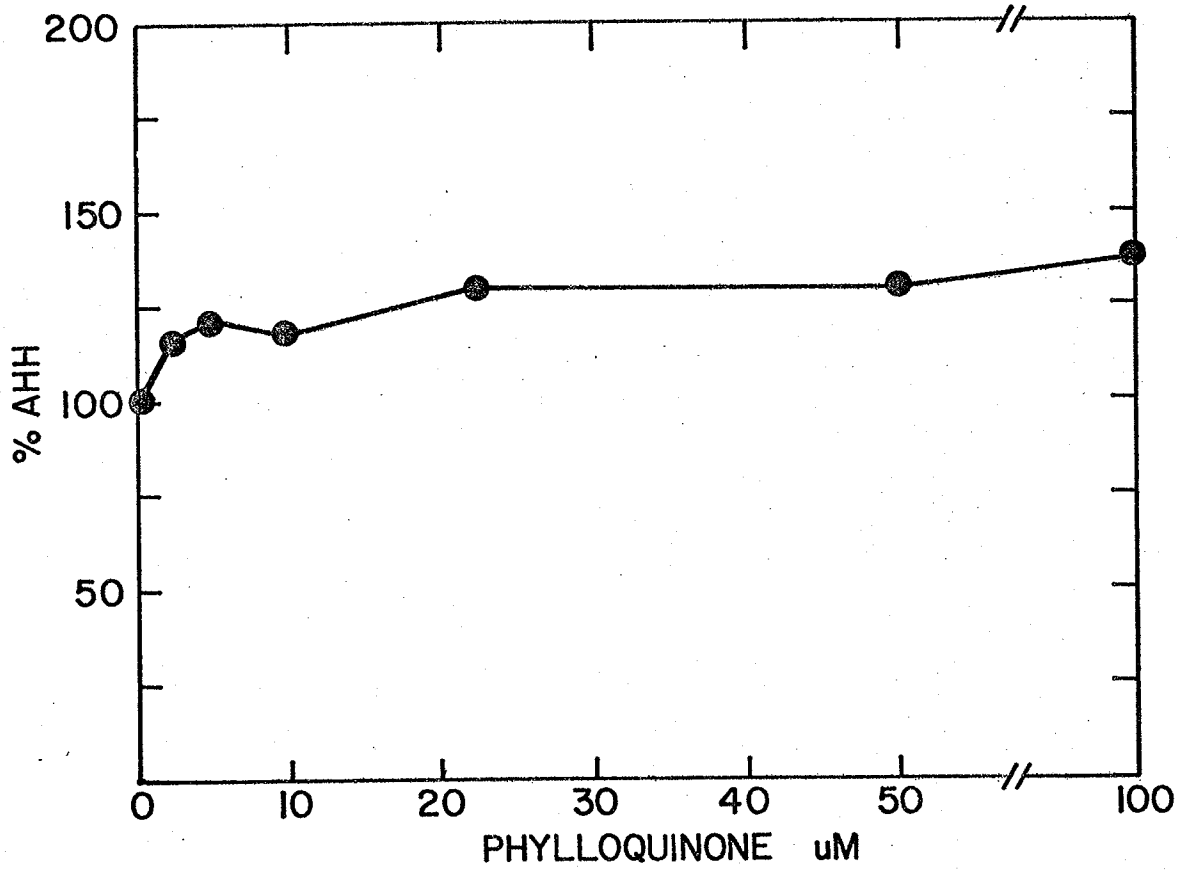
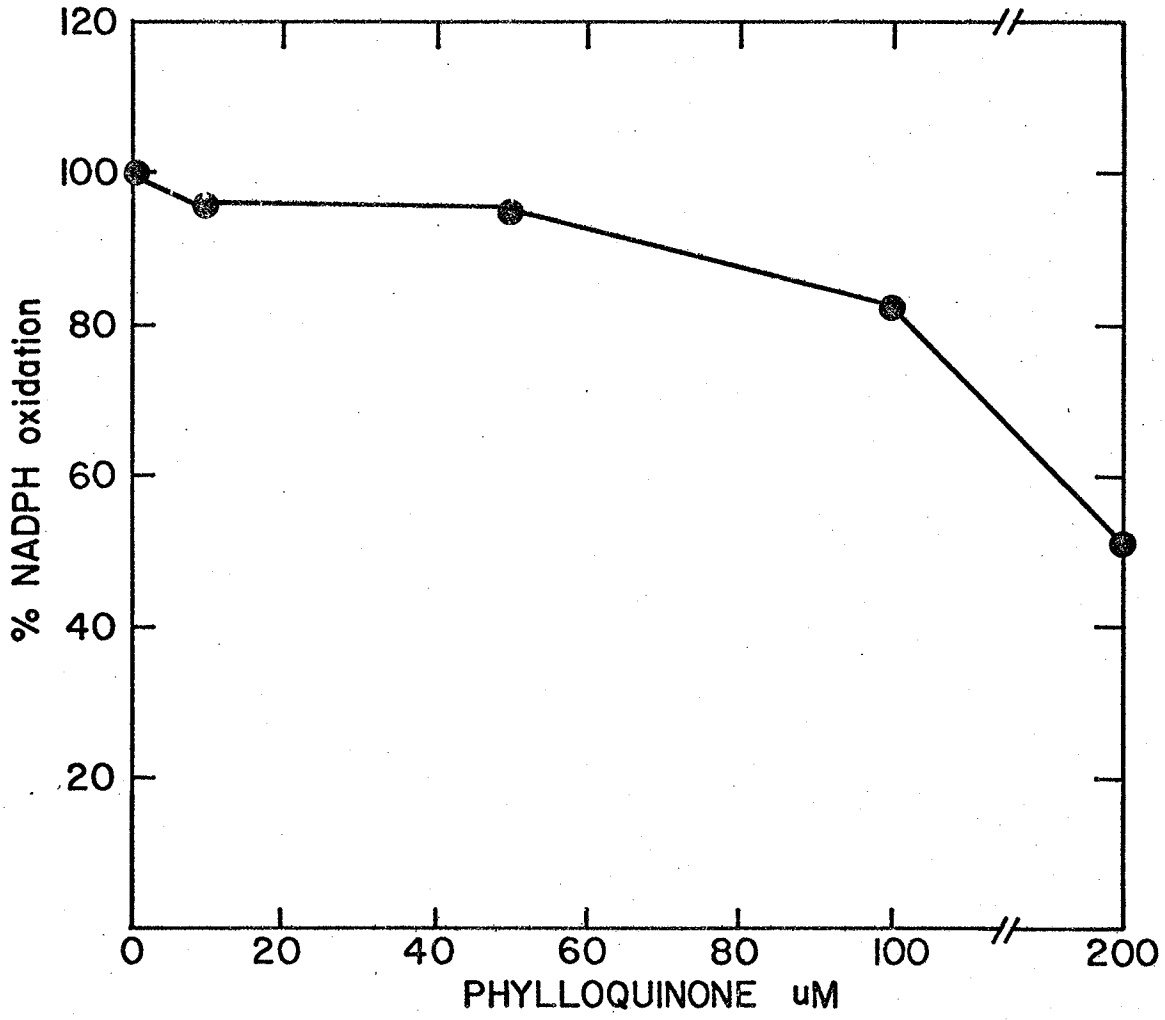


