STUDIES ON MECHANISMS INVOLVED IN THE INDUCTION OF MICROSOMAL BENZPYRENE HYDROXYLASE FOLLOWING PRETREATMENT WITH 3,4-BENZPYRENE AND AFLATOXIN B_1

by

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INTRODUCTION

The metabolism of compounds not normally present in living tissue is becoming increasingly important. Literally, it is vital to know more about how these compounds become metabolized since these materials represent nearly all drugs, carcinogens, pesticides and other foreign, non-nutrient compounds. Such compounds are now increasingly being called xenobiotics. In the future, we shall become more exposed to such compounds, not less. Furthermore, the interactions which become possible when several such compounds are administered as drugs or otherwise are obviously important.

The duration and the intensity of action of most xenobiotics, and especially the drugs, is largely dependent upon the rate at which they are metabolized in the body by liver microsomal enzymes. Activities of these enzymes can be greatly increased by prior administration of drugs, carcinogens and pesticides. This stimulatory effect of xenobiotics on liver microsomal enzymes is pharmacologically important for it leads to an accelerated biotransformation of drugs in vivo, thus altering the duration and the intensity of drug action in man and animals. The consequence of this altered drug metabolism depends upon the relative pharmacological activity of the drug and its metabolite. When the metabolite is pharmacologically inactive, the duration of action of the drug following increased microsomal enzyme activity is shortened. Conney et al. (1960) found that a high dose of zoxazolamine paralyzed rats for more than 11 hours but after treatment with phenobarbital for four days, the same dose paralyzed rats for only 102 minutes and after treatment with 3,4-benzpyrene 24 hours

before the test, the same dose of zoxazolamine paralyzed the rats for only 17 minutes. Other examples of similar nature are meprobamate and carisoprodal (Conney and Burns, 1960; Kato and Vassanelli, 1962; Kato et al., 1961). Conney and Burns (1960) found that the pretreatment of rats for four days with phenobarbital, barbital, phenylbutazone, orphenadrine or aminopyrine markedly decreased the duration of a paralytic dose of meprobamate or carisoprodal. Control rats were found to be paralyzed for an average of 1365 minutes by meprobamate while phenobarbital treated rats were paralyzed for an average of 175 minutes.

When the metabolite has the same quantitative effect as the parent compound or when the pharmacological effect seen following drug administration is due to that of its metabolite, the enzyme induction caused by prior administration of drugs may intensify the effect of the drug by accelerating the formation of its metabolite in the body. An example of this nature is shown by Tremorine and Guthion (the dimethoxy ester of benzotriazine dithiophosphoric acid). Murphy and DuBois (1958) observed that prior administration with 3-methylcholanthrene, which stimulates the enzymes that metabolize Guthion, increased the lethal effect of Guthion whereas inhibition of these enzymes with SKF-525A (a specific inhibitor of some microsomal enzymes) decreased the lethal effect of Guthion.

Another yet to be employed but pharmacologically important implication of inducers may be to rid the body of substances like insecticides which gain access to the body as contaminants and accumulate in the body fat. DDT given to rats previously fed dieldrin or heptachlor markedly decreased the storage of the latter compounds

in the fat (Street, 1964; Street and Blau, 1966; Street et al., 1966).

Another extremely important application of harmless chemicals which can act as inducers may be to block chemical carcinogenesis due to polycyclic aromatic hydrocarbons found in our environment. Treatment of rats with 3-methylcholanthrene or certain other polycyclic hydrocarbons has been shown to stimulate the metabolism of certain aminoazo dyes and 2-acetylaminofluorene to noncarcinogenic metabolites by liver microsomes (Conney et al., 1956; Cramer et al., 1960). These observations may explain why it has been shown that certain hydrocarbons inhibit the ability of aminoazo dyes and 2-acetylaminofluorene to cause liver cancer, mammary cancer and ear duct cancer (Conney, 1967).

Because of the role the metabolism of drugs and the associated phenomenon of induction play in the field of chemotherapy, the importance of the study of the phenomenon of induction cannot be understated. Taking this into consideration, the work to be included in this dissertation was planned and conducted so as to elucidate the mechanism of induction. Various authors have reported the induction to be solely due to increased microsomal enzyme protein synthesis; yet, there is no available evidence to exclude the possibilities of the modification of the kinetic properties of the microsomal enzymes following administration of foreign compounds, particularly the polycyclic aromatic hydrocarbons. The utilization of enzyme kinetics as the basis of the approach to elucidate the mechanism of induction is the essence of the work to be included in this dissertation.

REVIEW OF LITERATURE

Location of Drug Metabolizing Enzymes

Organs other than liver may show some so-called drug metabolizing activity; however, drug metabolizing enzymes are mostly localized in liver microsomes. This is entirely reasonable since liver is the chief detoxifying organ as well as handling more reactions of biochemical nature than any other organ in the body. For example, Axelrod (1955) while studying enzymatic conversion of codeine to morphine in different species found that liver was the only tissue showing activity of this nature. Similar findings have been reported by Conney et al. (1957), who found that the ability to metabolize 3,4-benzpyrene was restricted to liver in the case of rats. They have reported that the homogenates of spleen, kidney, lung, heart, small intestines and thymus from either untreated rats or those injected 24 hours previously with one milligram of 3,4-benzpyrene showed less ability to metabolize 3,4-benzpyrene as compared to liver preparations from the same animals. The chief purpose of these liver microsomal drug metabolizing enzymes is to detoxicate and preferentially convert foreign compounds, which gain access to the body either accidentally or purposefully, into more polar products, which are in general less toxic than the parent compounds and can be more easily eliminated from the body in urine. Berenblum and Schoental (1943) in their paper on the metabolism of 3,4-benzpyrene have quoted a great number of references to drive home the point that, in general, hydroxy or quinone derivatives of carcinogenic polycyclic aromatic hydrocarbons are far less carcinogenic than the parent compounds. For example, 2- and 3-hydroxy derivatives of methylcholanthrene

were found to be non-carcinogenic. Other reactions catalyzed by liver microsomal enzymes are O- and N-demethylations. Brodie and Axelrod (1950), while testing the quinidine-like action of a number of dialkylaminoethanols, found that their toxicities were related to the presence of methyl group(s). It is also a matter of common knowledge that methyl alcohol is toxic to the optic nerve compared to other alcohols in the homologous series.

A summary of the reactions catalyzed by these drug metabolizing microsomal enzymes of liver is as follows:

1. Aromatic hydroxylation
2. Aliphatic hydroxylation
3. N-hydroxylation
4. O-dealkylation
5. N-dealkylation
6. S-dealkylation
7. Sulfoxidation
8. Dehalogenation
9. Nitro-group reduction
10. Azo-link reduction
11. De-esterification
12. Glucuronidation, etc.

Factors Affecting Drug Metabolizing Enzymes

Activities of drug metabolizing liver microsomal enzymes are influenced by age, species, sex, strain, nutritional status of the animal, stress, and the administration of various xenobiotics.

Age. The liver microsomes of newborn animals have little or no

ability to metabolize drugs such as hexobarbital, aminopyrine, acetophenetidine, acetanilide, l-amphetamine, and chlorpromazine (Jondorf et al., 1959; Fouts and Adamson, 1959). This is confirmed by in vivo studies in which a prolonged duration of action of these drugs is observed. Fouts and Adamson (1959) have reported that both liver homogenates and 9000 x g supernatants from newborn rabbits are essentially unable to catalyze side chain oxidation of hexobarbital, N-dealkylation of pyrimidon, and the deamination of amphetamine, but the ability to do so begins to develop when the animals are two weeks old at which time it is still only 5-37% of the enzyme activity of the adult. The activity in the liver of four week old rabbits was found to be equal to that of the adult in most cases.

Species. Tremendous species differences have been reported in the metabolism of drugs. Phenylbutazone, an antirheumatic drug, is metabolized in man at a very slow rate with a half life averaging about three days. In the monkey, dog, rabbit, rat, guinea pig, and the horse, the drug is metabolized at a very rapid rate. Ethyl biscoumacetate, an anticoagulant, has a half life of one-half to two hours in man, whereas the same drug has a much longer half life in the dog. Similar species differences have been noted with various other drugs (Burns, 1962).

Individual Differences. In addition to species differences, individual differences within species have been noted in the metabolism of drugs. For example, the biological half life of bishydroxycoumarin (dicumarol) in man varies from 7-100 hours depending upon the individual (Burns, 1962).

Sex. The stimulating effect of foreign compounds on drug

metabolizing enzymes is more marked in the adult female rat than in the adult male rat (Conney, 1967). It has been reported that the duration and the intensity of drug action is often greater in the adult female rat than in the adult male rat (Holck, 1949; Holck et al., 1937; Poe et al., 1936). Quin et al. (1958) found that this difference in rats, when measured by the rate of hexobarbitone metabolism, was due to the fact that the enzyme which metabolized this drug was less active in females than in males. At birth, neither the male nor the female had much drug metabolizing enzyme activity and up to the age of four weeks the response of the two sexes was identical but between five and six weeks an increased activity of hexobarbitone metabolizing enzyme in males was manifested by an abrupt and lasting decrease in the action of this drug. Murphy and DuBois (1958) reported similar observations with respect to the enzyme which metabolizes Guthion. Sex hormones have been implicated as the probable cause of such sex differences. For example, testosterone given to female rats increased the activity of liver microsomal systems that metabolize hexobarbitone (Quin et al., 1958). In contrast to sex differences in rats, no such differences were observed for the metabolism of hexobarbitone in guinea pigs, rabbits, mice, and dogs (Holck et al., 1937; Quin et al., 1958).

Strain. Strain differences and even the differences between inbred lines of the same strain have been observed in drug metabolism. Quin et al. (1958) observed pronounced variations in the rate of metabolism of antipyrine in eight inbred strains of rats.

Various authors have reported differences in microsomal enzyme activities caused by nutritional and environmentally imposed stress

factors.

Prior Administration with Xenobiotics. The most significant effect is shown by prior administration with drugs, insecticides, polycyclic aromatic hydrocarbons, and various other foreign compounds. Numerous studies have shown that such pretreatment markedly increases the activities of drug metabolizing enzymes in liver microsomes. The stimulatory effect of foreign compounds on liver microsomal enzyme activity was first observed by Brown et al. (1954), who studied dietary factors that influence the activity of hepatic aminoazo dye N-demethylase. Several studies have shown that the injection of small amounts of polycyclic aromatic hydrocarbons such as 3-methylcholanthrene, 3,4-benzpyrene, and 1,2,5,6-dibenzanthracene into rats increased the activities of liver microsomal enzymes which (1) reduce the azo-linkage and N-demethylate aminoazo dyes (Conney et al., 1956), (2) hydroxylate benzpyrene (Conney et al., 1957) and several other drugs (Conney, et al., 1959), and (3) ring hydroxylate 2-acetylaminofluorene (Cramer et al., 1960). More than 200 drugs, insecticides and other chemicals are known to stimulate the activities of drug-metabolizing enzymes in liver microsomes. The characteristic pharmacological actions of these compounds, however, are diverse and there is no clear relationship between either their pharmacologic action or chemical structure and their ability to induce enzymes. The quantity of the foreign compound necessary to have an appreciable effect on the enzyme activity varies considerably. It is of interest that most of these compounds are lipid soluble at physiological pH.

Examples of some of the foreign compounds which increase

microsomal enzyme activities are shown in Table I.

Organophosphate insecticides are unlike the halogenated hydrocarbons in that they inhibit rather than stimulate the hydroxylation of drugs and steroids by liver microsomes when given chronically (Rosenberg and Coon, 1958; Welch et al., 1967; Welch et al., 1959). Biotransformations of some organo-phosphorous insecticides such as octamethylpyrophosphoramide (OMPA), p-nitrophenyldiethyl thionophosphate (Parathion), ethyl-p-nitrophenyl thionobenzene phosphonate (EPN), malathion, chlorothion, etc., into active or partially active cholinesterase inhibitors are affected by liver. Still other organophosphorous insecticides like the anticholinesterase tetraethyl pyrophosphate (TEPP) are not markedly activated or rapidly inactivated by liver. Rosenberg and Coon (1958) found that malathion and chlorothion produced the greatest prolongation of hexobarbital sleeping time while EPN and OMPA were also active in this respect. Their data suggest that these insecticides compete with hexobarbital for enzyme sites (that oxidize hexobarbital) in the liver microsomes, thereby leading to decreased rate of hexobarbital metabolism, which in turn results in prolonged sleeping time with hexobarbital. TEPP, which is not metabolized by liver to any great extent did not prolong the hexobarbital sleeping time of rats, indicating that competition for liver microsomal enzyme sites was not involved.

Morphology of Hepatic Microsomes

It has been recognized that microsomes isolated from liver by differential centrifugation do not consist of a single morphological entity, but there are at least three distinct components which have

Table I. Xenobiotics Capable of Microsomal Enzyme Induction

	Pharmacological Action	Some of the Drugs Tested
1.	Hypnotics and sedatives	Barbiturates, chloral hydrate, etc.
2.	Anesthetic gases	Nitrous oxide, ether, etc.
3.	CNS Stimulants	Amphetamine, coramine, etc.
4.	Anticonvulsants	Dilantin, etc.
5.	Tranquilizers	Meprobamate, etc.
6.	Antipsychotics	Chlorpromazine, etc.
7.	Hypoglycemic agents and related sulfonamides	Tolbutamide, sulfanilamide, etc.
8.	Anti-inflamatory agents	Phenylbutazone
9.	Muscle relaxants	Zoxazolamine, etc.
10.	Analgesics	Aminopyrine, aspirin, etc.
11.	Antihistiminics	Chlorcyclizine, etc.
12.	Insecticides	Chlordane, DDT, Aldrin, heptachlorepoxide, pyrethrins, etc.
13.	Steroid hormones and related substances	A number of steroids
14.	Carcinogenic polycyclic aromatic hydrocarbons	3-methylcholanthrene, 3,4-benzpyrene, 1,2,5,6-dibenzanthracene

been identified. These are as follows: (1) free ribosomes; (2) rough surfaced vesicles, which are recognized by the presence of electron dense ribosomes attached to the outer surfaces; and (3) smooth surfaced vesicles which are devoid of ribosomes.

Conney et al. (1957) have reported that pre-incubation of microsomes with trypsin destroyed the drug metabolizing activity whereas similar pre-incubation with ribonuclease had no effect on this activity.

Recently, Gillette (1966) discussed his earlier work wherein he demonstrated that incubation of hepatic microsomes with ribonuclease had no effect on drug metabolizing enzymes while treatment of microsomes with deoxycholate, pancreatic lipase, or organic solvents, procedures which solubilize or disrupt microsomal membrane phospholipids, caused inactivation of these enzymes. Thus, it has been concluded that the drug metabolizing enzymes are associated with the microsomal membranes and not with the ribosomes. Now the question arises whether these enzymes are associated with the rough surfaced or smooth surfaced fractions of the endoplasmic-reticulum. This question has been answered by Fouts (1961) who indicated that drug metabolizing enzymes in liver microsomes are concentrated in the smooth surfaced membranes of the endoplasmic-reticulum. He found that TPNH oxidase and drug metabolizing enzyme activities of smooth surfaced microsomes were three to five times greater than the values obtained for rough surfaced microsomes. Recent reports from Gram and Fouts (1968) and Gram et al. (1967) have shown that the degree of concentration of several drug metabolizing enzymes in the smooth membranes of

endoplasmic-reticulum depends upon the animal species studied and the method used for the preparation of smooth membranes. Under all conditions studied the metabolism of several drug substrates was concentrated in the smooth membrane fraction but some drugs were metabolized at the same rate by rough and smooth surfaced membranes. Remmer and Merker (1963) showed that treatment of rabbits with phenobarbital for several days increased drug metabolizing enzymes, protein, and lipid in both the rough surfaced and smooth surfaced endoplasmic-reticulum but the increases were greater in the smooth surfaced fraction. Ernster and Orrenius (1965) confirmed these results. These workers found that (1) TPNH-cytochrome c reductase activity, (2) oxidative demethylase activity, and (3) carbon monoxide binding pigment (P_{450}) were higher in the smooth than in the rough vesicles after daily injections of phenobarbital for two, three, four and five days; however, a different picture was observed during the initial phase of induction. They observed no change for the first three hours following phenobarbital administration. After three hours, enzyme levels began to increase rapidly in the rough vesicles and reached a maximum after six hours. Increases in the smooth fraction began only slowly at six hours and were maintained thereafter. It appears that the enzymes are synthesized first in the rough vesicles, then the ribosomes of these rough vesicles leave to attach themselves to new membranes, thereby transforming those originally rough vesicles into enzyme-rich, smooth vesicles. Gram and Fouts (1968) reported that both NADPH oxidase activity and a cytochrome, referred to as P_{450}, are markedly concentrated in smooth surfaced microsomes. Both of

these activities are related to components of the microsomal electron transport chain which is responsible for the hydroxylation of drug substrates.

Mechanism of Microsomal Hydroxylation

Although TPNH or a TPNH generating system is required for all oxidative, hydroxylating or demethylating reactions catalyzed by liver microsomes, the actual mechanism still awaits elucidation.

The possibility that L-ascorbic acid may be involved in the metabolism of drugs by liver microsomes has been considered by several workers, since vitamin C deficient guinea pigs were found to be more sensitive to phenobarbital and procaine (Richards et al., 1941; Richards, 1947). Axelrod et al. (1954) have shown that the rate of in vivo hydroxylation of either acetanilide or aniline was decreased in vitamin C deficient guinea pigs. They found that the half lives of these compounds in vitamin C deficient animals were greater than the controls. Conney et al. (1961) found that the duration of zoxazolamine paralysis in guinea pigs receiving ascorbic acid supplements was 156 minutes whereas it was 309 minutes in guinea pigs not receiving ascorbic supplements. Drugs, such as 3-methylcholanthrene and phenobarbital, which stimulate ascorbic acid synthesis in the rat, are also potent in stimulating the activities of drug metabobolizing enzymes of liver microsomes (Conney and Burns, 1959; Conney et al., 1961). For example, the duration of action of zoxazolamine in control rats was 730 minutes while in phenobarbital or 3,4-benzpyrene (both ascorbic acid inducers) treated rats it was 102 minutes and 17 minutes, respectively. Several workers (Udenfriend et al., 1954; Brodie et al., 1954; Dalgliesch, 1955) have reported that

an in vitro system consisting of L-ascorbic acid, ethylenediaminetetra-acetic acid (EDTA), ferrous ions, and O_2 catalyzed the hydroxylation of such aromatic compounds as acetanilide, aniline, antipyrine, anthrallic acid, etc., to yield products identical with those formed in the body. However, the importance of this model system and the other in vivo observations regarding vitamin C remains to be established, since no vitamin C is needed for the hydroxylation of drug substrates in in vitro systems in the presence of microsomal enzymes, whereas O_2 and TPNH or a TPNH generating system is an essential requirement for such hydroxylations.

In 1949, Calcutt claimed that 3,4-benzpyrene was able to react non-enzymatically with H_2O_2 at 55° C to yield metabolites resembling diols formed during the enzymatic conversion of 3,4-benzpyrene by liver subfractions. Several papers have suggested a perhydroxylation mechanism (i.e., direct involvement of 2(OH) groups of H_2O_2 on the basis of Feiser's work with dibenzanthracene). In our laboratory we substituted low levels of H_2O_2 for the TPNH generating system and found no hydroxylation of 3,4-benzpyrene even with our most active enzyme preparation. Further evidence against the view that the drug oxidative systems of liver microsomes utilize free hydrogen peroxide is the failure of cyanide to appreciably affect the oxidation of hexobarbital or the demethylation of monomethyl-4-aminoantipyrine (Brodie et al., 1958).

In a review paper, Brodie et al. (1958) have described several reactions of an oxidative nature catalyzed by enzyme systems located in liver microsomes. A common requirement of TPNH and O_2 suggests that these reactions are closely related. They have suggested that the

various oxidative pathways in microsomes may all be assumed to be hydroxylation reactions involving the direct substitution of a hydroxyl group for a hydrogen. To explain some microsomal reactions like dealkylations, sulfoxidation, etc., on the basis of this hypothesis, they have suggested the intermediate formation of an unstable hydroxy derivative, as indicated in some of the following reactions:

O-Dealkylation:

$$R\text{-}O\text{-}CH_3 \xrightarrow{[OH]} [R\text{-}O\text{-}CH_2\text{-}OH] \longrightarrow R\text{-}OH + HCHO$$

Unstable

N-Dealkylation:

$$R\text{-}NH\text{-}CH_3 \xrightarrow{[OH]} [R\text{-}NH\text{-}CH_2OH] \longrightarrow R\text{-}NH_2 + HCHO$$

Unstable

Sulfoxide formation:

$$R\text{-}S\text{-}CH_3 \xrightarrow{[OH]} [R\text{-}S\text{-}CH_2OH] \longrightarrow R\text{-}SO\text{-}CH_3$$

Unstable

Hydroxylation of aromatic rings:

$$CH_3\text{-}CO\text{-}NH\text{-}C_6H_5 \xrightarrow{[OH]} CH_3\text{-}CO\text{-}\overset{H}{N}\text{-}C_6H_4\text{-}OH$$

Oxidation of side chains:

$$R\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}CH_3 \xrightarrow{[OH]} R\text{-}CH_2\text{-}CH_2\text{-}CH_2OH\text{-}CH_3$$

Mason (1957) had earlier called enzyme systems catalyzing such oxidative reactions as "mixed function oxidases".

In 1952, C. Strittmatter and Ball discovered a new hemochromogen (later called cytochrome b_5) from liver microsomes. P. Strittmatter and Velic (1956; 1956a) established the existence of cytochrome b_5

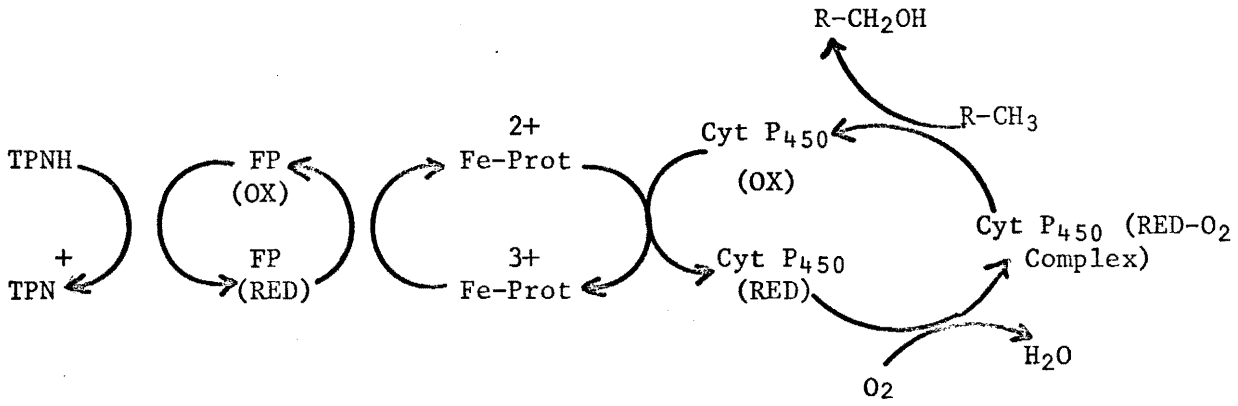
and noted that microsomes contained a flavoprotein (specific for DPNH as a reductant) and also a reductase activity oriented to TPNH as well; the latter seemed to be associated with a different enzyme system. This cytochrome b_5 can reduce cytochrome c and, therefore a system composed of flavoprotein, cytochrome b_5 and cytochrome c can serve as a DPNH-cytochrome c reductase. The significance and the role of this DPNH-cytochrome c reductase system remains obscure at the present time. Earlier, Horecker (1950) had isolated a TPNH-cytochrome c reductase from whole liver acetone powder, but his method did not permit him to ascribe its localization to any particular part of the cell. Phillip and Langdon (1962) and various other groups have described a TPNH oxidase system from microsomes.

In 1958, Klingenberg and Garfinckel separately described the existence of an additional cytochrome (later called P_{450}) in liver microsomes. P_{450} complexes with carbon monoxide showing spectral changes unlike cytochrome b_5.

Omura and Sato (1964) isolated and purified this cytochrome P_{450} and described the location of a soret band of the reduced material at 450 mμ when reacted with carbon monoxide. Later, cytochrome P_{450} was established by Omura et al. (1965) to be an active component required for the hydroxylation of steroids in adrenal cortex tissue. Estabrook's and Omura's laboratories working in collaboration then proceeded to purify the system from adrenal cortex and succeeded in reconstituting a steroid hydroxylation system using well characterized enzymes. The results of their study led to the scheme shown in Figure 1.

Figure 1. Electron transport in adrenal particles.

(In Enzymatic Oxidation of Toxicants, p. 9, Hodgson, 1968)


R-CH2OH
R-CH3
2+
Fe-Prot
Cyt P450
(OX)
TPNH
+
TPN
FP
(OX)
FP
(RED)
3+
Fe-Prot
Cyt P450
(RED)
Cyt P450 (RED-O2
Complex)
H2O
O2

Ernster and Orrenius (1965) and several others showed that the increased microsomal hydroxylation of chemical substrates following pretreatment paralleled increased contents of cytochrome P_{450} and the TPNH-cytochrome c reductase system. There was no increase in the DPNH-cytochrome b_5 reductase system or of cytochrome b_5 by itself. Thus it appeared from this indirect evidence that TPNH, TPNH-cytochrome c reductase and cytochrome P_{450} were indeed involved in the hydroxylation of drugs. One more component of the microsomal electron transport described by Mason (1965) and called Fe_x, which gives a distinctive ERP signal, has been implicated in microsomal hydroxylations. The scheme shown in Figure 2 summarizes the current concept of microsomal electron transport in liver tissue.

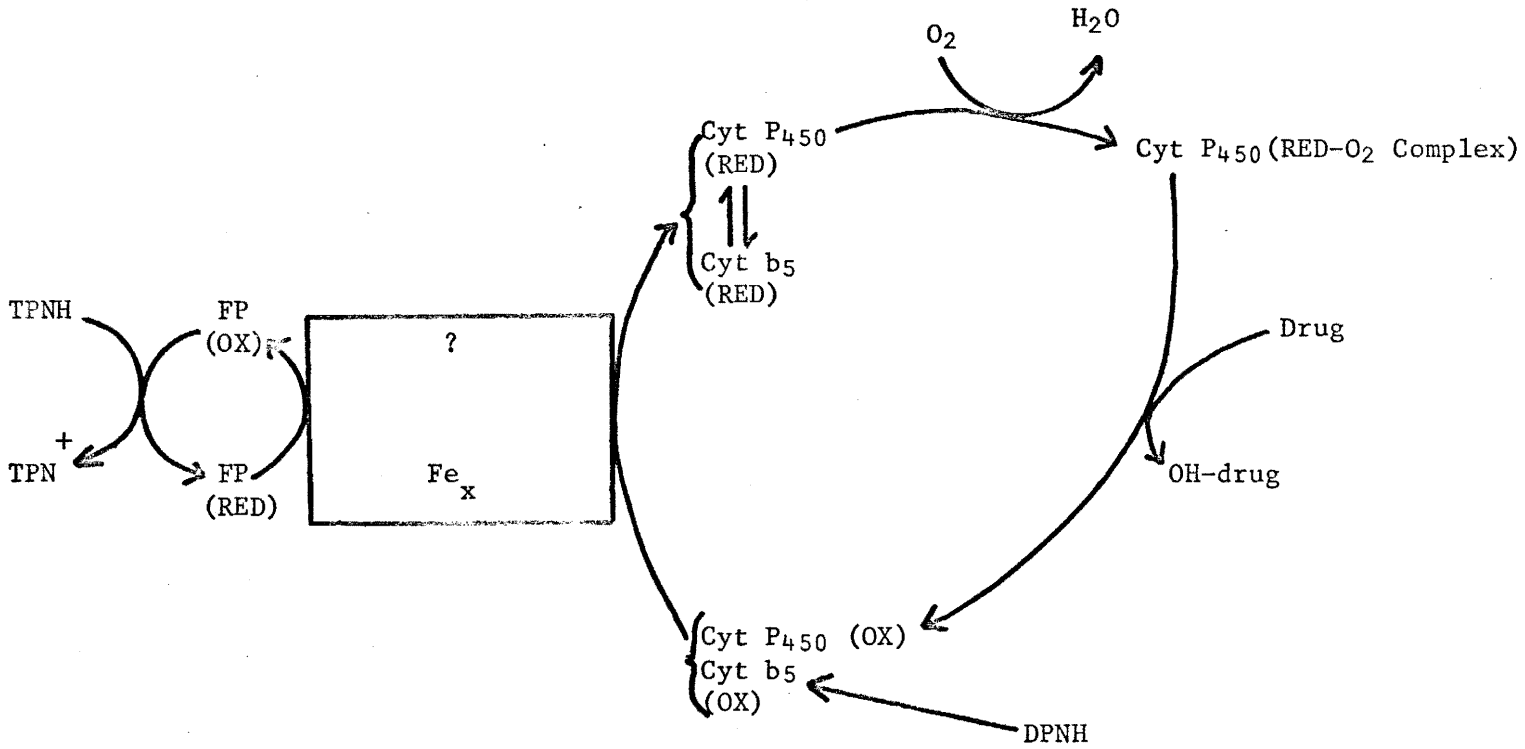
Classification of Inducers

Stimulation of drug metabolizing enzymes by various chemical compounds does not appear to be mediated through a single mechanism. Evidence for the existence of more than one mechanism has been comprehensively reviewed by Conney (1967). In his extensive review of this field he has categorized these compounds into two different classes: (1) inducers that stimulate the metabolism of many drugs, and (2) inducers that stimulate the metabolism of only a few drugs.

Many compounds are like phenobarbital and stimulate the enzymatic conversion of many drugs whereas aromatic polycyclic hydrocarbons typified by 3-methylcholanthrene (3-MC) and 3,4-benzpyrene (BP) stimulate a more limited group of reactions. Conney and Burns (1960) reported that liver microsomes obtained from rats that were pretreated for four days with phenobarbital, barbital, orphenadrine, phenylbutazone,

Figure 2. Electron transport for liver microsomes.

(Taken in part from Enzymatic Oxidation of Toxicants, p. 10, Hodgson, 1968)


O_2
H_2O
Cyt P_{450} (RED)
Cyt P_{450} (RED-O_2 Complex)
Cyt b_5 (RED)
TPNH
FP (OX)
?
Drug
+
TPN
FP (RED)
Fe_x
OH-drug
Cyt P_{450} (OX)
Cyt b_5 (OX)
DPNH

or aminopyrine had an increased ability to metabolize hexobarbital, aminopyrine, phenylbutazone, zoxazolamine, 3-methyl-4-monomethylaminoazobenzene and 3,4-benzpyrene whereas the administration of 3,4-benzpyrene enhanced the metabolism of 3,4-benzpyrene, zoxazolamine, and 3-methyl-4-monomethylaminoazobenzene, but had no effect on the metabolism of hexobarbital, aminopyrine or phenylbutazone.

In addition to this rather arbitrary classification, there are a number of features which are characteristic of polycyclic aromatic hydrocarbons and some of these features help to differentiate them from phenobarbital (PB) type compounds in so far as the induction of drug metabolizing enzymes is concerned. Some of these special features and the differences between these two classes of inducers are listed below.

1. Administration of PB to adult male rats increased the 7-α, 16-α, and 6-β-hydroxylation of testosterone. In contrast, 3-MC treatment stimulated the formation of 7-α-hydroxytestosterone but markedly inhibited the rate of 16-α-hydroxylation and had little or no effect on the formation of 6-β-hydroxytestosterone (Kuntzman et al., 1967).

2. SKF 525-A (2-diethylaminoethyl 2,2-diphenylvalerate), an inhibitor of many drug metabolizing enzymes, inhibits the N-demethylation of 3-methyl-4-methylaminoazobenzene by hepatic microsomes from both control and PB treated rats, but has little effect on the metabolism of this aminoazo dye by microsomes from the rats that have been treated with 3-MC (Sladek and Mannering, 1966 and 1966a).

3. 3-MC induces the formation of a new microsomal cytochrome with spectral properties different from those observed in liver

microsomes obtained from control or PB-treated rats (Alvares et al., 1967; 1968; Kuntzman et al., 1967).

4. The liver microsomes were more active in metabolizing drugs when BP and PB had been given together than when either had been given separately (Gillette, 1963). On the other hand, activity was about the same when the inducers used were BP and 3-MC together or separately. These results were checked under the conditions when the dose of the inducer given was maximal for its characteristic enzyme induction.

5. The two types of inducers also differ in the course and intensity of enzyme induction. On daily administration of PB to rats, maximal increase of enzyme activity is not reached for at least three days. On the other hand, after a single intraperitoneal injection of polycyclic hydrocarbons maximum increases are observed after 24 hours (Conney, 1967).

6. Among more than 57 polycyclic aromatic hydrocarbons studied with N-demethylation of an aminoazo dye as a test system, the optimal molecular size for enzyme induction ranged from 85 to 150 A° and coplanar hydrocarbons were more potent than non-coplanar (Arcos et al., 1961). These requirements suggest the importance of stearic fit between the hydrocarbon and the biological structure with which it interacts.

7. Enzyme induction by these two classes of compounds is also attended by different ultra-structural effects on the hepatocyte (Fouts and Rogers, 1965). PB causes marked proliferation of the smooth endoplasmic-reticulum while 3-MC treatment causes no significant effect on this organelle.

8. PB induction is associated with significant increases in total microsomal protein whereas 3-MC does not affect this parameter very much (Conney and Gilman, 1963).

9. Thioacetamide administration to animals blocks (Sladek and Mannering, 1966, cited by Gram and Fouts in Hodgson, 1968, p. 55) PB induction of microsomal enzymes but has no effect on induction produced by 3-MC.

Kinetic Basis of Induction

Before reviewing the very limited number of references that use kinetic parameters to measure the phenomenon of microsomal enzyme induction following drug administration it becomes obligatory to mention something about the kinetic basis of induction. To do so one cannot escape from mentioning the simple kinetics of an enzyme catalyzed reaction using a steady state assumption.

Considering a unimolecular enzyme catalyzed reaction, we may have:

$$A + E \underset{k_2}{\overset{k_1}{\rightleftharpoons}} X \underset{k_4}{\overset{k_3}{\rightleftharpoons}} P + E$$

Considering only the forward reaction or measuring the reaction rate when the backward reaction does not become important, the above equation reduces to:

$$A + E \underset{k_2}{\overset{k_1}{\rightleftharpoons}} X \xrightarrow{k_3} P + E$$

The rate laws for this equation are:

$$\frac{-dA}{dt} = k_1\ (A)(E) - k_2X$$

$$\frac{dX}{dt} = k_1\ (A)(E) - k_2 + k_3)X$$

$$\frac{dE}{dt} = (k_2 + k_3)X - k_1\ (A)(E)$$

$$v = \frac{dP}{dt} = k_3X \text{ .....(1)}$$

A complete solution of these equations is quite complex and requires a computer but if we assume that the total enzyme is small compared with A, there will be established a steady state situation in which very nearly $dE/dt = \frac{-dX}{dt} = 0$.

Assuming the steady state situation, we now have:

$$\frac{dX}{dt} = k_1\ AE - (k_2 + k_3)X = 0\text{.....(2)}$$

We also know that:

$$E_t = E + X \quad \text{or} \quad E = E_t - X \text{ .....(3)}$$

E_t is the total enzyme, E is the free enzyme and X is the enzyme bound to the substrate. Substituting #3 in #2, we can solve for X:

$$k_1\ (A)\ (E_t - X) - k_2X - k_3X = 0$$

$$k_1\ AE_t - k_1AX - k_2X - k_3X = 0$$

$$k_1\ AE_t - X(k_1A + k_2 + k_3) = 0$$

or $$X = \frac{k_1AE_t}{k_1A + k_2 + k_3} \text{ .....(4)}$$

Substituting #4 into #1

$$v = \frac{dP}{dt} = \frac{k_3k_1AE_t}{k_1A + k_2 + k_3}$$

Dividing both the numerator and the denominator with k_1 we have:

$$v = \frac{(k_3 E_t) A}{\frac{k_2 + k_3 + A}{K_1}} = \frac{VA}{K_m + A}$$

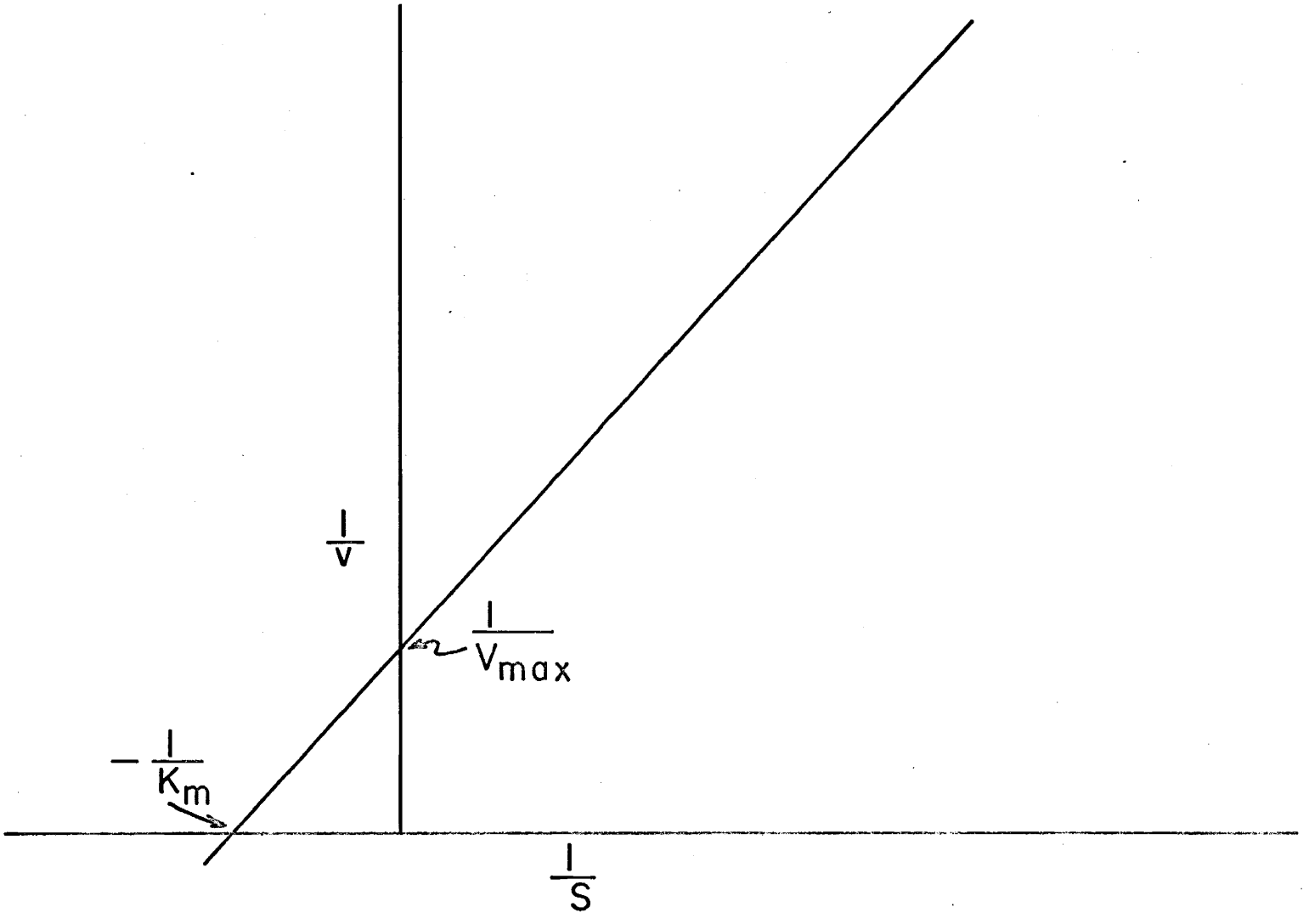
V = maximum velocity and is the function of rate constant(s) and the total enzyme concentration ($V = k_3 E_t$).

K_m = Michaelis Constant. This is the function of rate constants alone ($K_m = \frac{k_2 + k_3}{k_1}$). It is defined as the substrate concentration that gives half the maximum velocity. If substrate A is saturating, i.e., if A is large as compared to K_m, then the above equation reduces to v = V. This means that the velocity of the reaction at saturating substrate concentration is the same as the maximum velocity.

Both V_{max} and K_m are known as kinetic constants. V_{max} ($= k_3 E_t$) is a quantitative measurement of the enzyme whereas K_m ($= \frac{k_2 + k_3}{k_1}$) is a qualitative measurement.

If the velocity of an enzyme catalyzed reaction is measured at different substrate concentrations and the graph of V versus A is plotted, it will be a hyperbola going through the origin; however, if both sides of the equation $v = \frac{VA}{K_m + A}$ are inverted we have $\frac{1}{v} = \frac{K}{V}\frac{(1)}{(A)} + \frac{1}{V}$ and on plotting $\frac{1}{v}$ versus $\frac{1}{A}$ we will have a straight line where $K_{/V}$ is the slope and $1_{/V}$ is the Y intercept. Such a plot is called the Lineweaver-Burke plot and will look like the one shown in Figure 3. From these values of the X and Y intercept, the kinetic constants V_{max} and K_m can be determined and we can get the quantitative and qualitative measurements of the enzyme studied.

Figure 3. Lineweaver-Burke plot for a simple enzyme catalyzed reaction.


1/v
1/Vmax
-1/Km
1/S

However, all biological reactions are not unimolecular. Most of them involve two, three or more reactants and, when a constant amount of enzyme is used in an experiment, and all other substrates but one are held at constant concentration, an enzymatically-catalyzed reaction shows a variation of initial rate with the varied substrate concentration that follows the equation:

$$v = \frac{VA}{K_m + A}$$

The hydroxylation of BP by BP hydroxylase requires $TPNH_2$ and O_2 in addition to BP. In our experiments $TPNH_2$ and O_2 were kept constant and only BP levels were varied. The kinetic constants for reactions involving more than one substrate are also the functions of the rate constants involving the steps that catalyze the reactions for these substrates. Thus, the V_{max} and the K_m values obtained for BP hydroxylase in our study become apparent V_{max} and apparent K_m values. These parameters are also "apparent" because of the absence of enzyme purity.