FIGURE 54: Effect of phylloquinone on NADPH oxidation by cytochrome P-450 reductase. Reactions contained 0.4 units/ml reductase, 1 mM $MgCl_2$, 0.1 mM EDTA, 0.01% deoxycholate, 0.2 mM NADPH, 25 uM menadione, and 50 mM Hepes buffer pH 7.4. Phylloquinone was added in 10 ul acetone/ml reaction. Control reactions contained an equivalent amount of acetone. Reactions were initiated by the addition of NADPH. NADPH oxidation was measured at 37°C, by monitoring the change in absorbance at 340 nm. Data shown is an average of 2 experiments, the mean standard error was +/- 2.6%.

FIGURE 54

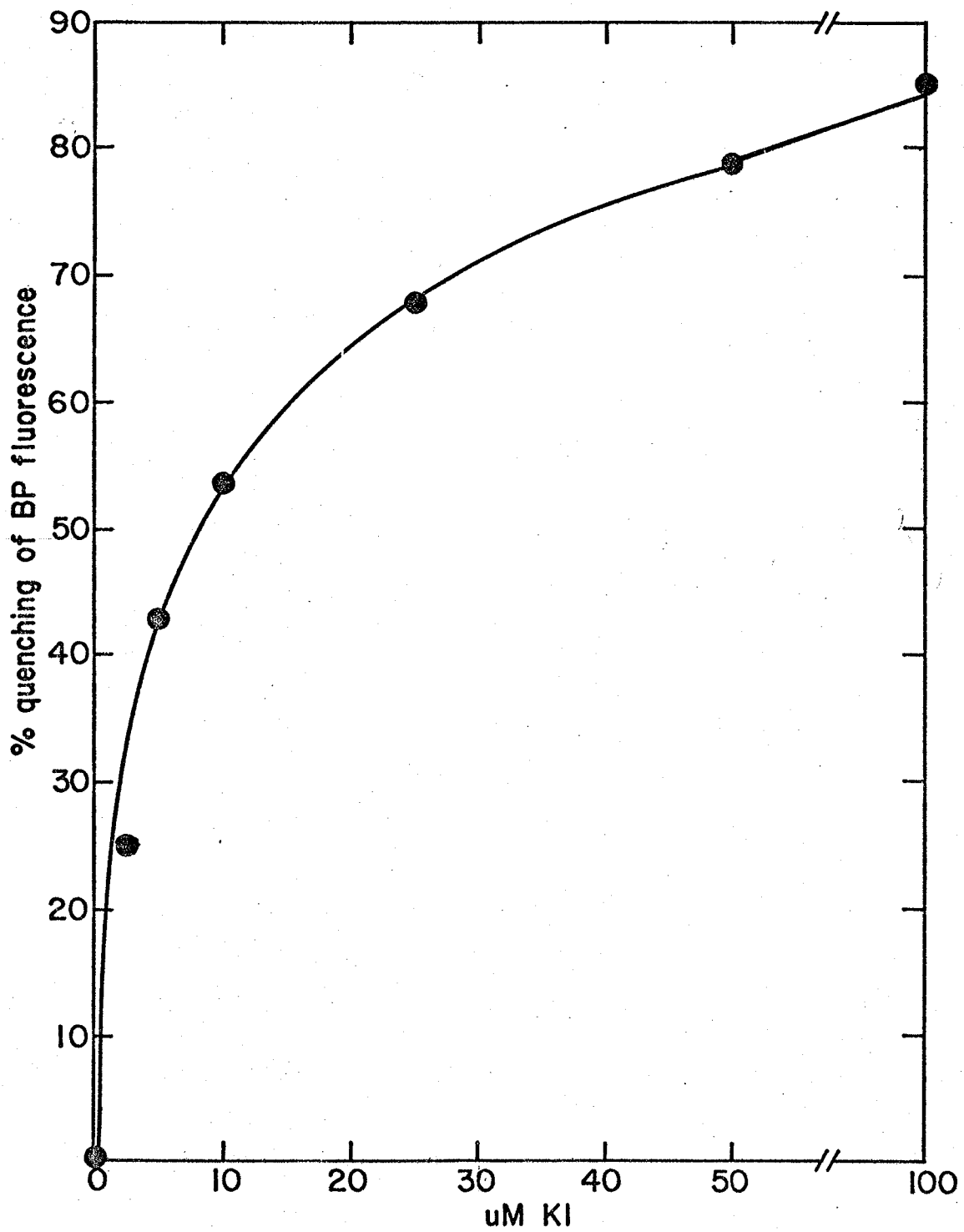

120
100
80
60
40
20
% NADPH oxidation
0
20
40
60
80
100
200
PHYLLOQUINONE uM

Since phylloquinone is known to transport electrons in many bacterial electron chains, an attempt was made to reduce cytochrome P-448 with the reduced form of K1. Reduction of cytochrome P-448 can be measured by monitoring the change in absorbance at 448 nm in the presence of carbon a monoxide atmosphere (Peterson et al, 1977). These experiments did not prove successful however (data not shown). It must be noted that only the introduction of the first electron could be measured using the technique of measuring binding of carbon monoxide at 448 nm, and not the second electron in the P-450 reaction cycle (Figure 1). Many activators of cytochrome P-450 act by altering the spin state and consequently the absorbance spectra of the enzyme (Cinti, 1978). Experiments were done to determine if K1 was working in this manner. These results were also negative (data not shown). A final hypothesis was tested. K1 could possibly act by increasing the binding potential of the hydrophobic substrate to the P-448 catalytic site. Experiments were done in a similar manner to those shown in Figures 36 and 37. K1 did not alter the binding of benzo(a)pyrene at the heme site on the P-448 molecule, however. Another method which can be used to measure the binding of BP is that of Imai (1982). Polycyclic aromatic hydrocarbons are highly fluorescent in suspension. Specific binding of BP to protein results in a quenching of this

fluorescence. Vitamin K1 was found to produce the quenching of BP fluorescence in the presence of the P-448 preparation. The quenching appeared to be saturable with respect to Vitamin K1 concentration. These results are shown in Figure 55. The Ka for quenching of BP fluorescence, as estimated from a Lineweaver Burk plot, was determined to be 14 uM K1. Vitamin K1 did not quench the fluorescence of BP when the enzyme preparation was absent, nor did it quench BP fluorescence in the presence of the P-450 reductase preparation. These results suggest that K1 could be mediating enhanced binding of BP to a protein present in the cytochrome P-448 preparation.