Conney et al. (1957, 1960), Conney and Gilman (1963), Fujimoto and Plaa (1961), and several others have reported that increased microsomal enzyme activity following drug administration results from a net synthesis of microsomal enzyme protein based upon the observation that such induction is blocked by the administration of protein inhibitors like ethionine, puromycin or actinomycin D. Conney et al. (1957) observed that the intraperitoneal injection of 0.3 mmole of DL-ethionine half an hour before the injection of BP prevented the increase in BP metabolizing activity. However, simultaneous injection of an equimolar amount of DL-methionine blocked the effect of ethionine. Fujimoto and Plaa (1961) observed that urethan and phenobarbital

administration shortened the duration of hexobarbital sleeping time and this response could be blocked if 200 milligrams per kilogram of ethionine was administered simultaneously with urethan or phenobarbital. Orrenius and Ernster (1964), and Ernster and Orrenius (1965), reported that simultaneous administration of actinomycin D or puromycin depressed phenobarbital induced enhancement of the drug hydroxylating activity, TPNH-cytochrome c reductase activity and the concentration of carbon monoxide binding pigment (P_{450}) of liver microsomes.

As far as the induction due to phenobarbital type drugs is concerned, there is some kinetic evidence that the increased activity may be due to higher enzyme protein synthesis and not due to any change in the kinetics of the enzyme studied. Rubin et al. (1964) found that PB administration increased the V_{max} of ethylmorphine N-demethylase, chlorpromazine sulfoxidase, and hexobarbital oxidase but did not influence the K_m of the susceptibility of these enzyme systems to various inhibitors. Netter and Seidel (1964) reported that both PB and BP increased the V_{max} of p-nitroanisole O-demethylase without influencing the K_m. Although such reports could be explained on the premise that induction of a number of microsomal enzyme systems is in fact the result of increased enzyme protein formation, they do not exclude the possibilities of modifications of some enzyme systems specifically involved in the transformation of polycyclic aromatic hydrocarbons. To the best of our knowledge no attention had been given to measure the kinetic properties of these enzymes until recently, and we felt that simple enzyme synthesis appeared to be an oversimplification of the

data presented in previous reports.

PURPOSE OF STUDY

Lack of enough literature on the kinetics of microsomal drug metabolizing enzymes and the apparent inability of the existing reports to explain satisfactorily the mechanism of induction of these enzymes following pretreatment with polycyclic hydrocarbons led us to investigate the kinetic properties of BP-hydroxylase of rat liver microsomes following pretreatment with polycyclic hydrocarbons like BP and aflatoxin B_1 (B_1).

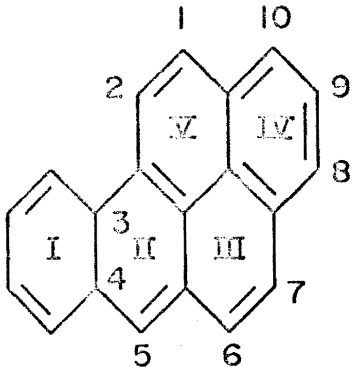
Aflatoxin B_1 and BP alone or in combination were used for pretreatment in our studies for the following reasons:

1. Both are hydroxylated (Conney et al., 1957; Berenblum and Schoental, 1946; de Iongh et al., 1964 and Holzapfel et al., 1966).
2. Both are polycyclic hydrocarbons with the aromatic nucleus.
3. Both are carcinogens, but vary in relative potency.
4. Whereas enzyme inducibility had been previously ascribed to BP (Conney et al., 1957), very little was known about the capacity of aflatoxin B_1 as a microsomal enzyme inducer. Furthermore, B_1 was reported to be a protein inhibitor (Smith, 1963) and the resulting BP enzyme kinetics should reveal information on the B_1 mechanism.

It is pertinent here to mention something about BP and the aflatoxins.

Benzpyrene is a polycyclic aromatic hydrocarbon (Figure 4) capable of inducing skin cancer when applied to the skin of rats. It is hydroxylated at the 8 and 10 positions by liver microsomal enzymes in the body (Berenblum and Schoental, 1946 and Conney et al., 1957). In addition, 5,8-quinone and 5,10-quinone derivatives have been isolated

Figure 4. Structure of 3,4-benzpyrene.

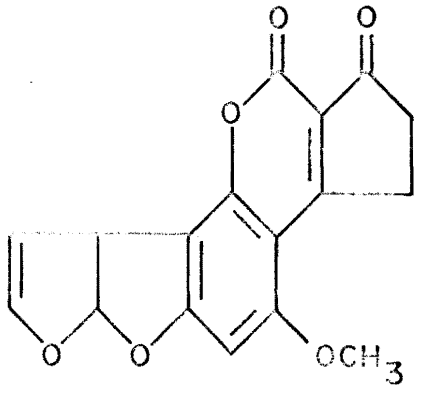


3,4-BENZPYRENE

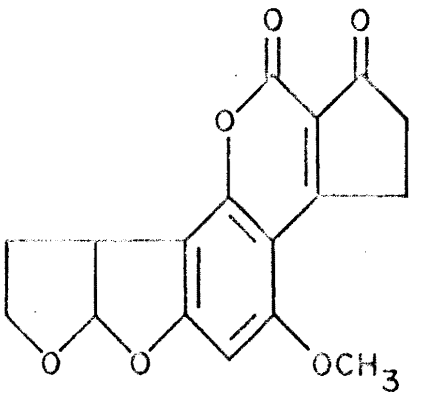
from the feces of treated rats but these metabolites are believed to arise from the non-metabolic oxidation of corresponding hydroxy derivatives of BP, which are sensitive to light.

Aflatoxin (Figure 5) is a name given to a group (four) of metabolites produced by Aspergillus flavus. These toxins as contaminants in imported Brazilian peanut meal were implicated in the death of 100,000 turkey poults in Britain in 1960 (Blount, 1961). Later, this group of compounds was resolved into four components, B_1, B_2, G_1 and G_2. Aflatoxin B_1 is the major metabolite and has been established over the past several years as probably the most potent chemical carcinogen ever known.

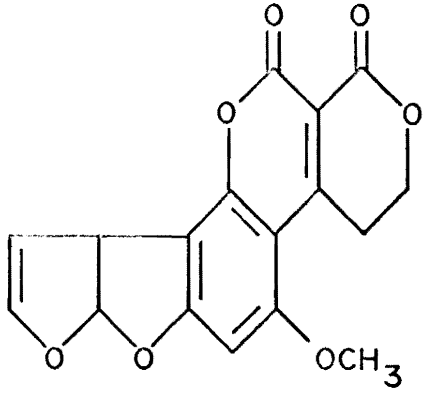
Figure 5. Structures of the four metabolites of aflatoxin.



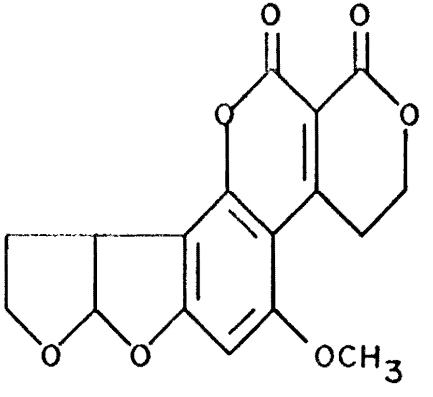
AFLATOXIN B_1



AFLATOXIN B_2



AFLATOXIN G_1



AFLATOXIN G_2

MATERIALS

Rats: Sprague-Dawley derived males (Dublin Rat Laboratories, Dublin, Virginia).

Feed: Wayne* laboratory chow--the commercial rat feed used where indicated. Composition of semipurified diet used in feeding the rats in the second and third series of experiments is given in Table II.

Tritium-labelled Benzpyrene: Stock solution of ^{3}H-BP in ethanol prepared by diluting ^{3}H-BP of specific activity 4 mc/μmole (Nuclear Chicago) to a specific activity of 1000 cpm/mμmole with cold BP. All appropriate dilutions used in the series of incubations were made from this stock solution.

Aflatoxin B_1 (B_1): Isolated and purified according to the method of Hanna and Campbell (1968).

Other Reagents: NADP, glucose-6-phosphate (disodium salt), yeast glucose-6-phosphate dehydrogenase and DL-ethionine (ET) were procured from Sigma. Baker's toluene (reagent grade) was used for making scintillation fluid. Hexane, redistilled in our laboratory, was used to extract unmetabolized BP.

Samples were counted in a Packard Scintillation Counter model 314-A.

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* Wayne Lab Blox for Rats, Mice and Hamsters, Allied Mills, Inc., Chicago, Illinois.

Table II. Composition of Semipurified Diet

Constituent	Percentage Composition
Casein	20.00
Sucrose	69.60
Mazola oil (corn oil)	4.00
Jones Foster Mineral Mix[1]	4.00
Vitamin Mix[2]	2.20
Methionine or Cystine	0.20
	100.00

[1] Jones Foster Mineral Mixture, Nutritional Biochemical Corporation.

[2] Vitamin Diet Fortification Mixture, Nutritional Biochemical Corporation.

METHODS

Three series of experiments were conducted. For the first series of experiments, 52 day old rats were used. Upon arrival the rats were caged individually and fed laboratory chow ad libitum for four days. On the fourth day, weights were recorded and the animals were randomly allocated to three groups with each group comprised of three rats. These rats were then injected intraperitoneally (I.P.) with the quantities of B_1, BP, or the diluent (20% Tween 80 in saline) according to the protocol shown in Table III. All rats received the same volume of diluent or solution (carrying the chemical) on a milligram per kilogram body weight basis. The weights of the rats are given in Table IV. After injection, the animals were decapitated 24 hours later, livers were excised, pooled for each group, and homogenized. Microsomes were isolated according to the procedure discussed later in this section.

For the second series of experiments, 45 day old rats were used. Rats upon arrival were caged individually and fed a 20% (semipurified) casein diet (Table II) ad libitum for 20 days. On the twentieth day, body weights were recorded (Table V) and the rats were distributed at random into ten groups, each group comprised of four rats. These animals were given various pretreatments as indicated in Table VI. All rats received the same volume of diluent (20% Tween 80 in saline) or solution (carrying the chemical) per kilogram of body weight. The rats were decapitated 24 hours following pretreatment, livers were excised, pooled for each group, and homogenized. Again, the microsomes were isolated as will be indicated in greater detail in the later

Table III. Protocol for First Experimental Series

Rats: Sprague-Dawley derived males

Age: 52 days (220-243 gms) old at the time of pretreatment

Group	Dosage, mg/kg (I.P.)
B_1 (1X)	0.67
BP (1X)	10.00
Control[1]	3.00 ml/kg

[1] Control received diluent alone. Diluent used was 20% Tween 80 in saline. Both BP and B_1 were carried in the same diluent and all dosages were equivalent volumes, i.e., 3.0 ml/kg body weight.

Table IV. Weight of Rats Used in First Experimental Series

Group	Weight Range (gms)	Number of Rats
Control	220-242	3
BP-treated	225-243	3
Aflatoxin B_1-treated	222-243	3

Table V. Weight of Rats Used in Second Experimental Series

Group	Weight Range (gms)	Number of Rats
Control	171-178	4
BP(1X)	174-180	4
BP(2X)	177-192	4
BP(3X)	170-179	4
B_1(1X)	171-184	4
B_1(2X)	172-179	4
B_1(3X)	176-183	4
BP(1X)+ET	178-194	4
BP(1X)+B_1(1X)	187-192	4
ET alone	174-188	4

Table VI. Protocol for Second Experimental Series

Rats: Sprague-Dawley derived males.

Age: 45 days (170-194 gms) old at the time of pretreatment.

Group	Dosage, mg/kg (I.P.)		
	BP	B_1	Ethionine (ET)
BP(1X)	10.0	-	-
BP(2X)	20.0	-	-
BP(3X)	30.0	-	-
B_1(1X)	-	0.67	-
B_1(2X)	-	1.34	-
B_1(3X)	-	2.01	-
BP(1X)+B_1(1X)	10.0	0.67	-
BP(1X)+ET	10.0	-	700.00
ET alone	-	-	700.00
Control (Basal diet)	Received diluent[1] alone (6.0 ml/kg)		

[1] 20% Tween 80 in saline. BP, B_1 and ET were all carried in the same diluent and all dosages were equivalent volumes, i.e., 6.0 ml/kg B.W.

part of this section.

For the third series of experiments, 42 day old rats were used. Rats upon arrival were caged individually and fed a 20% (semipurified) casein diet (Table II) ad libitum for ten days except for one group which was fed laboratory chow ad libitum. On the tenth day weights were recorded (Table VII) and the rats were distributed at random into five groups, each group comprised of four rats. They were then given various pretreatments as indicated in Table VIII. All rats received the same volume of diluent per kilogram body weight per 24 hour period. The rats were decapitated at the specified time intervals following pretreatment (Table VIII).

In all the above series of experiments, the procedures from the time of decapitating rats to the time of isolation of microsomes were carried out at 4° C in the cold room, followed up by using ice buckets or refrigerated instruments.

The following procedure modified from that of Silverman and Talalay (1967) was used in isolating microsomes from all groups of rats.

Livers were excised, weighed, chilled, pooled for each group and then homogenized in two to three volumes of cold 0.05 M potassium phosphate buffer containing 0.25 M sucrose (pH 7.4). Homogenates were centrifuged at 20,000 x g for 15 minutes to remove nuclei and mitochondria. The microsomal fractions were collected as pellets of the 105,000 x g centrifugation. The pellets were resuspended in buffer and washed twice with the phosphate buffer. The final washed pellets were then resuspended in the phosphate buffer to give a final concentration of microsomes equivalent to one gram of liver per milliliter.

Table VII. Weight of Rats Used in Third Experimental Series

Group	Weight Range (gms)	Number of Rats
Control (Basal diet)	166-172	4
Lab Chow	169-176	4
BP(3X)	167-169	4
BP(3X)+BP(1X)	155-159	4
BP(3X)+ET[1]	155-160	3

[1] One rat was found dead 36 hours after receiving the initial dose of BP(3X); hence, only three were used.

Table VIII. Protocol for Third Experimental Series

Rats: Sprague-Dawley derived males.

Age: 42 days (155-176 gms) old at the time of pretreatment.

Group	Dosage, mg/kg (I.P.)			Hours[1]
	1st dose	2nd dose		
		BP	ET	
BP(3X)	30.00	-	-	24
BP(3X)+BP(1X)[2]	30.00	10.00	-	48
BP(3X)+ET[2]	30.00	-	700.00	48
Control (Basal diet)	Received[3] diluent alone (6.0 ml/kg B.W.)			24
Lab Chow	Received[3] diluent alone (6.0 ml/kg B.W.)			24

[1] Time (in hours) of decapitation after receiving the initial dose of the chemical.

[2] BP(1X) or ET was given 24 hours after the initial dose of BP(3X) and the animals killed at 48 hours.

[3] 20% Tween 80 in saline. Both BP and ET were carried in the same diluent and all dosages were equal volumes (6.0 ml/kg B.W.) per 24 hour period.

Small volumes of these microsomal suspensions were stored in vials at -20° C for future use. Microsomal protein was estimated by the method of Lowry et al. (1951).

The composition of the 1.5 milliliter incubation mixture which was used to measure the initial velocities of microsomal BP hydroxylase for different substrate (^{3}H-BP) concentrations is given in Table IX. The incubation mixture without ^{3}H-BP and the microsomal protein suspension was equilibrated at 37° C for 30 minutes, then ^{3}H-BP in ethanol was added and four minutes later the reaction was started by the addition of the microsomal enzyme protein suspension. The TPNH generating system (Table IX) was kept constant and the incubations were carried out for appropriate times at atmospheric pressure in a Dubnoff Metabolic Shaker at 37° C. The reaction was stopped with 3.5 milliliters of 0.25 N KOH in 50% ethanol. Unmetabolized ^{3}H-BP was extracted with redistilled hexane and counted according to the method of Silverman and Talalay (1967). All incubations and extractions were carried out in near darkness to avoid photo-degradation of ^{3}H-BP or its hydroxy derivatives.

Initial velocities were recorded for different substrate ^{3}H-BP concentrations. Each concentration was run in quadruplicate in the first series of experiments. In the second and third series, the following procedure was adopted.

Twenty-five and 50 milliliter Erlenmeyer flasks were used for the incubations. Reactions were stopped by pipetting out 1.5 milliliter aliquots into KOH. Duplicate flasks were used for each time interval and two aliquots were removed from each duplicate flask giving four

Table IX. Composition of 1.5 Milliliter Incubation Mix

Glucose-6-P	3.00 mM
NADP	0.48 mM
Glucose-6-P-dehydrogenase	2.00 I.U.
Sucrose	83.50 mM
$K\text{-}PO_4$ Buffer pH 7.4	16.70 mM
^3H-BP in ethanol	0.05 ml
Microsomal protein as suspension	0.20 ml

aliquots for each time interval. The time interval in stopping the reaction between the two aliquots from the same flask was three to five seconds. The velocities were corrected for this time gap only for the one and two minute incubations. A common source of enzyme obtained from the pooled liver microsomes for each group was used in all cases.

RESULTS

Velocity as Function of Enzyme Concentration

In the second and third series of experiments B_1 and BP(1X)+ET pretreatments (Table VI) recorded fairly low velocities as compared to BP (different dosages) and BP(3X)+ET pretreatments (Tables VI and VIII) or the combination of BP+B_1 (Table VI). The latter groups showed very high velocities. In the case of pretreated groups where the velocities of BP disappearance were low, thus difficult to measure, a higher amount of microsomal protein was used for assaying the enzyme activity. These included both control groups (second and third experimental series), laboratory chow group, B_1(1X), B_1(2X), B_1(3X), and BP(1X)+ET groups. A level of 0.59 milligram microsomal protein was used in each of these groups, with the exception of BP(1X)+ET. Velocities remained linear for more than nine minutes at all the substrate concentrations used to assay the enzyme activity although an initial lag at the reported substrate concentrations (Figure 6) was found for the control (second experimental series) microsomes alone. In the case of the BP(1X)+ET group, 0.48 milligram of microsomal protein was used to assay enzyme activity for which the velocity was found to be linear up to four minutes at all substrate concentrations used. For this group, velocities were found to be a linear function of enzyme concentration between 0.48 and 0.59 milligram microsomal protein (Table X). In some of these low velocity groups enzyme assays were run to see if the velocity was a function of enzyme concentration. On account of the measurable velocities being attained at longer periods at lower protein levels, the initial velocities expressed as mμmoles BP disappearance

per milligram microsomal protein per hour are compared (Table XI).

In the case of high velocity groups which include BP(1X), BP(2X), BP(3X), BP(3X)+BP(1X), BP(3X)+ET, and BP(1X)+B_1(1X), it was difficult if not impossible to measure with any fair degree of accuracy the initial velocities using 0.59 milligram microsomal protein. The velocity was so high that it fell even after a one minute interval (Table XII), so it became necessary to use lower levels of microsomal protein. Higher substrate levels were tried in order to increase the linearity beyond one minute intervals but such efforts failed as no increase in velocity was noticed at the higher substrate concentration used. For example, at a substrate concentration of 14.00 μM, the velocity at one minute intervals for 0.59 milligram of BP(2X) microsomal protein was found to be 148 ± 14 in contrast to a value of 152 ± 15 reported in Table XII for 8.00 μM. In order to find out whether the use of low microsomal protein levels for assaying enzyme activity was valid for comparison with the control microsomes, it was decided to study the relationship between the velocity and microsomal protein concentration at a fixed substrate level. The result for such an experiment is shown in Table XIII and Figure 7. Velocities were found to be a linear function of the microsomal protein concentration up to 0.40 milligram microsomal protein, but a fall, although not significant (Table XIV), was observed between 0.40 to 0.59 milligram (0.59 milligram microsomal protein was used for control group). Although such a fall as reported in Table XIV is statistically not significant, in actual practice velocity of microsomal BP hydroxylase is a curvilinear function of microsomal protein. Such an observation

has also been made by Silverman and Talalay (1967), whose data suggest approximately a 40% fall in linearity between 0.3 milligram and 0.6 milligram of microsomal protein although they have recorded linear velocity up to 0.6 milligram of microsomal protein for their microsomal fractions solubilized with detergent. For oxidative demethylation of aminopyrine (another TPNH dependent oxidase system), Ernster and Orrenius (1965) have reported the velocity as the linear function of microsomal enzyme protein concentration up to three milligrams. Now if the slope ($K_{/V}$) of the Lineweaver-Burke plot remains constant when different microsomal protein concentrations (between 0.20 milligram and 0.59 milligram) are used to assay the BP hydroxylase activity, then this trend of decreased velocity with increasing enzyme concentration (between 0.20 and 0.59 milligram) should not invalidate the comparison between the pretreated and the control groups as the subsequent parameters of K_m and V_{max} obtained (when higher microsomal protein, i.e., 0.59 milligram, is used) will become even less than the values which were determined. Such a situation will actually strengthen the conclusions presented herein as well as the mechanism proposed, as will become clear later. In order to ascertain the pretreatment effect on the slope ($K_{/V}$) it was possible to assay the BP(1X)+B_1(1X) group at two microsomal protein concentrations, namely, 0.23 milligram and 0.59 milligram. The results are reported in Table XV and Figure 8. It can be seen that the value for the slope does not significantly change. With the above mentioned considerations in mind, 0.19-0.22 milligram microsomal protein (0.22 milligram for BP(1X) and 0.19 milligram for the rest of the groups) was used for assaying the enzyme

activity in the high velocity groups mentioned earlier. At such levels of microsomal protein, velocities were found to be linear in all groups up to two to three minutes at the substrate concentrations used.

In the first series of experiments (52 day old rats), 0.64-0.70 milligram microsomal protein was used to assay the enzyme activity and the velocities were found to be linear up to a 10-15 minute period in all groups, namely, BP(1X), B_1(1X) and control. All the rats used in the first series of experiments were maintained on laboratory chow before pretreatment with diluent, BP, or B_1 and the significance of this will be indicated later. It is essential to add here that in the study of the relationship between velocity and microsomal enzyme concentration, one should be careful to use the substrate concentration which is not <u>inhibitory</u> at a low enzyme protein concentration and at the same time not <u>limiting</u> to a high enzyme protein level; otherwise, misleading results could be obtained since this enzyme is subjected to "substrate inhibition" as frequently noted during the course of these studies.

Table X. Relationship between Microsomal Protein and Initial Velocity for BP(1X)+ET Group from Second Experimental Series

Group	Protein[1] in mg	Specific Velocity[2] ± S.D.[3]	Substrate Conc.[4]
	0.48	9.03 ± 0.77 (4)[5]	
BP(1X)+ET	0.50	8.43 ± 0.39 (4)[5]	0.40
	0.59	8.07 ± 0.48 (4)[5]	

[1] Microsomal protein in milligrams.

[2] Velocity at 3 minute interval expressed in mμmoles BP disappearance per milligram microsomal protein per hour.

[3] Standard deviation.

[4] Substrate concentration in μM.

[5] Numbers in parentheses are numbers of sample aliquots analyzed.

Table XI. Relationship between Microsomal Protein and Initial Velocity for Certain Groups from Second Experimental Series

Group	Protein[1] in mg	Specific Velocity[2] ± S.D.[3]	Substrate Conc.[4]
	0.26	4.45 ± 0.57 (2)[5]	
Control			2.00
	0.59	4.14 ± 0.58 (4)[5]	
	0.26	2.65 ± 0.35 (2)[5]	
Control			1.00
	0.59	3.37 ± 0.67 (4)[5]	
	0.20	5.17 ± 1.15 (4)[5]	
B_1 (2X)			2.00
	0.59	4.37 ± 1.14 (4)[5]	

[1] Microsomal protein in milligrams.

[2] Initial velocity expressed in mμmoles BP disappearance per milligram microsomal protein per hour.

[3] Standard deviation.

[4] Substrate concentration in μM.

[5] Numbers in parentheses are numbers of sample aliquots analyzed.

Table XII. Specific Velocity vs. Time for BP(2X) Group from Second Experimental Series

Group	Protein[1] in mg	Time in Minutes	Specific Velocity[2] ± S.D.[3]	Substrate Conc.[4]
		0.50	171 ± 25 (3)[5]	
		1.00	152 ± 15 (4)[5]	
BP(2X)	0.59	2.00	110 ± 18 (4)[5]	8.00
		3.00	82.2 ± 7.8 (4)[5]	

[1] Microsomal protein in milligrams.

[2] mμmoles BP disappearance per milligram microsomal protein per hour.

[3] Standard deviation.

[4] Substrate concentration in μM.

[5] Numbers in parentheses are numbers of sample aliquots analyzed.

Table XIII. Relationship between Microsomal Protein and Initial Velocity for Certain Groups from Second Experimental Series

Group	Protein[1] in mg	Total Velocity[2] at 3 min. ± S.D.[4]	Specific Velocity[3] ± S.D.[4]	Substrate Conc.[5]
	0.22	1.74 ± 0.12 (4)[6]	156 ± 11 (4)[6]	
BP(1X)	0.35	2.82 ± 0.09 (4)[6]	161 ± 5 (4)[6]	8.00
	0.40	3.09 ± 0.25 (4)[6]	155 ± 13 (4)[6]	
	0.12	0.85 ± 0.10 (2)[6]	144 ± 16 (2)[6]	
BP(2X)				3.00
	0.20	1.58 ± 0.14 (3)[6]	160 ± 14 (3)[6]	

[1] Microsomal protein in milligrams.

[2] mμmoles BP disappearance in 3 minutes.

[3] mμmoles BP disappearance per milligram microsomal protein per hour.

[4] Standard deviation.

[5] Substrate concentration in μM.

[6] Numbers in parentheses are numbers of sample aliquots analyzed.

Table XIV. Relationship between Initial Velocity and Microsomal Protein for BP(2X) Group from Second Experimental Series (See Table XII also.)

Group	Protein[1] in mg	Specific Velocity[2] ± S.D.[3]	Substrate Conc.[4]
	0.40	198 ± 64 (4)[5]	
BP(2X)			8.00
	0.59	152 ± 15 (4)[5]	

[1] Microsomal protein in milligrams.

[2] Velocity at 1 minute interval expressed as mμmoles BP disappearance per milligram microsomal protein per hour.

[3] Standard deviation.

[4] Substrate concentration in μM.

[5] Numbers in parentheses are numbers of sample aliquots analyzed.

Table XV. Kinetic Parameters of BP(1X)+B_1(1X) Group at Two Different Microsomal Protein Concentrations Used to Measure the Enzyme Kinetics

Parameter	BP(1X)+B_1(1X)	BP(1X)+B_1(1X)
Microsomal Protein[1] (mg)	0.23	0.59
K_m + S.E.[2]	1.25 $\pm$ 0.30	0.82 $\pm$ 0.37
V_{max} $\pm$ S.E.[2]	102.1 $\pm$ 10.3	62.15 $\pm$ 8.80
$K_{/V}$ $\pm$ S.E.[2]	0.0122 $\pm$ 0.0018	0.0132 $\pm$ 0.0042
$\frac{1}{V}$ at A = [3]	0.0139 (A = 3)	0.0205 (A = 3)

[1] Amount of microsomal protein in milligrams used to assay the enzyme activity (Lineweaver-Burke plot).

[2] Standard errors obtained from the computer analysis of the initial velocity data.

[3] Substrate concentration in μM.

Figure 6. Velocity observed in control group from second experimental series. Note the lag which is discussed in the text.

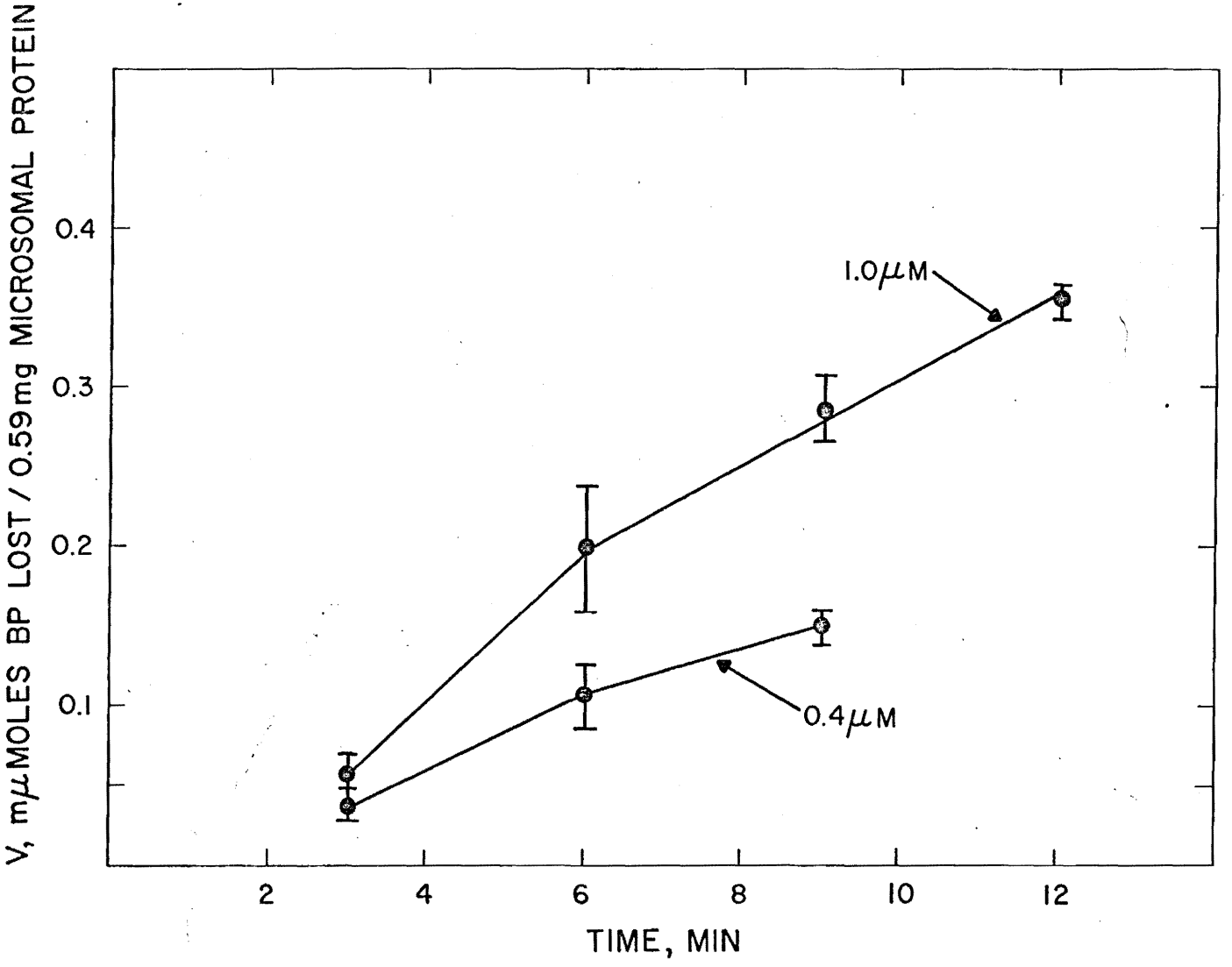

V, mμMOLES BP LOST / 0.59mg MICROSOMAL PROTEIN
0.4
0.3
0.2
0.1
1.0μM
0.4μM
2
4
6
8
10
12
TIME, MIN

Figure 7. Relationship between the velocity and microsomal protein as reported in Table XIII.

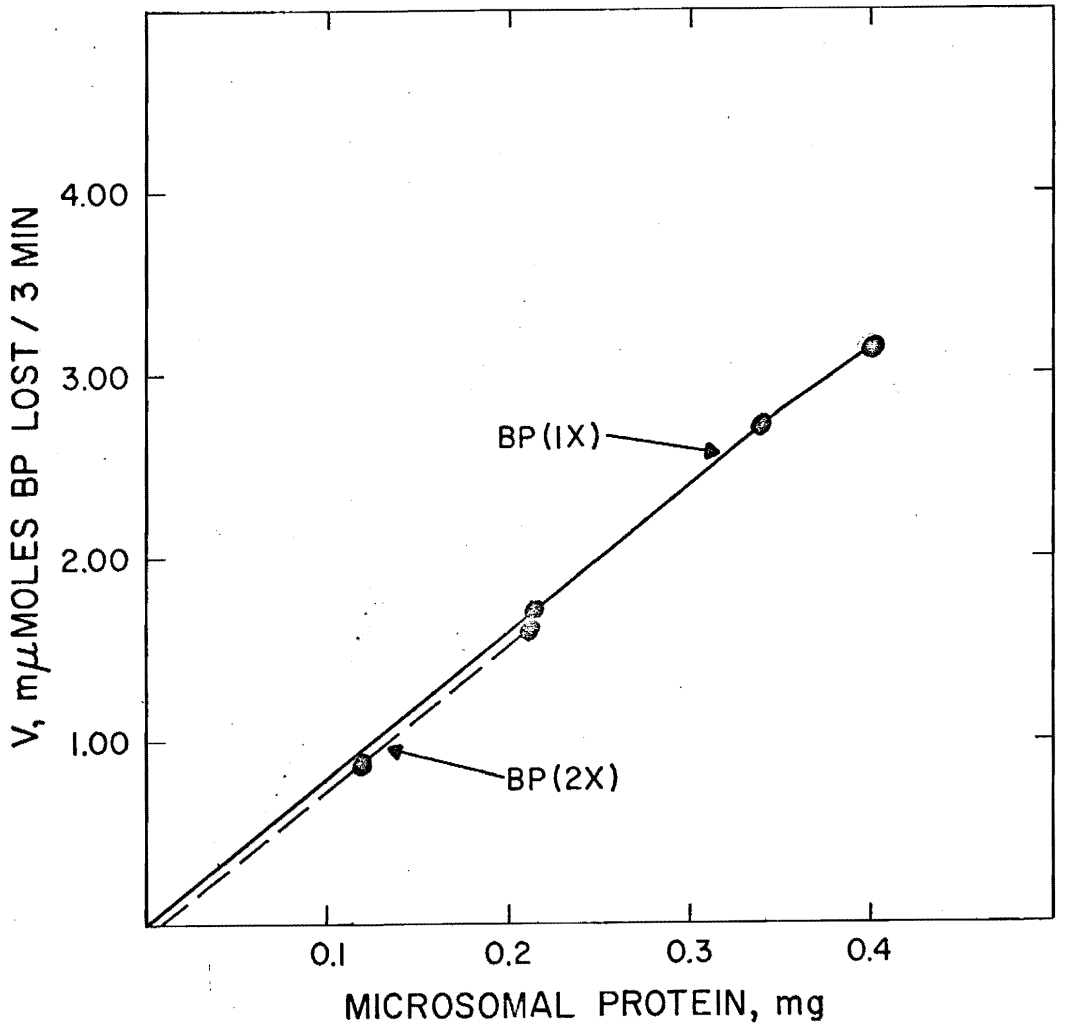
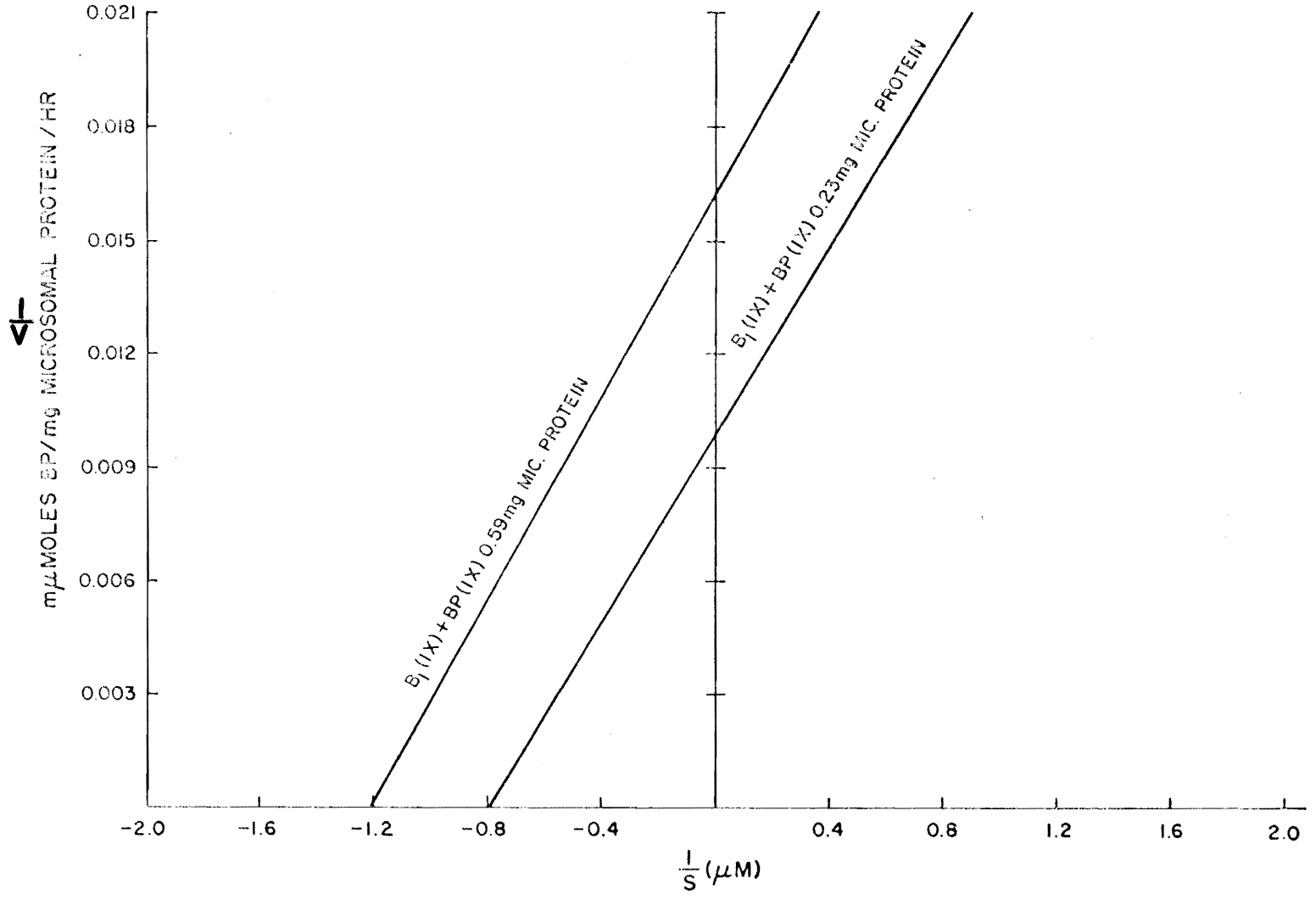

4.00
3.00
2.00
1.00
V, mμMOLES BP LOST / 3 MIN
BP(1X)
BP(2X)
0.1
0.2
0.3
0.4
MICROSOMAL PROTEIN, mg

Figure 8. Slope effect when different concentrations of microsomal protein are used to measure the enzyme kinetics (see text and Table XV).


0.021
0.018
0.015
0.012
0.009
0.006
0.003
1/V
mμMOLES BP/mg MICROSOMAL PROTEIN / HR
B; (IX) + BP (IX) 0.59mg MIC. PROTEIN
B; (IX) + BP (IX) 0.23mg MIC. PROTEIN
-2.0
-1.6
-1.2
-0.8
-0.4
0.4
0.8
1.2
1.6
2.0
1/S (μM)

Studies on Enzyme Kinetics

Experimental data and various kinetic parameters (obtained by the computer analysis of initial velocity data according to the program of Cleland, 1963) for different groups are given in Tables XVI through XL. The computed best fit lines for the Lineweaver-Burke plot for different groups are shown in Figures 9 through 15.

Table XVI. Experimental Data and Kinetic Parameters for Control Group from First Experimental Series

Substrate Conc.[1]	2.20	11.00	15.40	22.00	44.00
Initial Velocity[2] ± S.D.[3]	7.08 ± 0.75 (4)[4]	16.4 ± 1.61 (4)[4]	19.2 ± 1.12 (4)[4]	37.7 ± 1.97 (3)[4]	38.5 ± 2.75 (3)[4]
	$K/_V$ ± S.E.[5]	0.428 ± 0.073			
	K_m ± S.E.[5]	28.6 ± 10.0			
	V_{max} ± S.E.[5]	67.00 ± 12.2			

[1] Substrate concentration in μM.

[2] mμmoles BP disappeared per milligram microsomal protein per hour.

[3] Standard deviation of the sample mean.

[4] Numbers in parenthesis are numbers of samples analyzed.

[5] Standard error obtained from computer analysis of data.

Table XVII. Experimental Data and Kinetic Parameters for B_1(1X) Group from First Experimental Series

Substrate Conc.[1]	2.20	11.00	15.40	22.00	44.00
Initial Velocity[2] ± S.D.[3]	7.15 ± 3.56 (4)[4]	12.1 ± 1.56 (4)[4]	11.5 ± 2.20 (4)[4]	13.4 ± 6.58 (4)[4]	14.8 ± 2.80 (4)[4]
	$K/_V$ ± S.E.[5]	0.175 ± 0.085			
	K_m ± S.E.[5]	2.61 ± 1.48			
	V_{max} ± S.E.[5]	14.90 ± 1.60			

[1] Substrate concentration in μM.

[2] mμmoles BP disappeared per milligram microsomal protein per hour.

[3] Standard deviation of the sample mean.

[4] Numbers in parenthesis are numbers of samples analyzed.

[5] Standard error obtained from computer analysis of data.

Table XVIII. Experimental Data and Kinetic Parameters for BP(1X) Group from First Experimental Series

Substrate Conc.[1]	2.20	11.00	15.40	22.00	44.00
Initial Velocity[2] ± S.D.[3]	15.9 ± 3.22 (4)[4]	30.6 ± 4.30 (4)[4]	30.3 ± 5.98 (4)[4]	40.9 ± 7.03 (3)[4]	38.5 ± 8.05 (4)[4]
	$K/_V$ ± S.E.[5]	0.095 ± 0.027			
	K_m ± S.E.[5]	4.04 ± 1.43			
	V_{max} ± S.E.[5]	42.60 ± 3.40			

[1] Substrate concentration in μM.