FIGURE 55: Quenching of fluorescence of benzo(a)pyrene by phylloquinone. Cytochrome P-448 was diluted to 0.03 nmoles/ml in P-450 dialysis buffer (5 mM potassium phosphate, 20% glycerol, 0.1 mM DTT, 0.1 mM EDTA and 0.05% cholate). Benzo(a)pyrene was added in 20 ul acetone/ml P-450 buffer to give a final concentration of 5 uM BP. The fluorescence of the sample was allowed to stabilize at 37°C before measuring. Phylloquinone was then added in 10 ul acetone/ml P-450 buffer to the concentrations indicated. The fluorescence was again allowed to stabilize before measuring. The percent decrease in fluorescence was calculated. Phylloquinone did not decrease the fluorescence of BP in dialysis buffer without P-448, nor did it decrease BP fluorescence in the presence of the P-450 reductase preparation. Data shown is an average of 2 experiments, the mean standard error was +/- 1.8%.

FIGURE 55


90
80
70
60
50
40
30
20
10
% quenching of BP fluorescence
0
10
20
30
40
50
100
uM KI

# DISCUSSION AND CONCLUSIONS

Regulation of Drug Metabolism is an important aspect in determining the role of foreign organic compounds in the mechanism of mutagensis and carcinogenesis. It has long been known that many compounds are not mutagenic or carcinogenic in their parent form, and require "activation" before they become operative. Polycyclic aromatic hydrocarbons are such compounds, of which benzo(a)-pyrene is an experimental prototype (Figure 2). Although the primary function of the drug metabolism system is the excretion of these hydrophobic molecules, the same system mediates their activation to reactive intermediates. For excretion and detoxification to occur, the PAH must be rendered polar by the Phase I mixed function oxidase system and subsequently conjugated by the Phase II enzymes. Phase I metabolism of PAHs generates reactive intermediates that can potentially induce toxic and mutagenic events in the cell. Conjugation is largely a detoxification mechanism for these compounds. In this thesis it is proposed that a fine balance exists between the two phases of drug metabolism and the fate of the parent compound towards detoxification or activation. This discussion generally applies to polycyclic aromatic hydrocarbons, although other compounds that are activated by Phase I and detoxified by

Phase II may also be considered. Shifting the balance in favor of Phase I or Phase II will result in a predominance of activation or detoxification respectively. The K series of vitamins may affect this balance by modulating the activity of the cytochrome P-450 enzyme system. The two analogs, Vitamin K1 and K3, are shown to operate at different sites and to have different effects.

Earlier studies in this laboratory using microsomes, tissue fragments, and whole animals, indicated that the K vitamins modulate the activity of the mixed function oxidase system (Israels et al, 1983). Both K1 and K3 were shown to inhibit MFO activity in microsomes, menadione at very low concentrations, and K1 only at concentrations greater than 100 uM. Whole animal experiments showed that K1 and K3 had opposite effects in enhancing and delaying tumor progression respectively. It was proposed that the quinone vitamins were altering BP metabolism by interfering with the supply of reduced NADPH. Menadione was thought to inhibit by mediating the oxidation of NADPH making it unavailable for mixed function oxidation. Similarily phylloquinone was proposed to be altering the NADPH supply through competing reactions of the K cycle. To dissect these phenomena, both whole cell and subcellular systems were utilized to study BP metabolism and the role of NADPH.