[2] mμmoles BP disappeared per milligram microsomal protein per hour.

[3] Standard deviation of the sample mean.

[4] Numbers in parenthesis are numbers of samples analyzed.

[5] Standard error obtained from computer analysis of data.

Table XIX. Experimental Data and Kinetic Parameters for Control (Basal Diet) Group from Second Experimental Series

Substrate Conc.[1]	1.00	2.00	3.00	8.00
Initial Velocity[2] ± S.D.[3]	3.37 ± 0.67 (4)[4]	4.14 ± 0.59 (4)[4]	5.29 ± 0.62 (4)[4]	8.25 ± 0.87 (3)[4]
	$K/_V$ ± S.E.[5]	0.268 ± 0.035		
	K_m ± S.E.[5]	2.95 ± 0.66		
	V_{max} ± S.E.[5]	11.02 ± 1.13		
	Mic. protein[6]	14.02		
	Velocity/gm liver[7]	154.5 ± 15.8[8]		

[1] Substrate concentration in μM.

[2] mμMoles BP disappeared per milligram microsomal protein per hour.

[3] Standard deviation of sample mean.

[4] Numbers in parentheses are numbers of sample aliquots analyzed.

[5] Standard error obtained from computer analysis of data.

[6] Milligrams microsomal protein per gram liver tissue.

[7] Maximum velocity per gram of liver tissue (obtained by multiplying V_{max} x Mic. protein).

[8] Calculated standard error.

Table X. Experimental Data and Kinetic Parameters for B_1(1X) Group from Second Experimental Series

Substrate Conc.[1]	0.40	1.00	2.00	3.00	8.00
Initial Velocity[2] ± S.D.[3]	2.03 ± 0.03 (4)[4]	3.35 ± 0.34 (4)[4]	4.55 ± 0.98 (4)[4]	4.84 ± 1.31 (4)[4]	5.49 ± 1.35 (3)[4]
	$K/_V$ ± S.E.[5]	0.128 ± 0.031			
	K_m ± S.E.[5]	0.78 ± 0.24			
	V_{max} ± S.E.[5]	6.12 ± 0.55			
	Mic. protein[6]	15.68			
	Velocity/gm liver[7]	95.80 ± 8.62[8]			

[1] Substrate concentration in μM.

[2] mμMoles B_1 disappeared per milligram microsomal protein per hour.

[3] Standard deviation of sample mean.

[4] Numbers in parentheses are numbers of sample aliquots analyzed.

[5] Standard error obtained from computer analysis of data.

[6] Milligrams microsomal protein per gram liver tissue.

[7] Maximum velocity per gram of liver tissue (obtained by multiplying V_{max} x Mic. protein).

[8] Calculated standard error.

Table XXI. Experimental Data and Kinetic Parameters for B_1(2X) Group from Second Experimental Series

Substrate Conc.[1]	0.40	0.70	1.00	1.50
Initial Velocity[2] ± S.D.[3]	1.92 ± 0.13 (4)[4]	2.70 ± 0.49 (4)[4]	4.16 ± 0.22 (4)[4]	4.83 ± 0.36 (4)[4]
	$K/_V$ ± S.E.[5]	0.176 ± 0.021		
	K_m ± S.E.[5]	2.07 ± 0.68		
	V_{max} ± S.E.[5]	11.76 ± 2.56		
	Mic. protein[6]	17.83		
	Velocity/gm liver[7]	209.7 ± 45.6[8]		

[1] Substrate concentration in μM.

[2] mμMoles BP disappeared per milligram microsomal protein per hour.

[3] Standard deviation of sample mean.

[4] Numbers in parentheses are numbers of sample aliquots analyzed.

[5] Standard error obtained from computer analysis of data.

[6] Milligrams microsomal protein per gram liver tissue.

[7] Maximum velocity per gram of liver tissue (obtained by multiplying V_{max} x Mic. protein).

[8] Calculated standard error.

Table XXII. Experimental Data and Kinetic Parameters for B_1(3X) Group from Second Experimental Series

Substrate Conc.[1]	0.40	0.70	1.00	1.50
Initial Velocity[2] ± S.D.[3]	2.70 ± 0.18 (4)[4]	3.84 ± 0.30 (4)[4]	5.53 ± 0.46 (4)[4]	6.58 ± 1.12 (4)[4]
	$K/_V$ ± S.E.[5]	0.123 ± 0.017		
	K_m ± S.E.[5]	1.82 ± 0.66		
	V_{max} ± S.E.[5]	14.82 ± 3.43		
	Mic. protein[6]	19.37		
	Velocity/gm liver[7]	287.1 ± 66.4[8]		

[1] Substrate concentration in μM.

[2] mμMoles BP disappeared per milligram microsomal protein per hour.

[3] Standard deviation of sample mean.

[4] Numbers in parentheses are numbers of sample aliquots analyzed.

[5] Standard error obtained from computer analysis of data.

[6] Milligrams microsomal protein per gram liver tissue.

[7] Maximum velocity per gram of liver tissue (obtained by multiplying V_{max} x Mic. protein).

[8] Calculated standard error.

Table XXIII. Experimental Data and Kinetic Parameters for BP(1X) Group from Second Experimental Series

Substrate Conc.[1]	1.00	2.00	3.00	8.00
Initial Velocity[2] ± S.D.[3]	86.1 ± 1.07 (4)[4]	123.0 ± 3.18 (4)[4]	145.0 ± 9.37 (4)[4]	159.0 ± 11.2 (4)[4]
	$K/_V$ ± S.E.[5]	0.0055 ± 0.0005		
	K_m ± S.E.[5]	1.01 ± 0.13		
	V_{max} ± S.E.[5]	182.4 ± 6.13		
	Mic. protein[6]	16.81		
	Velocity/gm liver[7]	3066.0 ± 103.0[8]		

[1] Substrate concentration in μM.

[2] mμMoles BP disappeared per milligram microsomal protein per hour.

[3] Standard deviation of sample mean.

[4] Numbers in parentheses are numbers of sample aliquots analyzed.

[5] Standard error obtained from computer analysis of data.

[6] Milligrams microsomal protein per gram liver tissue.

[7] Maximum velocity per gram of liver tissue (obtained by multiplying V_{max} x Mic. protein).

[8] Calculated standard error.

Table XXIV. Experimental Data and Kinetic Parameters for BP(2X) Group from Second Experimental Series

Substrate Conc.[1]	0.40	1.00	2.00	3.00
Initial Velocity[2] ± S.D.[3]	48.6 ± 0.14 (4)[4]	95.0 ± 1.33 (4)[4]	124.0 ± 6.06 (4)[4]	165.0 ± 15.6 (4)[4]
	$K/_V$ ± S.E.[5]	0.0070 ± 0.007		
	K_m ± S.E.[5]	1.80 ± 0.34		
	V_{max} ± S.E.[5]	255.2 ± 23.4		
	Mic. protein[6]	18.95		
	Velocity/gm liver[7]	4837.0 ± 444.0[8]		

[1] Substrate concentration in μM.

[2] mμMoles BP disappeared per milligram microsomal protein per hour.

[3] Standard deviation of sample mean.

[4] Numbers in parentheses are numbers of sample aliquots analyzed.

[5] Standard error obtained from computer analysis of data.

[6] Milligrams microsomal protein per gram liver tissue.

[7] Maximum velocity per gram of liver tissue (obtained by multiplying V_{max} x Mic. protein).

[8] Calculated standard error.

Table XXV. Experimental Data and Kinetic Parameters for BP(3X) Group from Second Experimental Series

Substrate Conc.[1]	0.40	1.00	2.00	3.00
Initial Velocity[2] ± S.D.[3]	49.6 ± 0.25 (4)[4]	93.7 ± 1.46 (4)[4]	127.6 ± 9.42 (4)[4]	189.9 ± 10.1 (4)[4]
	$K/_V$ ± S.E.[5]	0.0086 ± 0.0009		
	K_m ± S.E.[5]	3.25 ± 0.86		
	V_{max} ± S.E.[5]	377.8 ± 60.5		
	Mic. protein[6]	19.98		
	Velocity/gm liver[7]	7548.0 ± 1209.0[8]		

[1] Substrate concentration in μM.

[2] mμMoles BP disappeared per milligram microsomal protein per hour.

[3] Standard deviation of sample mean.

[4] Numbers in parentheses are numbers of sample aliquots analyzed.

[5] Standard error obtained from computer analysis of data.

[6] Milligrams microsomal protein per gram liver tissue.

[7] Maximum velocity per gram of liver tissue (obtained by multiplying V_{max} x Mic. protein).

[8] Calculated standard error.

Table XXVI. Experimental Data and Kinetic Parameters for BP(1X)+ET Group from Second Experimental Series

Substrate Conc.[1]	0.40	1.00	2.00	3.00
Initial Velocity[2] ± S.D.[3]	9.03 ± 0.77 (4)[4]	11.1 ± 1.64 (3)[4]	12.4 ± 2.35 (4)[4]	14.2 ± 3.32 (3)[4]
	$K/_V$ ± S.E.[5]	0.018 ± 0.006		
	K_m ± S.E.[5]	0.27 ± 0.11		
	V_{max} ± S.E.[5]	14.62 ± 1.23		
	Mic. protein[6]	13.90		
	Velocity/gm liver[7]	203.1 ± 17.1[8]		

[1] Substrate concentration in μM.

[2] mμMoles BP disappeared per milligram microsomal protein per hour.

[3] Standard deviation of sample mean.

[4] Numbers in parentheses are numbers of sample aliquots analyzed.

[5] Standard error obtained from computer analysis of data.

[6] Milligrams microsomal protein per gram liver tissue.

[7] Maximum velocity per gram of liver tissue (obtained by multiplying V_{max} x Mic. protein).

[8] Calculated standard error.

Table XXVII. Experimental Data and Kinetic Parameters for BP(1X)+B_1(1X) Group from Second Experimental Series (Using 0.23 milligram microsomal protein for enzyme assay)

Substrate Conc.[1]	0.40	1.00	2.00	3.00
Initial Velocity[2] ± S.D.[3]	29.7 ± 0.60 (4)[4]	42.8 ± 1.88 (4)[4]	53.5 ± 0.42 (2)[4]	76.0 ± 7.06 (4)[4]
	$K/_V$ ± S.E.[5]	0.0122 ± 0.0018		
	K_m ± S.E.[5]	1.25 ± 0.30		
	V_{max} ± S.E.[5]	102.1 ± 10.3		
	Mic. protein[6]	14.24		
	Velocity/gm liver[7]	1454.0 ± 147.0[8]		

[1] Substrate concentration in μM.

[2] mμMoles BP disappeared per milligram microsomal protein per hour.

[3] Standard deviation of sample mean.

[4] Numbers in parentheses are numbers of sample aliquots analyzed.

[5] Standard error obtained from computer analysis of data.

[6] Milligrams microsomal protein per gram liver tissue.

[7] Maximum velocity per gram of liver tissue (obtained by multiplying V_{max} x Mic. protein).

[8] Calculated standard error.

Table XXVIII. Experimental Data and Kinetic Parameters for BP(1X)+B1(1X) Group from Second Experimental Series (Using 0.59 milligram microsomal protein for enzyme assay)

Substrate Conc.[1]	1.00	2.00	3.00
Initial Velocity[2] ± S.D.[3]	36.2 ± 2.43 (4)[4]	39.0 ± 0.85 (4)[4]	52.9 ± 6.96 (3)[4]
	$K/_V$ ± S.E.[5]	0.013 ± 0.004	
	K_m ± S.E.[5]	0.822 ± 0.37	
	V_{max} ± S.E.[5]	62.15 ± 8.80	
	Mic. protein[6]	14.24	
	Velocity/gm liver[7]	885.0 ± 125.0[8]	

[1] Substrate concentration in μM.

[2] mμMoles BP disappeared per milligram microsomal protein per hour.

[3] Standard deviation of sample mean.

[4] Numbers in parentheses are numbers of sample aliquots analyzed.

[5] Standard error obtained from computer analysis of data.

[6] Milligrams microsomal protein per gram liver tissue.

[7] Maximum velocity per gram of liver tissue (obtained by multiplying V_{max} x Mic. protein).

[8] Calculated standard error.

Table XXIX. Experimental Data and Kinetic Parameters for Laboratory Chow Group from Third Experimental Series

Substrate Conc.[1]	0.40	1.00	2.00	3.00	4.00
Initial Velocity[2] ± S.D.[3]	3.05 ± 0.42 (4)[4]	5.41 ± 0.66 (4)[4]	9.07 ± 1.51 (4)[4]	14.1 ± 1.54 (4)[4]	13.6 ± 3.48 (4)[4]
$K_{/V}$ ± S.E.[5]		0.135 ± 0.023			
K_m ± S.E.[5]		3.81 ± 1.54			
V_{max} ± S.E.[5]		28.29 ± 6.85			
Mic. protein[6]		15.71			
Velocity/gm liver[7]		444.4 ± 108.0[8]			

[1] Substrate concentration in μM.

[2] mμMoles BP disappeared per milligram microsomal protein per hour.

[3] Standard deviation of sample mean.

[4] Numbers in parentheses are numbers of sample aliquots analyzed.

[5] Standard error obtained from computer analysis of data.

[6] Milligrams microsomal protein per gram liver tissue.

[7] Maximum velocity per gram of liver tissue (obtained by multiplying V_{max} x Mic. protein).

[8] Calculated standard error.

Table XXX. Experimental Data and Kinetic Parameters for Control (Basal Diet) Group from Third Experimental Series

Substrate Conc.[1]	0.40	1.00	2.00	3.00	8.00
Initial Velocity[2] ± D.S.[3]	1.06 ± 0.30 (4)[4]	1.37 ± 0.33 (4)[4]	1.59 ± 0.66 (3)[4]	4.10 ± 1.83 (4)[4]	6.80 ± 0.92 (3)[4]
$K/_V$ ± S.E.[5]		0.60 ± 0.06			
K_m ± S.E.[5]		8.96 ± 2.83			
V_{max} ± S.E.[5]		14.86 ± 3.64			
Mic. protein[6]		14.50			
Velocity/gm liver[7]		215.5 ± 52.8[8]			

[1] Substrate concentration in μM.

[2] mμMoles BP disappeared per milligram microsomal protein per hour.

[3] Standard deviation of sample mean.

[4] Numbers in parentheses are numbers of sample aliquots analyzed.

[5] Standard error obtained from computer analysis of data.

[6] Milligrams microsomal protein per gram liver tissue.

[7] Maximum velocity per gram of liver tissue (obtained by multiplying V_{max} x Mic. protein).

[8] Calculated standard error.

Table XXXI. Experimental Data and Kinetic Parameters for BP(3X) Group from Third Experimental Series

Substrate Conc.[1]	1.00	2.00	3.00	3.50	4.00
Initial Velocity[2] ± S.D.[3]	103.0 ± 1.50 (4)[4]	138.0 ± 14.1 (4)[4]	177.0 ± 9.10 (4)[4]	193.0 ± 20.7 (4)[4]	214.0 ± 2.93 (4)[4]
$K/_V$ ± S.E.[5]		0.008 ± 0.0008			
K_m ± S.E.[5]		2.69 ± 0.55			
V_{max} ± S.E.[5]		345.0 ± 33.7			
Mic protein[6]		18.45			
Velocity/gm liver[7]		6364.0 ± 621.0[8]			

[1] Substrate concentration in μM.

[2] mμMoles BP disappeared per milligram microsomal protein per hour.

[3] Standard deviation of sample mean.

[4] Numbers in parentheses are numbers of sample aliquots analyzed.

[5] Standard error obtained from computer analysis of data.

[6] Milligrams microsomal protein per gram liver tissue.

[7] Maximum velocity per gram of liver tissue (obtained by multiplying V_{max} x Mic. protein)

[8] Calculated standard error.

Table XXXII. Experimental Data and Kinetic Parameters for BP(3X)+BP(1X) Group from Third Experimental Series

Substrate Conc.[1]	1.00	1.50	2.00	2.50	3.00
Initial Velocity[2] ± S.D.[3]	155.0 ± 5.66 (4)[4]	184.0 ± 5.16 (4)[4]	234.0 ± 20.1 (4)[4]	221.0 ± 14.4 (4)[4]	232.0 ± 56.5 (4)[4]
	$K/_V$ ± S.E.[5]	0.003 ± 0.0008			
	K_m ± S.E.[5]	1.00 ± 0.37			
	V_{max} ± S.E.[5]	317.9 ± 40.2			
	Mic. protein[6]	17.07			
	Velocity/gm liver[7]	5427.0 ± 686.0[8]			

[1] Substrate concentration in μM.

[2] mμMoles BP disappeared per milligram microsomal protein per hour.

[3] Standard deviation of sample mean.

[4] Numbers in parentheses are numbers of sample aliquots analyzed.

[5] Standard error obtained from computer analysis of data.

[6] Milligrams microsomal protein per gram liver tissue.

[7] Maximum velocity per gram of liver tissue (obtained by multiplying V_{max} x Mic. protein).

[8] Calculated standard error.

Table XXXIII. Experimental Data and Kinetic Parameters for BP(3X)+ET Group from Third Experimental Series

Substrate Conc.[1]	1.00	2.00	2.50	3.00	3.50
Initial Velocity[2] ± S.D.[3]	106.0 ± 7.67 (4)[4]	126.0 ± 4.80 (4)[4]	150.0 ± 8.51 (4)[4]	170.0 ± 10.3 (4)[4]	211.0 ± 16.1 (4)[4]

$K/_V$ ± S.E.[5]	0.009 ± 0.0014
K_m ± S.E.[5]	3.45 ± 1.21
V_{max} ± S.E.[5]	383.6 ± 77.2
Mic. protein[6]	15.41
Velocity/gm liver[7]	5912.0 ± 1189.0[8]

[1] Substrate concentration in μM.

[2] mμMoles BP disappeared per milligram microsomal protein per hour.

[3] Standard deviation of sample mean.

[4] Numbers in parentheses are numbers of sample aliquots analyzed.

[5] Standard error obtained from computer analysis of data.

[6] Milligrams microsomal protein per gram liver tissue.

[7] Maximum velocity per gram of liver tissue (obtained by multiplying V_{max} x Mic. protein).

[8] Calculated standard error.

Table XXXIV. Kinetic Parameters for Various Groups from First Experimental Series

Group	$K_m \pm$ S.E.[1]	$V_{max} \pm$ S.E.[1]	$K_m/V \pm$ S.E.[1]
Control	28.6 ± 10.0	67.0 ± 12.2	0.428 ± 0.073
B_1(1X)	2.61 ± 1.48	14.9 ± 1.60	0.175 ± 0.085
BP(1X)	4.04 ± 1.43	42.6 ± 3.40	0.095 ± 0.027

[1] Standard error obtained from computer analysis of data.

Table XXXV. Kinetic Parameters for Various Groups from First Experimental Series

Group	$K_m \pm$ S.E.[1]	$\frac{1}{K_m}$[2]	$V_{max} \pm$ S.E.[1]	$\frac{1}{V_{max}}$	$\frac{1}{V}$ at (A^3=)
Control	28.6 ± 10.0	0.035	67.00 ± 12.2	0.015	0.0246 (A=44)
B_1(1X)	2.61 ± 1.48	0.383	14.90 ± 1.60	0.067	0.0712 (A=44)
BP(1X)	4.04 ± 1.43	0.247	42.60 ± 3.40	0.023	0.0256 (A=44)

[1] Standard error obtained from computer analysis of data.

[2] $\frac{1}{K_m}$ has been obtained from K_m value by calculation. The rest of the values, i.e., V_{max}, $\frac{1}{V_{max}}$, K_m and $\frac{1}{V}$ at (A =), have been obtained from computer analysis of data.

[3] A is the substrate concentration in μM.

Table XXXVI. Kinetic Parameters for Various Groups from Second Experimental Series

Group	$K_m \pm$ S.E.[1]	$V_{max} \pm$ S.E.[1]	$K/V \pm$ S.E.[1]	Mic. protein[2]
Control	2.95 ± 0.66	11.02 ± 1.13	0.268 ± 0.035	14.02
B_1(1X)	0.78 ± 0.24	6.12 ± 0.55	0.128 ± 0.030	15.68
B_1(2X)	2.07 ± 0.68	11.76 ± 2.56	0.176 ± 0.021	17.83
B_1(3X)	1.82 ± 0.66	14.82 ± 3.43	0.123 ± 0.017	19.37
BP(1X)	1.01 ± 0.13	182.4 ± 6.13	0.0055 ± 0.0005	16.81
BP(2X)	1.80 ± 0.34	255.2 ± 23.4	0.0070 ± 0.0007	18.95
BP(3X)	3.25 ± 0.86	377.8 ± 60.5	0.0086 ± 0.0009	19.98
BP(1X)+ET	0.27 ± 0.11	14.62 ± 1.23	0.018 ± 0.006	13.90
BP(1X)+B_1(1X)	1.25 ± 0.30	102.1 ± 10.3	0.0122 ± 0.0018	14.24

[1] Standard error obtained from computer analysis of data.

[2] Milligrams microsomal protein per gram of pooled liver.