Initial investigations were performed with the H4IIE rat hepatoma cell line. Induction of the aryl hydrocarbon hydroxylase (AHH) enzyme in this cell line was first examined by Benedict et al (1973). The H4IIE line in our laboratory showed very different induction kinetics than these previous data. Maximum activity was attained at 16-20 hours after 3-methylcholanthrene was added to the cells, as assayed by 3-OH production. Unlike Benedict's previous studies with these cells, at 24 hours post induction, replenishment of the inducer did not result in a substantial increase in activity (Figure 9). A marked difference is apparent in the metabolism of BP depending on the method of assay (Figures 9 and 10). Activity as measured by the production of total water soluble products does increase after this medium change (Figure 10). This is not seen in the 3-OH BP assay. Since these assays were performed with intact cells, conjugation of primary metabolites does occur. As the 3-OH assay measures only unconjugated product, and the level of 3-OH produced by the cells remained constant following 16 hours induction, a stability in the amount of conjugation relative to total activity was reached at this point in induction. The increase in total BP metabolism as measured by the radioactive BP assay suggests an increase in conjugated product during this interval. The capacity of liver cells to generate NADPH

is enhanced markedly by induction with 3MC. This enhanced NADPH supply could account for the more rapid growth rate of the cells cultured with the inducer (Figure 11).

The development of an assay for AHH in permeabilized cells allowed for the analysis of the effect of NADPH supply on mixed function oxidation. Permeabilization of the cells caused a marked increase in BP metabolism (Figures 12, 13 and 15). BP metabolism in these cells was linear with respect to both cell density and time (Figures 14, 15 and 16). The permeabilization process probably facilitates a greater access of both NADPH and substrate to the mixed function oxidase system. However, in the presence of 1 mM NADPH the permeabilized cells were able to maintain a maximum rate of BP hydroxylation for at least 35 minutes. Without external NADPH the initial reaction was slightly slower, and deviated from linearity 20-25 minutes after the start of the reaction (Figure 15). In cells of higher passage number the initial reaction rate was not enhanced with NADPH, but without 1 mM NADPH, the high rate of BP metabolism could not be maintained as the reaction stopped after 15 minutes. These data indicate that in the whole cell system, NADPH is an essential rate limiting factor of Phase I metabolism of BP. NADPH has been implicated as being a rate limiting factor in BP metabolism since Nebert and Gelboin first characterized

the AHH enzyme (1968). Studies by Thurman and Kauffman (1977) demonstrated that decreases in metabolism of the mixed function oxidase dependant substrate, p-nitroanisole, in a perfused liver system could not be correlated with product conjugation, decreases in substrate or limiting oxygen. Since microsomes prepared from these livers could maintain a steady rate of p-nitroanisole demethylation for at least 30 minutes in the presence of an NADPH generating system, it was concluded that the rate limiting factor was NADPH (Thurman et al, 1977). Studies by Miller and Whitlock (1983) using murine hepatoma cells showed that metabolism by intact cells was not enhanced by the addition of NADPH to the reaction medium, and reaction rates remained linear for only 10-15 minutes. Sonication of the cells and supplementation with NADPH produced reactions which were linear for at least 20 minutes (Miller and Whitlock, 1983). The data presented here is the first direct evidence that the crucial limiting factor in PAH metabolism is NADPH. The difference between the experiments shown in Figures 15 and 16 with cells of different passage number suggests that the NADPH generating capacity of the cell line had changed over the length of time the cells were maintained in culture. A related observation is that cells of low passage number grow more slowly than do those of higher passage. This too may relate to the energy producing capability of the

cells.

To study the hypothesis that the K vitamins modulate mixed function oxidase activity by altering the supply of reduced dinucleotides, whole intact unpermeabilized cells were used in order to utilize the limiting nature of that essential cofactor. In these experiments, menadione proved to be a potent inhibitor of both phenolic BP production and total BP metabolism (Figure 18). Phylloquinone, however, did not inhibit BP metabolism as was expected. Instead K1 was found to be an activator of mixed function oxidation (Figure 19). K1 is present in minute quantity in liver, certainly not higher than 100 uM (Suttie, 1978), which is the highest concentration at which K1 was used in these experiments. Previous microsomal data indicated that K1 inhibited BP metabolism, but only at concentrations in excess of 100 uM (Israels et al, 1983). Closer analysis of those results show that in fact at low concentrations K1 does activate BP metabolism. Despite the relative inconsistancy of the K1 experiments, it became evident that two factors were operating in the effect of K1 on BP metabolism. One was an inhibitory effect that became evident at higher concentrations, and the second and probably more important effect of activation at low concentrations. To test the possibility that the effect seen with the K vitamins was not due to a metabolic alteration peculiar to the

transformed state of the H4IIE cell line, the same studies were repeated using normal hepatocytes. Again K3 inhibited BP metabolism in these normal cells, and K1 also had the same effect of activating metabolism (Figures 21 and 22). The K1 data from the whole cell experiments tends to negate the possibility that normal K1 metabolism by the K cycling enzymes could inhibit MFO activity by NADPH depletion. Limiting NADPH is a prerequisite for this phenomenon to occur. The earlier data presented (Figures 15 and 16) suggests that this is in fact the situation, however, K1 does not inhibit at low concentrations. Inconsistancy was a problem with the K1 data, but the hepatoma cell line and the normal cell data seem to suggest that the level of induction is important for the activation effect of K1 to occur (Figures 20 and 23). Menadione on the other hand is a very potent inhibitor under limiting NADPH conditions. From the data in Figures 18 and 21 it would appear that K3 could inhibit by altering NADPH supply.

The data in Figure 25 suggest that normal K1 metabolism in the H4IIE cell line is altered. Carboxylation activity was much lower in the hepatoma preparations than in normal liver enzyme preparations. Since the effect of K1 on BP metabolism in normal cells and the hepatoma line is essentially the same, the alteration in BP metabolism caused by phylloquinone is probably independant of

the normal K cycling reaction. Carboxylase is only one of several enzymes involved in the K cycle;it is a crucial step in the process, however. Recycling of reduced Vitamin K to the quinone form will be limited in the absence of normal carboxylase and epoxidase activity (Figure 6). However, other enzymes of the cycle may still be present in normal amounts such as the quinone reductase, this enzyme may be related to the activation effect of K1 on the mixed function oxidase system. This point will be brought up again later in the discussion.

Microsomal BP metabolism is affected in essentially the same way as in the whole cell experiments by the K analogs. Menadione was a better inhibitor of microsomal BP metabolism than in cellular metabolism (Figure 26). Metabolism of BP by microsomes was measured under saturating conditions using an NADPH generating system. The fact that menadione inhibits better in microsomes where NADPH is saturating than in the limiting NADPH conditions of the whole cell experiments shows that a different mechanism is operating in menadione inhibition than simple depletion of NADPH. Menadione enhanced the oxidation of NADPH in the presence of microsomes, this oxidation was mediated by an NADPH-dependant reductase as shown in Figure 28. The data in Figures 28 and 29 indicate that the major enzyme responsible for reduction of menadione by microsomal pre-

parations is NADPH-dependant P-450 reductase. The affinity of microsomal P-450 reductase for menadione as determined by kinetic analysis was found to be 9.5 uM. These data provide insight into the possible mechanism of inhibition of the P-450 dependant activity by K3. Since no correlation between NADPH supply and inhibition capacity of menadione was observed, the inhibition is probably mediated by a more specific effect involving P-450 reductase. Interaction of reductase with menadione could reduce the electron flow to cytochrome P-450.

Vitamin K1 activated BP metabolism in microsomes, as well as in the whole cells, as shown in Figure 27. Other studies done in our laboratory analyzing the products of BP metabolism by microsomes showed that at 25 uM K1 all of the metabolites measurable by HPLC analysis were increased by 50-100%. However, at higher concentrations of Vitamin K1 (200 uM) BP metabolism was inhibited by 10-25% (Israels, personal communication). Unlike menadione, phylloquinone inhibits NADPH oxidation by microsomes (Figure 28). The background oxidation of NADPH is completely inhibited by both 10 and 25 uM K1. As will be shown later K1 inhibits oxidation of NADPH by P-450 reductase during reduction of artificial electron acceptors.

The microsomal drug metabolizing enzymes cytochrome P-450, and NADPH-dependant cytochrome P-450 reductase have

both been purified to apparent homogeneity (Dignam and Strobel, 1977; Strobel and Dignam, 1978; Ryan et al, 1978). The preparations used for this study, although only partially pure were adequate for the experiments undertaken. Following solubilization of the microsomes and treatment with protamine sulfate, the two proteins were separated on a DEAE A-25 column (Figure 31). The column flow through eluted P-448, whereas reductase was eluted at 0.1M KCl. Solubilization of the enzymes during chromatography is important for the resolution of the two proteins, thus the requirement of the non-ionic detergent emulgen 913. Reductase is fairly stable in the presence of the detergent, however P-448 is slowly converted to the inactive form P-420. The 20% glycerol in the purification buffers acts to stabilize the enzymes and reconvert P-420 to P-448 (Yamano and Ichikawa, 1978). Following chromatography the proteins were extracted from the column fractions with calcium phosphate, redissolved in the appropriate buffers and dialyzed (Tables 2 and 3). Further purification of P-448 was achieved with a 42-50% ammonium sulfate fractionation. SDS Polyacrylamide Gel electrophoresis of the enzyme preparations revealed that as many as 5 isozymes of P-450 were present in the P-448 preparation, with molecular weights ranging from approximately 46-55,000. Also there appeared to be a 55,000 nw P-450 contaminant in the reductase pre-

paration (Figures 33 and 34). Fortunately for purposes of this study the isozyme in question did not bind BP (Figure 35). The P-450 contaminant in the reductase preparation is probably a late eluting hemoprotein. Levin et al (1974) reported that during resolution of microsomal enzymes from a DEAE cellulose column, a P-450 eluted shortly before reductase. This particular isozyme was relatively unstable and readily converted to P-420 (Levin et al, 1974). The P-450 contaminant may have been inactivated by the purification process, and therefore did not bind BP, or it could be an uninduced form of the enzyme with little affinity for the substrate. The P-448 preparation also retained a small amount of cytochrome c reductase activity, however, the NADPH-dependant cytochrome P-450 reductase preparation was totally free of P-448 activity. The Ks for BP of the P-450 preparation was determined to be 17.2 uM as analyzed by binding studies. This constant in many cases can be correlated with the apparent Km for the substrate (Backes and Canady, 1981), although there is not absolute agreement. For the following experiments BP was always used at saturating or near saturating conditions (80 uM). Concentrations of substrate much higher than this cannot be used because of insolubility.