Table XXXVII. Kinetic Parameters for Various Groups from Second Experimental Series

Group	$K_m \pm$ S.E.[1]	$\frac{1}{K_m}$[2]	$V_{max} \pm$ S.E.[1]	$\frac{1}{V_{max}}$	$\frac{1}{V}$ at (A^3=)
Control	2.95 ± 0.66	0.339	11.02 ± 1.13	0.0910	0.1242 (A=8)
B_1(1X)	0.78 ± 0.24	1.275	6.12 ± 0.55	0.1634	0.1794 (A=8)
B_1(2X)	2.07 ± 0.68	0.482	11.76 ± 2.56	0.0850	0.2025 (A=1.5)
B_1(3X)	1.82 ± 0.66	0.548	14.82 ± 3.43	0.0675	0.1495 (A=1.5)
BP(1X)	1.01 ± 0.13	0.999	182.4 ± 6.13	0.0055	0.0062 (A=8)
BP(2X)	1.80 ± 0.34	0.556	255.2 ± 23.4	0.0039	0.0063 (A=3)
BP(3X)	3.25 ± 0.86	0.307	377.8 ± 60.5	0.0026	0.0055 (A=3)
BP(1X)+ET	0.27 ± 0.11	3.750	14.62 ± 1.23	0.0684	0.0745 (A=3)
BP(1X)+B_1(1X)	1.25 ± 0.30	0.800	102.1 ± 10.3	0.0098	0.0139 (A=3)

[1] Standard error obtained from computer analysis of data.

[2] $\frac{1}{K_m}$ has been obtained from K_m value by calculation. The rest of the values, i.e., V_{max}, $\frac{1}{V_{max}}$, K_m and $\frac{1}{V}$ at (A =), have been obtained from computer analysis of data.

[3] A is the substrate concentration in μM.

Table XXXVIII. Kinetic Parameters for Various Groups from Third Experimental Series

Group	$K_m \pm$ S.E.[1]	$V_{max} \pm$ S.E.[1]	$K/V \pm$ S.E.[1]	Mic. Protein[2]
Lab Chow[3]	3.81 ± 1.54	28.29 ± 6.85	0.135 ± 0.023	15.71
Control (Basal diet)	8.96 ± 2.83	14.86 ± 3.64	0.60 ± 0.06	14.50
BP(3X)	2.69 ± 0.55	345.0 ± 33.7	0.008 ± 0.0008	18.45
BP(3X)+BP(1X)	1.00 ± 0.37	317.9 ± 40.3	0.003 ± 0.0008	17.07
BP(3X)+ET	3.45 ± 1.21	383.6 ± 77.2	0.009 ± 0.0014	15.41

[1] Standard error obtained from computer analysis of data.

[2] Milligrams microsomal protein per gram pooled liver.

[3] Laboratory Chow for Rats from Wayne.

Table XXXIX. Kinetic Parameters for Various Groups from Third Experimental Series

Group	$K_m \pm$ S.E.[1]	$\frac{1}{K_m}$[2]	$V_{max} \pm$ S.E.[1]	$\frac{1}{V_{max}}$	$\frac{1}{V}$ at (A^3=)
Lab Chow	3.81 ± 1.54	0.262	28.29 ± 6.85	0.0353	0.0690 (A=4)
Control (Basal diet)	8.96 ± 2.83	0.112	14.86 ± 3.64	0.0673	0.1426 (A=8)
BP(3X)	2.69 ± 0.55	0.372	345.0 ± 33.7	0.0029	0.0048 (A=4)
BP(3X)+BP(1X)	1.00 ± 0.37	0.997	317.9 ± 40.2	0.0031	0.0042 (A=3)
BP(3X)+ET	3.45 ± 1.21	0.290	383.6 ± 77.2	0.0026	0.0052 (A=3.5)

[1] Standard error obtained from computer analysis of data.

[2] $\frac{1}{K_m}$ has been obtained from K_m value by calculation. The rest of the values, i.e., V_{max}, $\frac{1}{V_{max}}$, K_m and $\frac{1}{V}$ at (A =), have been obtained from computer analysis of data.

[3] A is the substrate concentration in μM.

Table XL. Microsomal Protein

Group	No. of rats	Microsomal protein[1]	Mean ± S.D.[2]
Control (Basal diet)	3	13.37 14.03 18.11	15.17 ± 2.57
B_1 (1X)	4	13.41 17.24 14.89 13.68	14.80 ± 1.75
BP(1X)	4	16.06 18.89 15.77 17.04	16.94 ± 1.41

[1] Microsomal protein in milligrams per gram of liver.

[2] Standard deviation of the sample mean.

Figure 9. Lineweaver-Burke plots showing comparison among different groups from first experimental series.

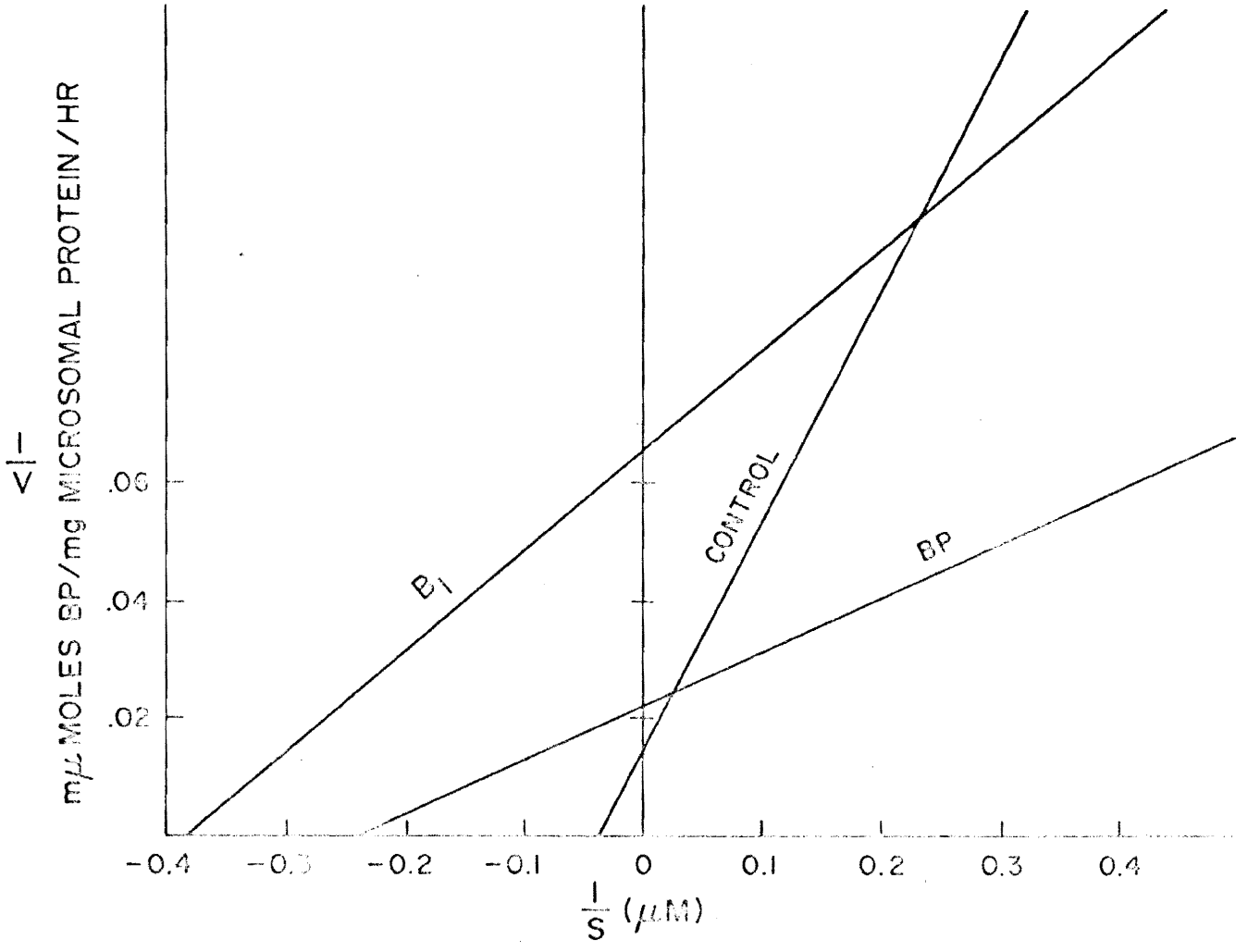

1/V
mμ MOLES BP/mg MICROSOMAL PROTEIN/HR
.06
.04
.02
B1
CONTROL
BP
-0.4
-0.3
-0.2
-0.1
0
0.1
0.2
0.3
0.4
1/S (μM)

Figure 10. Lineweaver-Burke plots showing comparison among the following groups from second experimental series:

Control (Basal diet)

B_1(1X)

B_1(2X)

B_1(3X)

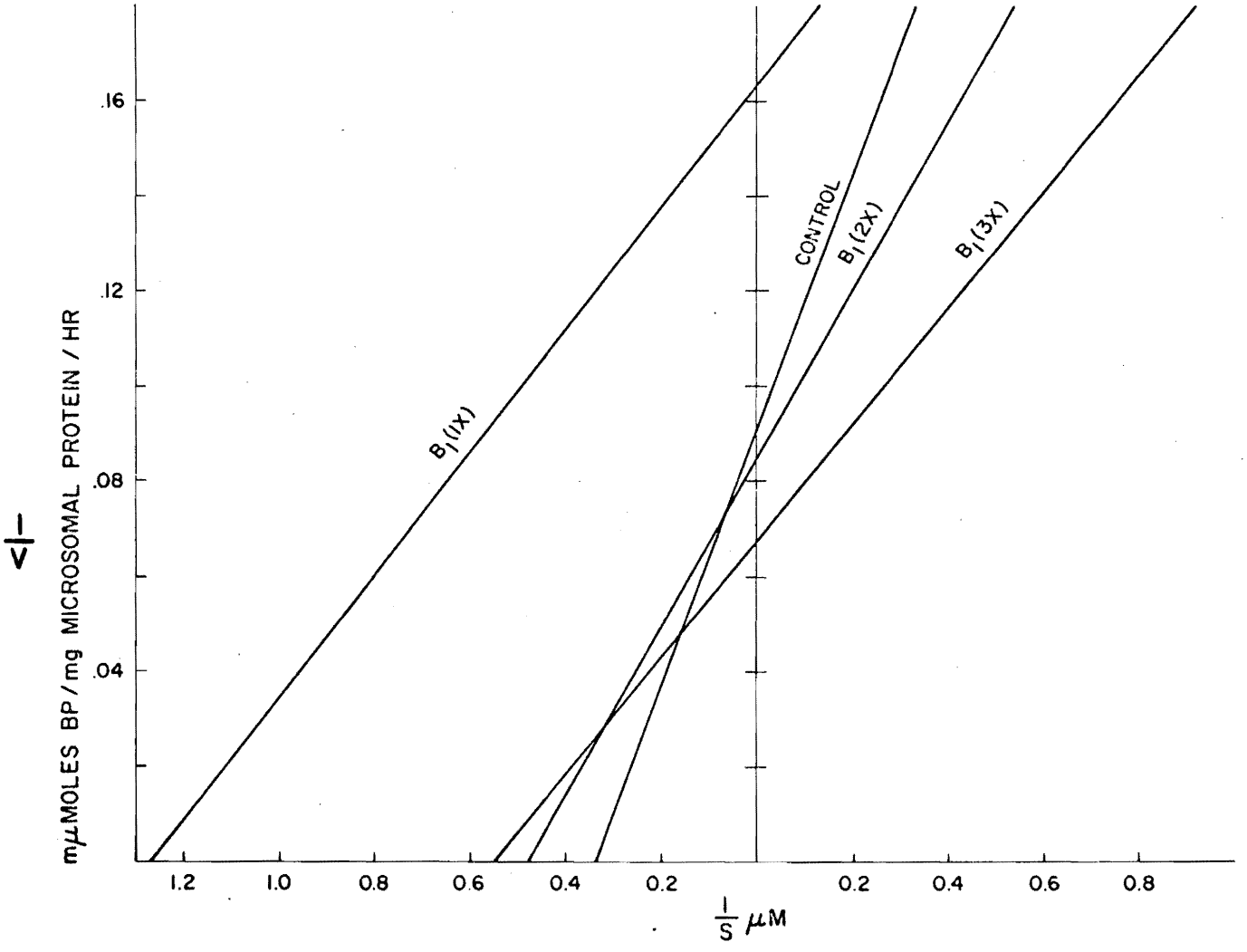

1/V
mμMOLES BP / mg MICROSOMAL PROTEIN / HR
.16
.12
.08
.04
1.2
1.0
0.8
0.6
0.4
0.2
0.2
0.4
0.6
0.8
1/S μM
B1(1X)
CONTROL
B1(2X)
B1(3X)

Figure 11. Lineweaver-Burke plots showing comparison among the following groups from second experimental series:

Control (Basal diet)

BP(1X)

BP(2X)

BP(3X)

BP(1X)+ET

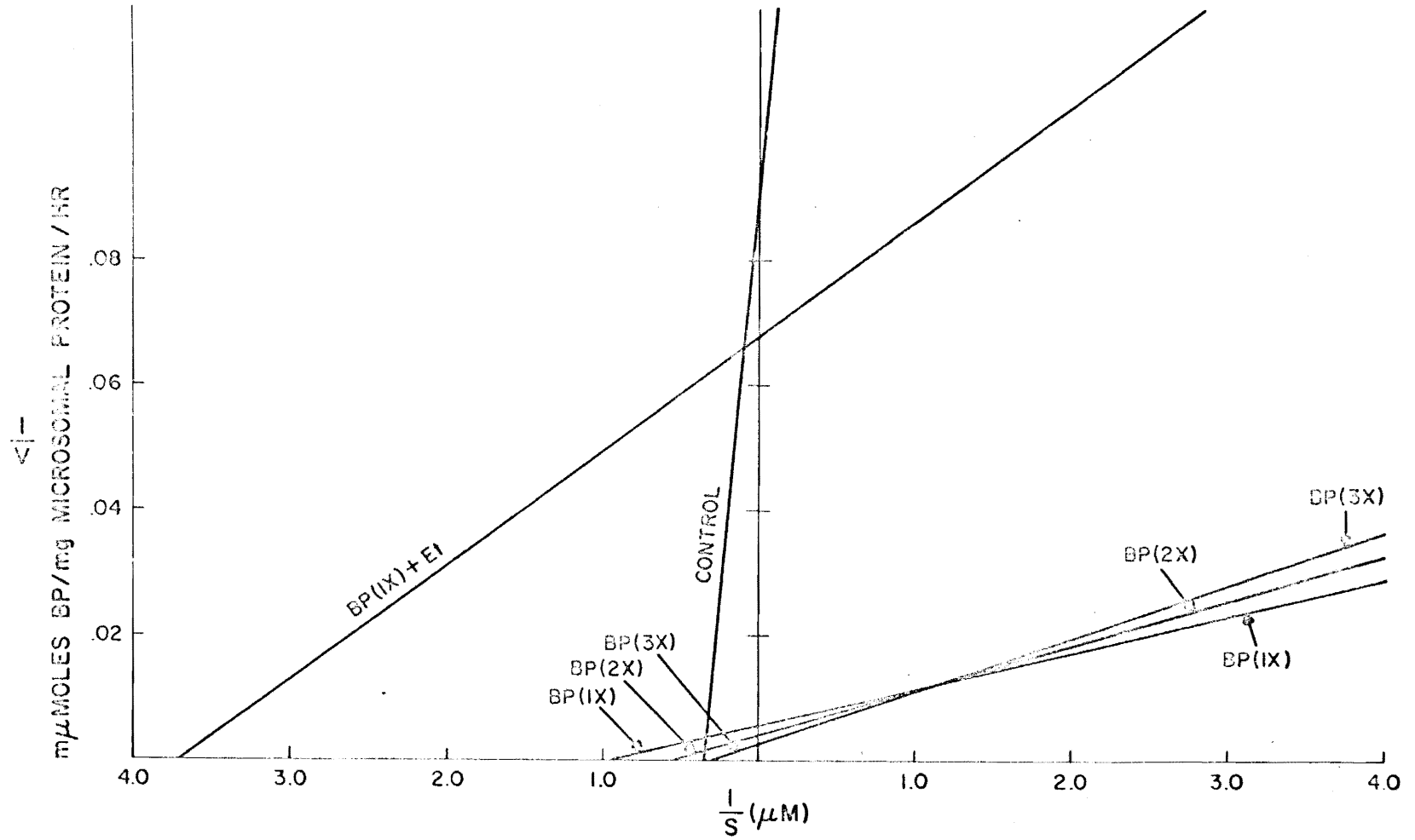

V
mμMOLES BP/mg MICROSOMAL PROTEIN / HR
.08
.06
.04
.02
BP(IX)+EI
CONTROL
BP(3X)
BP(2X)
BP(IX)
BP(3X)
BP(2X)
BP(IX)
4.0
3.0
2.0
1.0
1.0
2.0
3.0
4.0
1/S (μM)

Figure 12. Lineweaver-Burke plots showing comparison among the following groups from second experimental series:

BP(1X)

BP(2X)

BP(3X)

BP(1X)+B_1(1X)

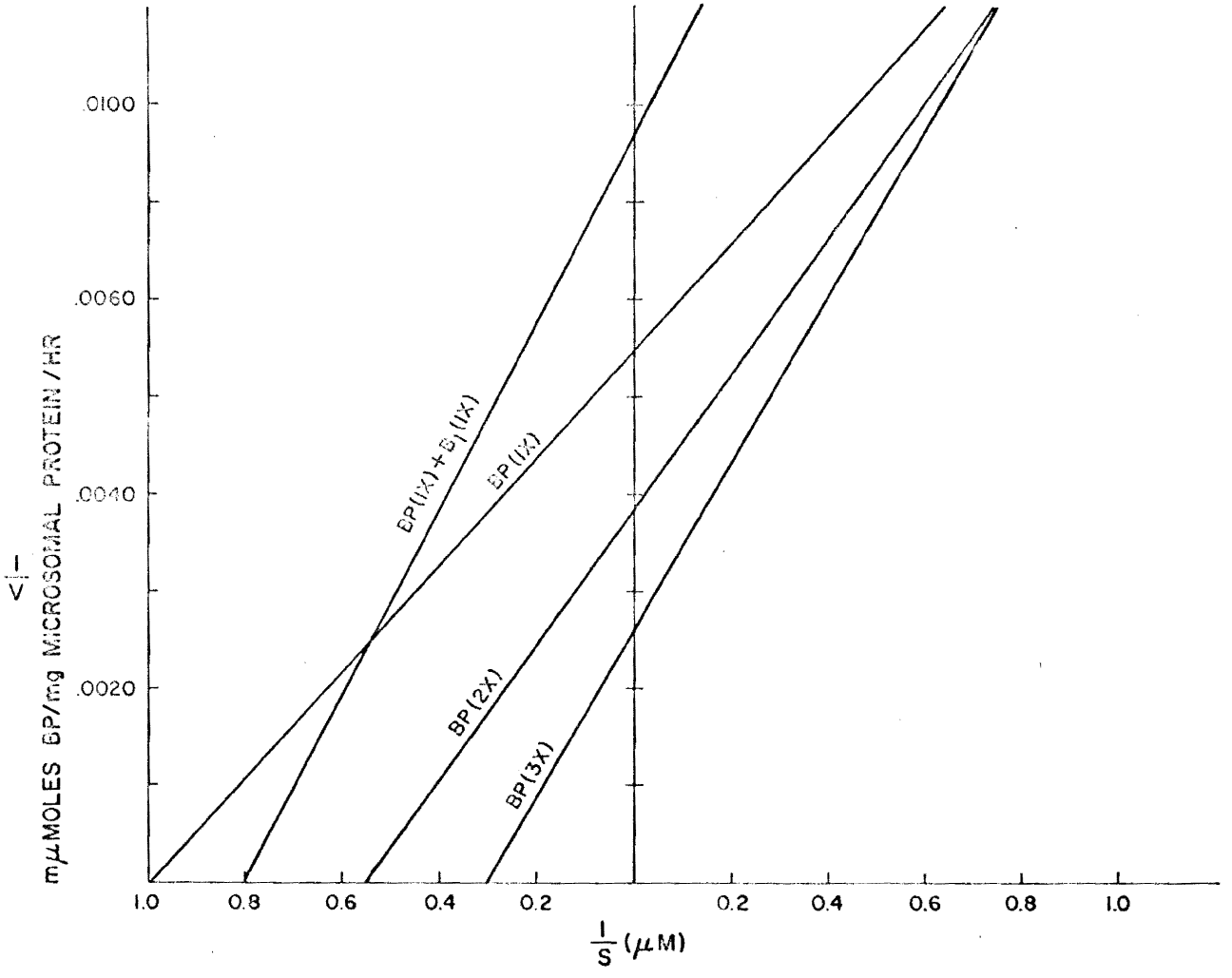

.0100
.0060
.0040
.0020
$\frac{V}{}$
mμMOLES 6P/mg MICROSOMAL PROTEIN / HR
BP(1X)+B(1X)
BP(1X)
BP(2X)
BP(3X)
1.0
0.8
0.6
0.4
0.2
0.2
0.4
0.6
0.8
1.0
$\frac{1}{S}$ (μM)

Figure 13. Lineweaver-Burke plots showing comparison among different groups from second experimental series.