Reconstitution of the MFO activity from protein purified to homogeneity requires the addition of dilauryl-

phosphatidylcholine for activity (Coon, 1978). The preparations used in this work are not totally dependant on externally added lipid. The dependance of the reconstituted system on external phosphatidylcholine increases with the relative purity of P-448. Mixed function oxidation of benzo(a)pyrene could be doubled by adding lipid to our purified enzymes (Figure 40). Cytochrome P-450 is dependant upon reductase for activity. Maximum activity of a reconstituted system is achieved when the ratio of P-450 reductase to P-450 is 1:1 (Miwa et al, 1979). However, in microsomal preparations the limiting enzyme component in drug metabolism has been shown to be the reductase (Ullrich et al, 1980; Miwa et al, 1978). Following induction by a PAH the ratio of P-450 to reductase can be as high as 30-100:1 (Nebert et al, 1981). The data in Figure 39 indicate that the P-448 preparation was contaminated with a small amount of reductase activity, as there was a low level of hydroxylation activity with no externally added reductase. The addition of reductase to the reconstituted system increased activity linearly until approximately 0.4 units reductase/ml, when further increases in concentration did not enhance BP hydroxylation activity.

In vivo, quinones are reduced by several reductases. DT-diaphorase is a cytosolic and microsomal NAD(P)H-dependant quinone reductase that reduces menadione and other

quinone molecules to hydroquinones (Lind et al, 1982; Ernster et al, 1962). Microsomal P-450 reductase also reduces many artificial electron acceptors, including Vitamin K3. P-450 reductase is a single electron donor however and reduces quinones to an unstable semiquinone form (Powis and Appel, 1980; Masters et al, 1965). This reactive intermediate rapidly reoxidizes while reducing oxygen to form a superoxide anion (Auclair et al, 1978; Prough and Masters, 1973). The reoxidized form can then be again reduced by reductase. The generation of superoxide has been implicated to be the cause of toxicity and oxidative stress in cells treated with menadione (Thor et al, 1982). Induction of DT-diaphorase by 3-methylcholanthrene has been shown to relieve toxicity by menadione, probably by reduction of the quinone to the fully reduced form (Lind et al, 1982; Schor et al, 1983). Reduction kinetics of the quinones 1, 4-napthoquinone, menadione and menadione bisulfite by P-450 reductase are shown here to be non-linear in Lineweaver Burk plots under conditions suitable for reconstitution of the MFO system (Coon, 1978). This deviation from linearity was not due to an impurity of the NADPH-dependant reductase activity as demonstrated by the linear velocity vs. enzyme concentration (Figure 47) and Lineweaver Burk plot of NADPH dependance for DCIP reduction (Figure 48). A possible explanation

for the non-linear reduction kinetics could be that superoxide anions may be inhibitory to the reductase. High concentrations of the quinone would generate a high proportion of the anion, possibly leading to inhibition of reduction. However, the addition of superoxide dismutase did not have a significant effect upon the curvature of the Lineweaver Burk plot for menadione reduction (Figure 50). Superoxide Dismutase did increase the Km for menadione, however. This suggests that the oxidation of NADPH may partially be mediated by the superoxide anion directly. This could affect the apparent Km for menadione.

Conditions of high ionic strength were found to generate linear plots, (Figure 51). Masters et al (1965) reported data similar to that in Figures 44 and 51 using P-450 reductase isolated from porcine liver, in which menadione reduction kinetics yielded curved double reciprocal plots. They postulated that a contaminant in the menadione preparation inhibited the enzyme at high concentrations. However, the experiments were done at low ionic strength (50 mM potassium phosphate buffer). Other studies on the effect of ionic strength have shown that an increase in the molarity of the reaction buffer results in an increase in the reduction rate of artificial electron acceptors by P-450 reductase (Phillips and Langdon, 1962). There was no stimulatory effect of any particular ion

observed, a non-specific ionic strength effect was acting for all artificial electron acceptors. An ionic strength of 0.8 molar was required for maximum reductase activity in these experiments. Phillips and Langdon suggested two possibilities for the mechanism of increased activity with ionic strength. These are that the binding of NADPH to the enzyme may be enhanced under these conditions, or that a stabilizing effect may be functioning at high salt concentration. The data in Figure 51 indicates that the apparent Km for menadione was increased in conditions of high ionic strength. At 0.3 M KCl the Km was 6.25 uM, compared to a Km of 3.75 uM at lower ionic strength. Nishibayshi-Yamashita and Sato observed a Km for menadione of 5 uM at an ionic strength of 0.34 molar (1970). The curvilinear reduction kinetics of NADPH-dependant cytochrome P-450 reductase at low ionic strength is probably a phenomenon unique to the solubilized enzyme preparation. The kinetics of menadione reduction by microsomal reductase was linear with respect to menadione concentration (Figure 30). The ionic strength in these experiments was only 50 mM. Solubilization and purification of the enzyme may alter the conformation of the enzyme as the Km for menadione in the microsomal data was much higher (9.5 uM) (Figure 30) than in the purified enzyme (3.75 uM) (Figure 44). Increasing ionic strength probably acts by stabilizing

the conformation of the enzyme as the Km and activity increase under these conditions (Figure 51). Menadione bisulfite also displayed non-linear reduction kinetics, however the curvature of the double reciprocal plot was concave downward (Figure 39) rather than concave upward as for menadione (Figure 37) and 1, 4-napthoquinone (Figure 38). The sulfite group on the molecule (Figure 4) renders the compound very water soluble, whereas the water solubility of the other two analogs is limited. Solubility in the reaction medium could account for the difference in reduction kinetics of the analogs. Conditions of high ionic strength were not used for the kinetic experiments with the quinones, so that the data could be compared to the reconstituted BP hydroxylation studies, which were done under conditions as described by Coon (1978). Increasing the salt concentration of the reaction medium induces the conversion of P-448 to the inactive form P-420 (Yamano and Ichikawa, 1978), therefore the ionic strength was kept at 50 mM, to retain maximum activity.

Menadione and other quinones have previously been shown to inhibit mixed function oxidation activities such as BP hydroxylation, demethylation of aminopyrene and p-chloro-N-methylaniline, cortisol metabolism, N-oxidation of dimethylaniline and biphenyl metabolism (Wills, 1972; Kupfer et al, 1968; Hlavica and Kehl, 1977; Stohs and Wu,

1982; Weibkin et al, 1979). Wills suggested that since menadione greatly enhanced the oxidation of NADPH in microsomal suspensions, NADPH would be unavailable for mixed function oxidase activities (Wills, 1972). Weibkin described the menadione effect as an uncoupling of NADPH oxidation by P-450 reductase from mixed function oxidation (Weibkin et al, 1979). Menadione and 1,4-napthoquinone are shown here to be very potent inhibitors of reconstituted BP hydroxylation. 50% inhibition was achieved at a concentration of about 3.5 uM (Figures 41 and 42). The estimated Kis were very similar to the apparent Kms for reduction by P-450 reductase (Table 4). The estimated Ki for menadione bisulfite was much higher at 28.6 uM. This value is close to the apparent Km for its reduction by P-450 reductase. The comparision of the data from the reductase kinetics and the inhibition studies indicates that inhibition by the quinone is related to the affinity for reductase. An interpretation of this data is that the mechanism of inhibition is a competition between the quinone and cytochrome P-450 for electrons from P-450 reductase. To demonstrate the competitive nature of menadione inhibition, the effect of the quinone was determined on the Km of reductase for P-450. This data is shown in Figure 52. Menadione is shown in this data to be a competitive inhibitor of BP metabolism. Replots of 1/Km vs. 1/menadione

concentration generated an average Ki of 4.97 +/- 1.56 uM. This value is similar to the estimated Ki of menadione (Figure 41), and also close to the Km of menadione reduction by P-450 reductase (Figure 44). The apparent affinity constant for reductase and P-450 was estimated to be 96 nM. This value is close to the Km of 80 nM as determined by Dignam and Strobel (1977).

In all of the systems used, menadione was shown to be a good inhibitor of BP metabolism. 50% inhibition was achieved at K3 concentrations of 23 uM in the H4IIE cells, 20 uM in normal hepatocytes, 6 uM in microsomal preparations and 3.5 uM in the purified enzyme preparations. Reduction of menadione inhibition could be caused in cellular systems by impermeability of the membrane to the quinone, and by other oxidoreductases with affinity for menadione. When the P-450 enzyme system is isolated from other cellular enzymes, inhibition by menadione increases. This suggests that in vivo, enzymes such as DT-diaphorase and NADH-dependant cytochrome b5 reductase, which both reduce menadione, will alter the inhibition potential of menadione.

Studies using the enzyme preparations for analysis of the effect of K1 show two opposing effects of phylloquinone. In Figure 53, K1 enhanced BP metabolism, as is seen in microsomes and the whole cells. However, phylloquinone also inhibited NADPH-cytochrome P-450 reductase mediated

NADPH oxidation (Figure 54). Part of the variability in the results with the lipid soluble vitamin may be due to these opposing effects. At high concentrations of K1, reductase inhibition would predominate. At 200 uM K1 reductase was inhibited to 50% of the control. This inhibition could explain the earlier data on the effect of K1 on microsomal BP metabolism (Israel et al, 1983). There is only one other report of a mixed function oxidase reaction being activated by Vitamin K1. In 1972, Wills reported that phylloquinone enhanced microsomal lipid peroxide formation to 138 and 161% of the control at 5 and 50 uM respectively. No indication was given as to the mechanism of this activation, and further reports on this phenomenon do not appear in the literature.

At this point, hypotheses on the mechanism of activation of the MFO system by K1 must remain speculative. Several mechanisms could be proposed. Vitamin K1 may be enhancing BP metabolism by altering the binding potential of BP for the enzyme. A small increase in the relative availability or affinity of BP for cytochrome P-450 would increase the rate of metabolism. Since K1 is a lipid soluble molecule, it may be interacting with the hydrophobic substrate to make it more available to the active site. The data in Figure 55 tends to suggest that K1 enhances the binding of the substrate to a protein. Since

the effect of K1 followed Michaelis-Menton kinetic parameters, it is possible that K1 is acting at a specific site on a protein to enhance BP binding. These studies do not indicate the protein to which BP is binding (Imai, 1980), but to increase metabolism the protein is probably a P-450. Studies were done to determine if K1 alters the binding kinetics of BP to P-450, but no change was observed in the presence of K1. A limitation of such an assay for BP binding to P-450 is that the isozymes in the preparation do not all generate the same type of spectra upon interaction with the substrate. This leaves open the possibility that K1 could interact with only one or two isozymes of P-450. Increased binding of BP to these isozymes in the presence of K1 could be masked by the large spectra generated by the remaining isozymes. This concept of specific interaction of K1 with various isozymes is interesting because it is known that the various forms of P-450 generate different spectra of BP metabolites (Gozukara et al, 1982). Interaction of K1 with a form generating predominately 7, 8 and 9, 10 position oxygenated metabolites could greatly increase the mutagenic potential of the metabolites. Data from the experiments with the H4IIE cells and normal hepatocytes would tend to support this hypothesis. It was observed with these cells that the level of induction was an important aspect in determining if K1 could activate

the mixed function oxidase system. If K1 was acting specifically with a single P-450, then the amount of that isozyme in the microsomes would be important in the action of phylloquinone. During induction, however, more than just an increase in the level and alteration of the types of P-450 in the microsomes occurs. Other enzymes are induced as well which alter NADPH metabolism and conjugation (Conway et al, 1983; Fagan et al, 1983; Reinke et al, 1980). The effects of induction on K1 activation could be due to other inducible factors.