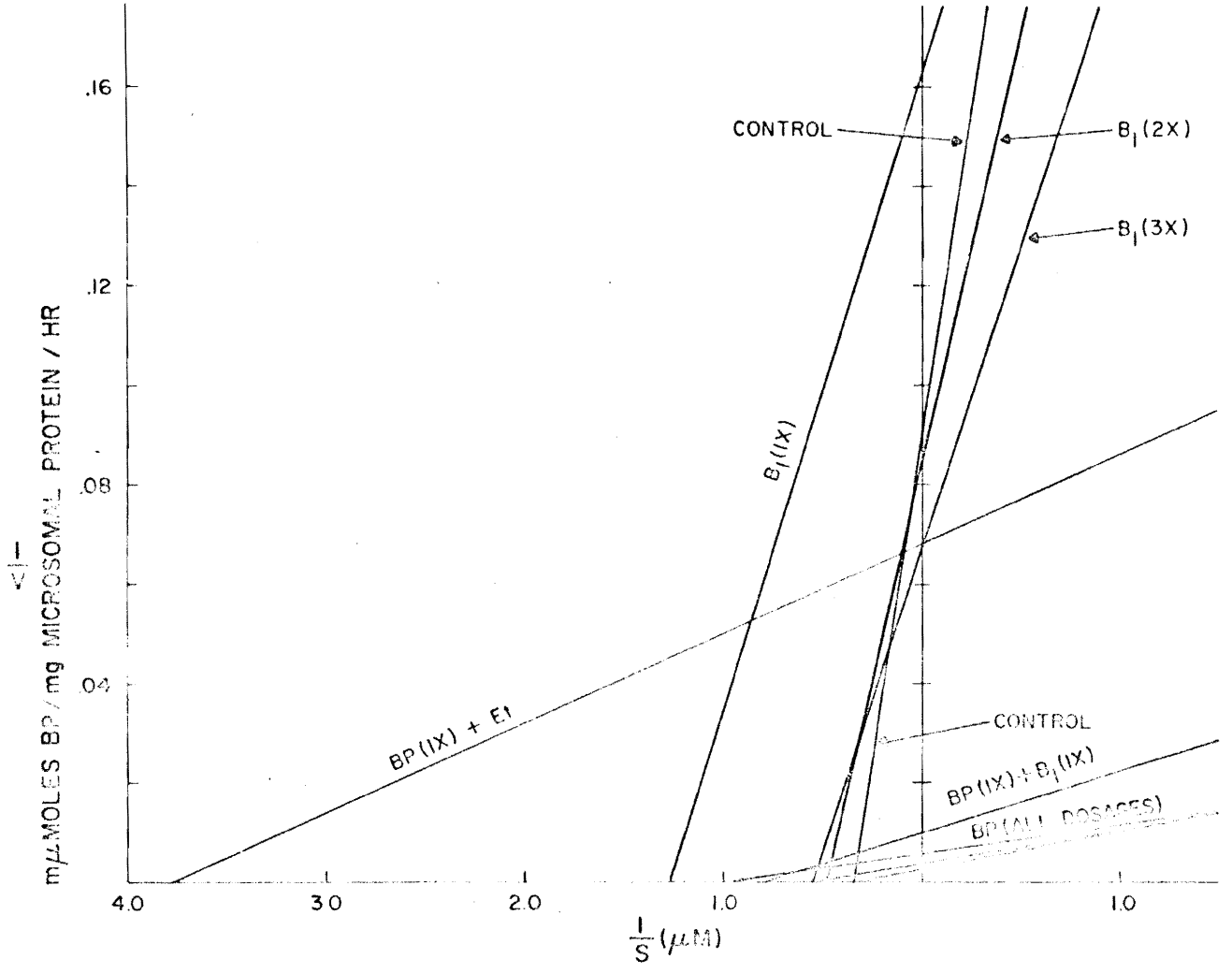

.16
.12
.08
.04
CONTROL
B_1(2X)
B_1(3X)
B_1(1X)
BP(1X) + E.
CONTROL
BP(1X)+B_1(1X)
BP(ALL DOSAGES)
4.0
3.0
2.0
1.0
1.0
$\frac{1}{S}$ (μM)
mμMOLES BP / mg MICROSOMAL PROTEIN / HR

Figure 14. Lineweaver-Burke plots showing a comparison of Laboratory Chow vs. Control (Basal diet) group from third experimental series.

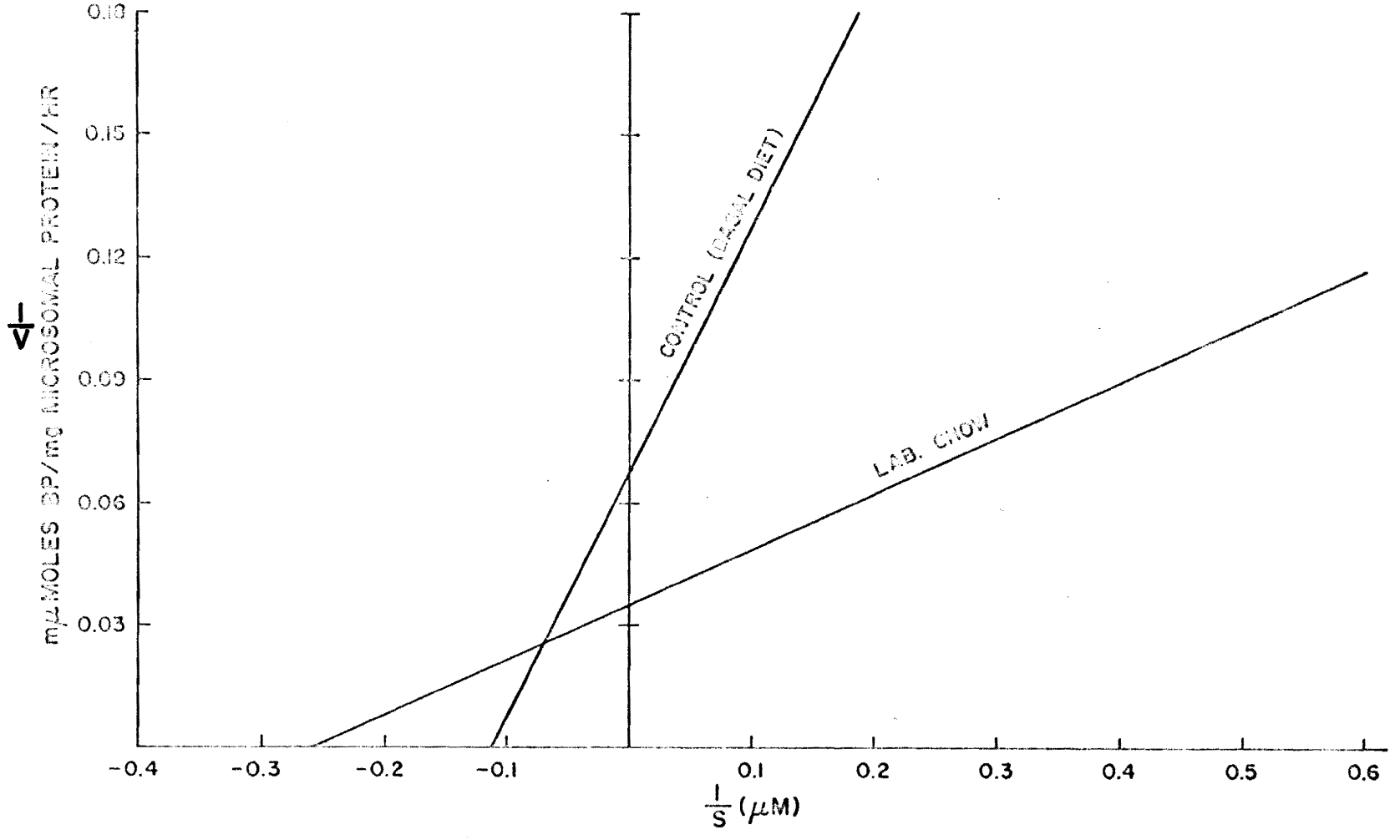
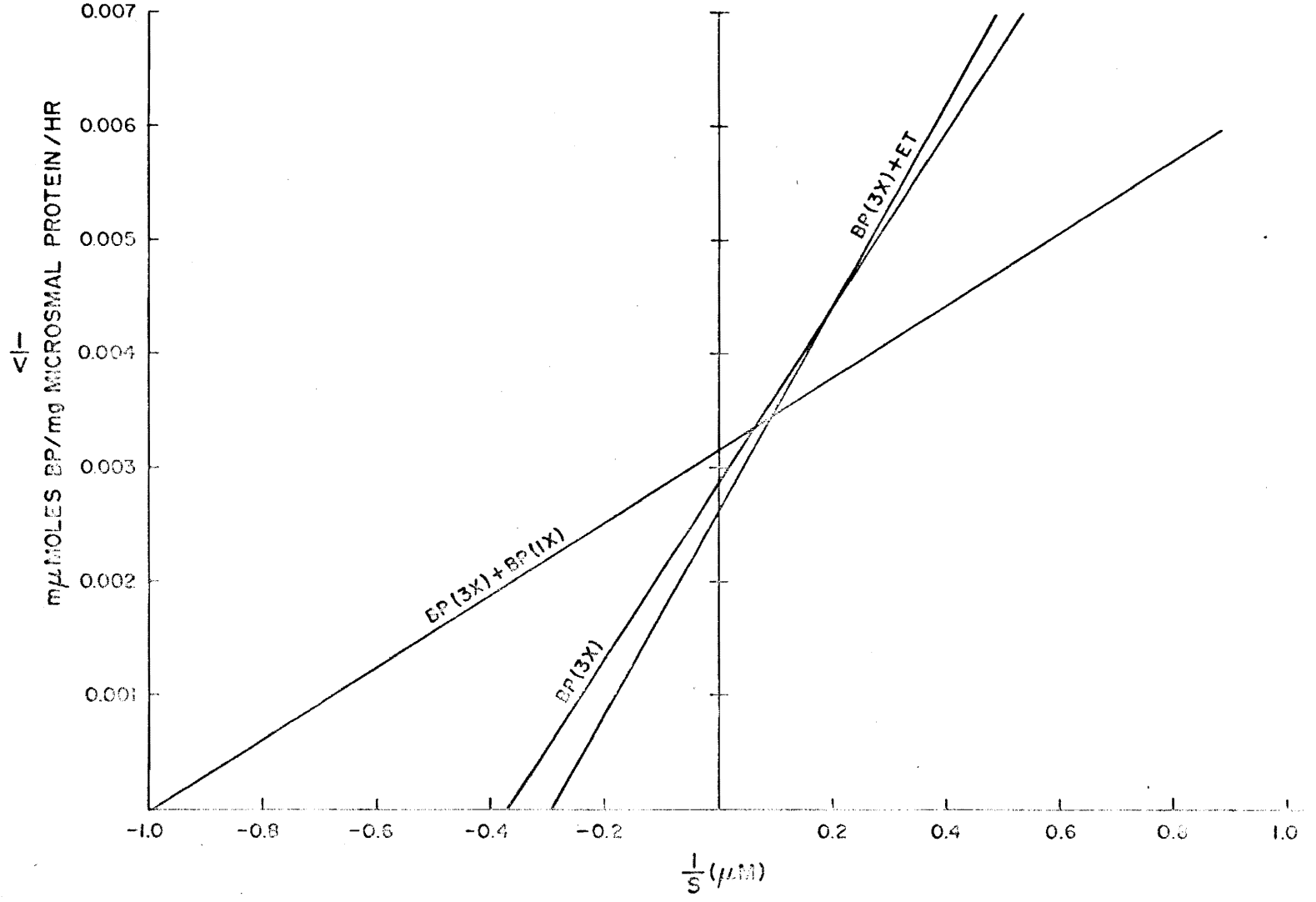

0.18
0.15
0.12
0.09
0.06
0.03
1/V
mμMOLES BP/mg MICROSOMAL PROTEIN/HR
CONTROL (ORAL DIET)
LAB. CHOW
-0.4
-0.3
-0.2
-0.1
0.1
0.2
0.3
0.4
0.5
0.6
1/S (μM)

Figure 15. Lineweaver-Burke plots showing comparison among the following groups from third experimental series:

BP(3X)

BP(3X)+BP(1X)

BP(3X)+ET


0.007
0.006
0.005
0.004
0.003
0.002
0.001
1/V
mμMOLES BP/mg MICROSMAL PROTEIN/HR
BP(3X)+BP(1X)
BP(3X)
BP(3X)+ET
-1.0
-0.8
-0.6
-0.4
-0.2
0.2
0.4
0.6
0.8
1.0
1/S (μM)

DISCUSSION

As already discussed under Review of Literature, increases in microsomal enzyme activities following prior administration with polycyclic hydrocarbons differ in a number of characteristic features from similar increases in activities caused by prior administration with phenobarbital type drugs. One of the features that helps to differentiate these two types of drugs is the effect on microsomal protein and liver size. The results reported by Conney *et al.* (1960) after pretreatment of rats with ten milligrams of sodium phenobarbital for four days are shown in Table XLI. Also, Silverman and Talalay (1967) have reported 10-15 milligrams of microsomal protein per gram of liver.

Conney and Gilman (1963) have reported that intraperitoneal injection of 37 milligrams per kilogram of sodium phenobarbital twice daily for four days increased the weight of livers by 20-30% and the microsomal protein per gram of liver was increased by 39%, whereas injection of 20 milligrams per kilogram of 3-MC did not increase the amount of microsomal protein in the liver significantly at 24 hours; however, administration of this latter hydrocarbon daily for four days did stimulate liver growth resulting in a 20 to 40% increase in microsomal or total liver protein. Microsomal protein per gram of liver, however, was not consistently increased by treatment of rats with 3-MC. Studies by Gelboin and Sokoloff (1961) with 3-MC and phenobarbital and by Von Der Decker and Hultin (1960) with 3-MC have shown that the marked effect of these agents on the synthesis of microsomal enzymes *in vivo* was paralleled by increased incorporation *in*

Table XLI. Effect of Phenobarbital Pre[illegible]eatment on Liver Size and Microsomal Protein [illegible]Conney et al., 1961)

Group	Total Wet Weight of Liver g/100 gm rat	Microsomal Liver Protein in mg/100 gm rat
Control	4.64 ± 0.57	234 ± 18
PB-treated	5.35 ± 0.32	348 ± 29

vitro of amino acids into microsomal protein. Studies from the second experimental series (Table XXXVI) have shown that there is an increasing trend in the synthesis of microsomal protein as the dosage of BP and B_1 administered increases. These values are for pooled livers for each group; hence statistical analyses are not possible. Values in Table XL are given for individual rats from the control, B_1(1X) and BP(1X) groups and the statistical analysis shows that although the values obtained for the BP(1X) group are somewhat higher than the control, they are not statistically significant ($P > 0.05$). The slightly lower values for B_1(1X) are also not significantly different from the control value ($P > 0.05$). The explanation for this non-significant effect of pretreatment with polycyclic hydrocarbons on microsomal protein has been provided by Fouts and Rogers (1965). They noticed massive proliferation of smooth endoplasmic-reticulum (SER) with phenobarbital and chlordane pretreatment; whereas BP and 3-MC did not cause any significant proliferation of SER. They believe that such morphological findings are consistent with the fact that phenobarbital and chlordane are relatively nonspecific inducers of many microsomal enzymes and such a generalized induction is accompanied by increase in liver size and a net increase in measurable microsomal protein per gram of liver. It is likely that these large increases in microsomal protein are due to increased production of SER which is the major site for drug metabolizing enzymes. Remmer and Merker (1963) have shown that the marked increase in drug metabolizing enzyme activity following phenobarbital administration is associated primarily with the smooth surfaced microsome fraction rather than the rough surfaced fraction.

From this observation it would appear that phenobarbital and chlordane induce the synthesis of so many new enzymes that the liver must also make a lot more of SER vesicles and tubes to accommodate these enzymes. Such is not true for BP or 3-MC promoted induction. New enzyme synthesis caused by BP and 3-MC is not sufficient to increase the amount of measurable microsomal protein per gram of liver (Conney and Gilman, 1963). This is consistent with the fact that there is no great increase in SER from animals treated with BP or 3-MC (Fouts and Rogers, 1965) and may mean that the number of packages (tubes and vesicles of SER), containing the microsomal drug metabolizing enzymes, is sufficient to hold the new enzymesbeing made after BP administration--the packages may be more completely filled but they are not much more numerous than in untreated animals. However, in the second experimental series, the level of microsomal protein can be increased to some extent when the dosage level of either BP or B_1 is increased twofold and threefold over the 1(X) level.

Increase in microsomal enzyme activities following drug administration has been solely attributed to the de novo synthesis of microsomal enzyme protein as such induction can be blocked by the prior administration with protein inhibitors like ethionine, puromycin or actinomycin D (Conney et al., 1957, 1960; Conney and Gilman, 1963; Fujimoto and Plaa, 1961, Ernster and Orrenius, 1965, and several others). All these workers have used one substrate concentration to study the rates of conversion with or without the pretreatment of animals with the inducer and/or protein inhibitor. Comparison of Tables XIX, XXIII and XXVI will show that the velocity of disappearance

of BP at any one substrate level is greatly reduced when DL-ethionine is administered along with BP. This definitely supports the view that microsomal enzyme protein synthesis following drug administration is one important phase of induction but such studies by various workers, who have used but one substrate level to compare reaction rates, do not tell anything about another important phase of microsomal enzyme "induction", namely, enzyme activation. A look at Tables XXXIV and XXXVI and Figures 9, 11 and 13 will reveal that not only is the V_{max} increased by BP(1X) pretreatment (Table XXXVI and Figures 11 and 13) but also the values for K_m are decreased. An increase in V_{max} is a quantitative measurement representing enzyme synthesis whereas a decrease in K_m represents some form of activation of the enzyme system which does not include de novo synthesis of enzyme. The various aspects of V_{max} and K_m and the limitations of these parameters have already been discussed under the Review of Literature. Until recently only a few references dealing with the kinetic aspects of microsomal enzyme induction were available and these mostly concerned phenobarbital type drugs and the enzymes which metabolize such drug types. For example, Rubin et al. (1964) reported that phenobarbital administration increased the V_{max} of ethylmorphine N-demethylase, chlorpromazine sulfoxidase, and hexobarbital oxidase of rat liver but the K_m values for the metabolism of each drug were not different ($P > 0.05$) when determined in noninduced and phenobarbital induced systems. Netter and Seidel (1964) reported that both phenobarbital and BP pretreatment increased the V_{max} of p-nitroanisole O-demethylase of rat liver without influencing the K_m values. Such reports are consistent with the

interpretation that induction of a number of microsomal enzyme systems are in fact the result of increased enzyme protein formation; however, they do not exclude the possibilities of modifications of some enzyme systems specifically involved in the transformation of polycyclic hydrocarbons which differ in a number of (see Review of Literature) characteristic features from phenobarbital type drugs. To the best of our knowledge, until recently no attention had been given to the kinetic properties or the effect of polycyclic aromatic type inducers on the kinetic parameters, namely, the V_{max} and K_m of these enzyme systems. For the first time Alvares et al. (1968) and our group (Gurtoo et al., 1968) reported simultaneously the change in the kinetic behavior of BP hydroxylase following pretreatment. The publication of Alvares et al. (1968) showed the effects of 3-MC pretreatment, whereas our group studied the effects of BP and B_1 pretreatment. Both groups reported the activation of BP hydroxylase resulting at least in part from a decreased K_m. Alvares et al. (1968) observed a decrease in K_m from a control value of 1.33×10^{-5}M to 0.26×10^{-5}M when rats were pretreated with 3-MC, whereas pretreatment with phenobarbital did not cause any significant change in the K_m value (1.54×10^{-5}M). They also reported an increase in V_{max} following pretreatment with both 3-MC or phenobarbital. Part of the results published in our paper are shown in Table XXXIV and Lineweaver-Burke plots for these results are plotted in Figure 9. In Tables XVI, XVII and XVIII are given the experimental data and various kinetic parameters for these groups, namely, control, B_1(1X) and BP(1X), respectively (first experimental series). A K_m value of 28.6 ± 10 (μM) was obtained for the microsomes of the control

group. Silverman and Talalay (1967) have reported a K_m value of 28.5 μM for microsomal BP hydroxylase from rats weighing 250-300 grams. Rats used in our experiment ranged between 222-243 grams in body weight (Table III). Pretreatment of rats with BP(1X) decreased the K_m from a control value of 28.6 ± 10 to 4.40 ± 1.43, whereas a value of 2.61 ± 1.48 was obtained for B_1(1X) pretreatment (Table XXXIV). In contrast to the increase in V_{max} values obtained by Alvares et al. (1968), our results (Table XXXIV and Figure 9) show a significant decrease in V_{max} with B_1(1X) pretreatment, whereas the V_{max} values for controls and BP(1X) pretreated groups are within one standard deviation of each other--in fact, a slight decrease instead of a significant increase was observed with BP(1X) pretreatment in this experimental series. In our later experiments we have observed a significant increase in V_{max} with BP(1X) pretreatment. This discrepancy here, i.e., no increase in V_{max} with BP(1X), will be discussed when the laboratory chow and control (20% casein diet) groups are discussed. The rats used in the first series of experiments were maintained on laboratory chow.