A second hypothesis concerning the effect of K1 on mixed function oxidation is that K1 may be functioning as a lipid much like phosphatidylcholine does in the interaction of P-450 with P-450 reductase. The exact role of phosphatidylcholine in cytochrome P-450 activity is still unknown. Lipids such as dilaurylphosphatidylcholine interact with P-450 to generate an alteration in the absorbance spectra of the enzyme (Chiang and Coon, 1979; Gibson et al, 1980). This suggests that the spin state of the prosthetic heme group is altered in the presence of the lipid. It is generally thought that the lipid acts in a direct physical manner to facilitate the interactions between reductase and P-450 and the conformational changes ensuing during transfer of electrons (Lu et al, 1980; Tsong and Yang, 1978; Ingelman-Sundberg, 1977). Evidence seems to indicate

that K1 probably does not function in the same manner as phosphatidylcholine as K1 did not alter the difference spectra of cytochrome P-450. However, the activity of the enzyme preparations used for these experiments was quite high without adding external lipid. This made it difficult to determine the role K1 could have in functioning in this manner. Experiments were attempted to determine the effect of lipid concentration on K1 activation. K1 did not activate the enzyme system better in the presence of low lipid concentrations. These results would indicate that K1 does not function in the MFO system with a mechanism similar to dilaurylphosphatidylcholine.

A final hypothesis on the mechanism of K1 in mixed function oxidase activity is that the quinone may act as an electron carrier between the two protein components. Phylloquinone functions in some bacteria as an electron carrier in oxidative phosphorylation pathways (Brodie and Watanabe, 1966; Martius, 1954). K1 has been shown to transport electrons from single electron carrier flavoproteins to two electron acceptor hemoporteins (Brodie and Ballantie, 1960). Cytochrome P-450 reductase is such a flavoprotein (Vermillion et al, 1981; Iyanagi, 1980). The possibility exists that K1 transports electrons from P-450 reductase to P-450. This hypothesis would seem plausible as the level of activation by K1 is generally only 20-50%.

Efficient transport of electrons occurs in the absence of K1. If K1 were to facilitate electron transport, the level of activation would not be excessively high. However, the reductase does not reduce K1 (Figure 28), and in fact the opposite effect is observed (Figure 54). K1 inhibited oxidation of NADPH by reductase in both microsomes and the purified enzyme preparation. This inhibitory effect is manifest in the inhibition of MFO activity at high concentrations of K1 (Israels et al, 1983). Furthermore, artificially reduced Vitamin K1 did not reduce cytochrome P-450. It must be noted that sodium dithionite reduced K1 is a hydroquinone form, while reduction by a single electron carrier protein would produce a semiquinone. A semiquinone analog could possibly reduce P-450. The assay for P-450 reduction measures only the introduction of the first electron in the P-450 reaction cycle (Figure 1) (Peterson et al, 1977), therefore the results do not rule out the possibility of K1 semi or hydroquinone being capable of donating this second electron. This step has been shown to be rate limiting in the P-450 reaction cycle (Bjorkhem, 1977).

Because of the inability of NADPH-dependant cytochrome P-450 reductase to reduce K1, it is more likely that another enzyme is involved in this oxidation reduction process. Normal metabolism of Vitamin K1 requires the

cyclic conversion of the fully oxidized form to a reduced form then to an epoxide, and then back to the fully oxidized form (Figure 6) (Sadowski et al, 1976; Jackson and Suttie, 1977). Reduced K1 from the K cycle could in turn reduce cytochrome P-450. The two enzyme systems are closely associated in the microsomal membrane (Wallin and Suttie, 1982), therefore it is possible that K1 could be involved in both pathways. Since a hydroquinone form is required for catalysis of the carboxylase reaction, the enzyme involved in the reduction of the quinone would have to be a two electron carrier (Jackson and Suttie, 1977). The enzyme most likely to catalyze this reaction is DT-diaphorase. Normally in the microsomal membrane cytochrome P-450 reductase is the rate limiting enzyme component in the MFO system, this becomes even more pronounced in an induced situation where there can be as many as 30-100 molecules of P-450 per molecule of reductase (Miwa et al, 1978). Reduced K1 from the K cycle could bypass reductase completely in the metabolism of BP. Even if hydroxyvitamin K reduced the hemoprotein at a slow rate, this would be sufficient to cause an increase in total BP metabolism, certainly to a level 20-50% higher than the control. The whole cell data that suggest that K1 activation is dependant on the induction state of the system (Figures 20 and 23) supports this hypothesis for two reasons. First P-450 reductase would

become more limiting under these conditions (Miwa et al, 1978), and secondly because DT-diaphorase is also induced by 3-methylcholanthrene (Lind and Ernster, 1974). In the semi-purified P-448 preparation many microsomal enzymes are present, of which DT-diaphorase may be one.

In conclusion although the activating effect of K1 is rather small, this could be sufficient to cause an excess amount of activated metabolites accumulating in the cell. A slight enhancement of Phase I metabolism is probably the factor leading to the more rapid rate of tumor progression and tumor death in mice given K1 concurrently with BP (Israels et al, 1983). In contrast the inhibitory effect of menadione results in a protective effect against tumor induction by BP. These two analogs demonstrate the importance of regulation of the two phases of drug metabolism. Menadione shifts the balance in favor of detoxification, while phylloquinone allows activation to dominate. These studies demonstrate that K1 may have a function in the cell independant of it's known role in the synthesis of calcium binding proteins involved in coagulation. This previously unrecognized function of Vitamin K1 may be important in the regulation of cytochrome P-450 activity.

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