Individual experimental data and kinetic parameters for each group from the second series of experiments are given in Tables XIX through XXVIII. Kinetic parameters for all groups and Lineweaver-Burke plots are shown in Tables XXXVI and XXXVII and Figures 10, 11, 12 and 13, respectively. It will be seen (Table XXXVI) that with BP(1X) pretreatment, K_m is decreased from a control value of 2.95 ± 0.66 to 1.01 ± 0.13, whereas V_{max} is increased from a control value of 11.02 ± 1.31 to 182.37 ± 6.13 (14- to 17-fold). This increase in V_{max} represents

de novo synthesis of the enzyme protein and is consistent with the observation that "induction" caused with prior administration of drugs is due to the synthesis of enzyme protein. The decrease in the K_m value with BP(1X) pretreatment represents the enzyme activation phase of overall "induction". As can be observed (Table XXXVI and Figures 11 and 12), both the K_m and V_{max} show an increasing trend with the increase in dosage from BP(1X) to BP(3X). This increasing trend in V_{max} is paralleled by an increase in microsomal enzyme protein expressed in milligrams per gram of pooled liver (Table XXXVI). The effects of pretreatment with B_1 are similar but the picture becomes more complex due to the other properties of B_1 which will be discussed separately in detail, later. With B_1(1X) both the K_m and V_{max} are decreased (Table XXXVI and Figures 10 and 13). This is consistent with the observations made with the first experimental series (Table XXXIV and Figure 9). When the dosage of B_1 is increased to B_1(2X) and B_1(3X), a picture somewhat more complicated but similar to that of the BP pretreatment is observed. Both the K_m and V_{max} values increase and approach control values (Table XXXVI and Figures 10 and 13). When B_1(1X) is combined with BP(1X) (Table XXXVI and Figures 12 and 13) the expected results are obtained. The K_m value is 1.25 as against the control value of 2.95, whereas the V_{max} value of 182 obtained with BP(1X) is reduced to 102. When BP(1X) is combined with a dose of 700 milligrams per kilogram body weight of DL-ethionine, results (Table XXXVI and Figures 11 and 13) are obtained which are consistent with its protein inhibition properties, but the effect on the K_m would not appear to be due to this protein inhibitory property of ethionine. V_{max} does not increase as it

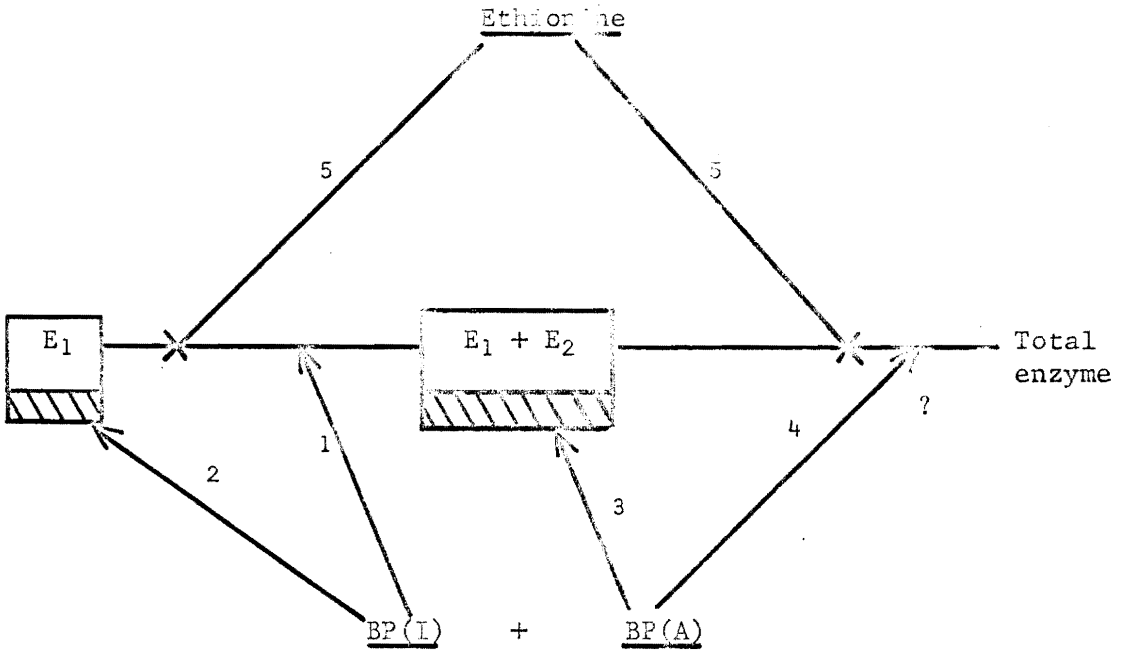
does with BP(1X) alone and as a result, a value (14.62) close to that of the control group is obtained. K_m is decreased from a control value of 2.95 to a value of 0.27. This decrease is greater than the decrease obtained with BP(1X) alone. From these results a question related to the properties of ethionine was posed. Does ethionine, in addition to inhibiting protein synthesis, also activate the enzyme? A control group which received ethionine alone (Table VI) was run in the second experimental series but the velocities were so low and erratic that no reasonable values could be obtained. Computer analysis of the initial velocity data at three substrate concentrations gave very high standard errors. The values obtained for K_m and V_{max} were 12 ± 7 and 49.7 ± 31, respectively. Although not much can be said from these values because of the standard errors, the values do suggest that ethionine has no effect on the K_m. The third series of experiments was conducted for the twofold purpose (1) of obtaining a better picture of the effects of ethionine on the K_m value and (2) of determining whether the activation (decrease in K_m) following BP pretreatment is a phenomenon related to the simultaneous presence of inducer. The K_m value of the new enzyme (increased V_{max} following administration with an initial dose of BP) should be expected to decrease with an additional dose of BP, if the activation is dependent upon the simultaneous presence of inducer. The results for this series of studies and the Lineweaver-Burke plots are given in Tables XXXVIII and XXXIX and Figures 14 and 15. The values of K_m and V_{max} for the control group (basal diet) from this series are 8.96 ± 2.83 and 14.86 ± 3.64, respectively. The values obtained for the BP(3X) group are similar

to the values obtained for BP(3X) of the second experimental series. When ethionine is given 24 hours after an initial dose of BP(3X) and the animals killed at 48 hours, the values obtained for K_m and V_{max} (BP(3X)+ET) are not different from the values for the BP(3X) group measured at 24 hours. This suggests that not only does the ethionine have no effect on K_m but also the turnover rate of microsomal BP hydroxylase is low during the 24 hour period. Low turnover rates for microsomal enzymes have been reported by Conney and Gilman (1963) and Kato et al. (1961). When a booster dose of BP(1X) is given 24 hours after the initial dose of BP(3X), i.e., the BP(3X)+BP(1X) group (animals killed at 48 hours), values obtained for V_{max} are not different from the original BP(3X) group, but the K_m value of 2.69 $\pm$ 0.55 for the BP(3X) group is reduced to a value of 1.00 $\pm$ 0.37. The significance of this reduction in K_m with a booster dose of BP(1X) becomes more evident when the average value (3.13) of K_m for the BP(3X) group (second experimental series) and BP(3X) and BP(3X)+ET (third experimental series) are compared with the K_m value of 1.00 $\pm$ 0.37 for the BP(3X)+BP(1X) group. Furthermore, the results obtained with laboratory chow (Table XXXVIII and Figure 14) are quite different from the control animals receiving semipurified 20% casein diet. The V_{max} is almost doubled whereas the K_m is significantly reduced although the latter falls within the range of values for control groups from the second and third experimental series, (compare Tables VI and VIII for weights and ages of rats used in second and third experimental series). These effects on V_{max} may help to explain at least in part why no significant increase in V_{max} was obtained with BP(1X) in the first

series of experiments (Table XXXIV and Figure 9), in which the rats received laboratory chow instead of 20% casein diet. It would appear that the V_{max} was already elevated to a plateau level (see also the control V_{max} value in Table XXXIV) and BP only affected the K_m, whereas B_1 decreased both K_m and V_{max} as is consistent with its properties.

On the basis of the results discussed so far it is tempting to formulate a hypothesis which may not only explain all the phases of induction due to administration of polycyclic aromatic hydrocarbons, including aflatoxin B_1, but also help to explain some of the inconsistent observations reported by other workers in this field. The schematic representation of the hypothesis is shown on the following page.

It can be seen from the scheme that BP(I) activates the already existing enzyme as well as induces the synthesis of new enzyme. BP(A) will also activate the enzyme already present but will cause no further increase in enzyme synthesis if the plateau in induction has been reached by BP(I). In the third series of experiments, the V_{max} of BP(3X) and BP(3X)+<u>BP(1X)</u> and BP(3X)+<u>ET</u> are not different from each other while the K_m of BP(3X)+<u>BP(1X)</u> is reduced significantly as compared to the K_m of BP(3X) or BP(3X)+<u>ET</u> (Table XXXVIII). These observations and the observations recorded with BP(1X)+ET (Table XXXVI) indicate that ethionine acts mainly as a protein inhibitor and does not cause any decrease in K_m, whereas the additional dose of BP decreases the K_m of the enzyme already present in the system. The question now arises of whether the K_m of the BP(1X)+ET group should be as low as 0.27 ± 0.11 in contrast to a value of 1.01 ± 0.13 obtained with the



▨ = Activation of existing enzyme by BP(I).

E_1 = Old enzyme (as in control).

E_2 = New enzyme synthesized after BP administration.

$E_1 + E_2$ = The proportion of old enzyme to new is low depending upon dosage of BP administered.

BP(I) = Initial dosage of BP.

BP(A) = Additional dosage of BP after a given time interval.

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[1] De novo synthesis of enzyme indicated by increase in V_{max}.

[2] Activation of enzyme by lowering its K_m.

[3] Additional dose of BP activates the enzyme already present in the system either directly or indirectly.

[4] Additional dose of BP has no effect on enzyme synthesis if plateau in induction has already been reached with BP(I).

[5] Inhibits new protein synthesis.

BP(1X) group (Table XXXVI). It can be seen from the same table that BP(1X) increased the V_{max} 14- to 17-fold, whereas the V_{max} of the BP(1X)+ET group is close to the control value. This suggests that since the total enzyme content of the BP(1X)+ET group is very much less than that of the BP(1X) group, the much greater reduction in K_m is due to the presence of a large amount of BP as compared to a low enzyme concentration. The same hypothesis, with some reservations, can explain the effects due to pretreatment with different dosages of B_1. The K_m and the V_{max} values which are lower at the B_1(1X) level of pretreatment approach the control values when the dosage of B_1 is increased from 1X to 3X (Table XXXVI and Figures 10 and 13). These results fit the above hypothesis if we postulate that the reduction of V_{max} at B_1(1X) level could result from a decrease in the rate constant(s) for the catalytic step(s). A decrease in the rate constants involved in the breakdown of the enzyme-substrate complex(es) would usually lead to a lower V_{max}. This would also augment any decrease in K_m that might result from the direct or indirect "interaction" of the inducer with the biological structure. In turn, the dissociation constant(s) might then be reduced, which would be expressed in terms of a decrease in K_m. This decrease in V_{max} due to the inhibition of rate constant(s) for the breakdown of enzyme substrate complex(es) could be compensated for if new enzyme synthesis was induced by the inducer at the same time. This is probably what happens when B_1(2X) and B_1(3X) are given. Even at the B_1(1X) level, there is most likely some induction of new enzyme but this probably is not enough to compensate for the decrease in V_{max} resulting from the reduction in rate

constant(s). The newly formed enzyme, which is comparable in properties to the old enzyme, will increase the K_m of the overall enzyme if there would be no "interaction" between the new enzyme and the inducer or if this "interaction" was a slow time dependent phenomenon. For such an interaction to take place, the assumption is made that the enzyme would presumably have to be already available in the system while the inducer is still present. Such an observation is the essence of the hypothesis.

Varied and conflicting reports are available regarding the effects of B_1 on *in vivo* and *in vitro* protein synthesis. Smith (1963) reported marked inhibition of leucine incorporation into the protein of rat and duckling liver slices incubated simultaneously with B_1 and labelled amino acid. Wogan and Friedman (1965) reported the inhibition with B_1 of rat liver tryptophan pyrrolase induction caused by hydrocortisone. Recently, however, Shank and Wogan (1966) have reported a biphasic effect on protein synthesis. In their *in vivo* studies they found an inhibition of leucine incorporation (following a single dose of B_1) during the first six hours after treatment followed by a return to the pretreatment level at 24 hours and a marked increase in incorporation was apparent on days two through five after dosing.

Now let us proceed to examine some of the observations reported by other workers in this field. Rubin *et al.* (1964) reported that phenobarbital increased the V_{max} of ethylmorphine N-demethylase, chlorpromazine sulfoxidase and hexobarbital oxidase, but did not influence the K_m. Netter and Seidel (1964) also reported an increase in V_{max} and no change in K_m of p-nitroanisole O-demethylase following

pretreatment with phenobarbital and BP. As far as BP is concerned, Netter and Seidel (1964) injected intraperitoneally more than 20 milligrams of BP per kilogram body weight. From our data it is clear that with such doses of BP the proportion of new enzyme formed is far in excess of the old enzyme with the result that the V_{max} is greatly increased whereas the K_m of the enzyme mixture containing only negligible quantities of old but activated enzyme is essentially not changed. In the studies reported in this thesis, BP(3X) has a very high V_{max} compared to controls but has the same K_m as controls (Table XXXVI). An alternative explanation might be that p-nitroanisole O-demethylase may be an exception to this hypothesis, especially if this enzyme does not metabolize polycyclic hydrocarbons. Also, the phenobarbital which is used shows somewhat different inducer properties from BP and may make this case an exception from the hypothesis referred to above. Arcos et al. (1961) studied the relationship between the structure of approximately 57 polycyclic aromatic hydrocarbons and their ability to induce enzyme systems that (a) N-demethylate 3-methyl-4-monomethylaminoazobenzene, (b) hydroxylate zoxazolamine, and (c) have the ability to shorten the duration of action of a paralytic dose of zoxazolamine. These hydrocarbons were injected on an equimolar basis. They found that the optimal size for enzyme induction ranged from 85 to 150 A° units and coplanar hydrocarbons were more potent than non-coplanar. This observation is consistent with our hypothesis as it makes apparent the importance of stearic fit between the hydrocarbon molecule and the biological structure with which it "interacts". However, Conney and Burns (1960) have reported that phenobarbital and BP

did not increase the activities of the microsomal enzymes when added *in vitro* and no evidence was found that either BP or phenobarbital altered the levels of a possible activator or an inhibitor in liver. Conney *et al.* (1957) have also reported that the preincubation of liver homogenates from untreated rats with BP at 37° C for 60 minutes before the assay had no effect on their activities. These reports in terms of enzyme studies do not prove or disprove anything as the authors have used only one time interval and one substrate level to study the velocity or conversion rates. If the time interval used were beyond the linear range of reaction and/or if the substrate level used were high enough (at or near V_{max}) then preincubation with BP *in vitro* will not increase the conversion rate contrasted to controls (since enzyme synthesis will not occur *in vitro*). Furthermore, the effect of a decreased K_m will not become evident at or near V_{max} substrate levels. It would be interesting to study the kinetics of a microsomal enzyme system in the presence of a constant amount of BP or 3-MC in order to find the effect on K_m as well as to see if the Lineweaver-Burke plot would be linear or either convex or concave up, which would mean either allostearic activation or the involvement of two enzyme systems with different K_m values. The possibility that polycyclic hydrocarbons may cause indirect activation by inducing the synthesis of an activator molecule of some nature, e.g., nucleic acid, cannot be excluded. Under such circumstances no effect may be seen by the *in vitro* addition of the compound. Axelrod (1955) has reported the existence of heat stable activator factors and heat labile inhibitory factors in rat liver microsomes. Alvares *et al.* (1967) have

mentioned in their paper that Bresnick et al. (1967), while investigating the distribution of labelled 3-MC within hepatocytes, observed some labelling of the microsomal fraction and the labelled material appeared to be protein bound. Ernster and Orrenius (1965) have reported both in vivo and in vitro binding of the labelled drugs such as C^{14}-phenobarbital and/or C^{14}-aniline to microsomes.

In a report by Gillette (1963), dosages of BP, 3-MC and phenobarbital that were maximal for their "inductive" effects were administered to rats either alone or in pairs. The liver microsomes were found to be more active in metabolizing drugs when BP and phenobarbital were given together than when either had been given separately. On the other hand, activity was about the same when the inducers used were BP and 3-MC, together or separately. Such an observation can be explained on the basis that when BP and phenobarbital are given together there is activation of the enzyme due to BP, in addition to de novo synthesis of enzyme by both inducers; however, the possibility that the de novo synthesis of microsomal enzymes is greater when maximal doses of both the inducers are given together than when the maximal dose of either is given alone cannot be excluded. Alvares et al. (1967) reported in their first paper a shift in the 450 mμ peak of dithionite reduced carbon monoxide binding pigment of liver microsomes to 448 mμ on treatment of rats with 3-MC and this they attributed either to the possible binding of 3-MC with microsomes in vivo or the synthesis of a new hemoprotein. In their subsequent paper, Alvares et al. (1968) studied the effects of 3-MC and phenobarbital treatment on the kinetic parameters of BP hydroxylase and reported a decrease in K_m following

pretreatment with 3-MC and not phenobarbital. From these studies they concluded that the shift to 448 mμ was possibly due to the formation of a new hemoprotein which was also responsible for the decrease in the K_m value of BP hydroxylase. Our data support the formation of a new enzyme in general and also the decrease in K_m following pretreatment with polycyclic hydrocarbons.

SUMMARY

Studies were undertaken to determine the effect of pretreatment with 3,4-benzpyrene (BP) and aflatoxin B_1, (B_1) (metabolite of Aspergillus flavus) on the subsequent kinetic properties of rat liver microsomal benzpyrene hydroxylase and to investigate the mechanism of inductive effects caused by such pretreatment. Different groups of rats were pretreated with different dosages of either BP or B_1 or a combination of BP and B_1 or BP and ethionine (ET). Rats were decapitated 24 or 48 hours following pretreatment and the livers were excised, weighed, pooled for each group, and homogenized in cold 0.05 M potassium phosphate buffer containing 0.25 M sucrose (pH 7.4). Microsomes were isolated as pellets by centrifugation at 105,000 x g for 90 minutes. Isolated microsomes were resuspended in buffer after being washed twice with buffer.

In the presence of a TPNH generating system and with ^{3}H-BP as the variable substrate, initial velocities for the disappearance of BP were determined for different groups of microsomes. Initial velocity data were analyzed by the computer program of Cleland (1963) and the values for K_m, V_{max} and $K_{/V}$ determined. The following observations are reported:

1. The great increase in the V_{max} (14- to 17-fold with BP(1X)) paralleled the increase in the dosage of BP administered.

2. BP, in addition to greatly enhancing the V_{max}, decreased the K_m values.

3. ET (protein inhibitor) when administered along with BP blocked the increase in V_{max} but did not block the effect on K_m.

4. The effect of BP on the qualitative properties (K_m) of the enzyme was a slow time dependent phenomenon which required the simultaneous presence of both the enzyme and the inducer.

5. The effects of B_1 pretreatment on the K_m and the V_{max} followed the same trend as BP except that the effect on V_{max}, although well correlated with dosage of B_1, was somewhat more attenuated compared to BP. An inhibition of V_{max} was obtained with the B_1(1X) dosage level and as the dosage of B_1 increased from B_1(1X) to B_1(3X), the V_{max} increased to a value not significantly higher than the control. The possible reasons for this V_{max} effect are elaborated.

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STUDIES ON MECHANISMS INVOLVED IN THE INDUCTION OF MICROSOMAL BENZPYRENE HYDROXYLASE FOLLOWING PRETREATMENT WITH 3,4-BENZPYRENE AND AFLATOXIN B_1

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ABSTRACT

Microsomes obtained from different groups of rats, following pretreatment with different dosages of 3,4-benzpyrene (BP), aflatoxin B_1 (B_1), ethionine (ET) and/or a combination of these, were used to obtain initial velocity data for BP disappearance. The values for K_m, V_{max} and $K_{/V}$ for different groups were obtained by the computer analysis of initial velocity data. V_{max} and K_m values were used to assess the quantitative and qualitative changes of microsomal BP hydroxylase, respectively, caused by pretreatment. BP pretreatment greatly enhanced the V_{max} and also decreased the K_m values. The increase in V_{max} paralleled the increase in the dosage of BP from BP(1X) to BP(3X). When ET, a protein inhibitor, was given in conjunction with BP it blocked the increase in V_{max} but did not block the effect of BP on the K_m. The effect of BP on the qualitative properties (K_m) of enzyme was observed to be a slow time dependent phenomenon which required the simultaneous presence of both the enzyme and the inducer.

The effects of B_1 pretreatment on the K_m and V_{max} values of microsomal benzpyrene hydroxylase followed the same general trend as BP except that the effect on V_{max} was somewhat more attenuated. An inhibition of V_{max} was observed with the B_1(1X) level and as the

dosage of B_1 increased from B_1(1X) to B_1(3X), the V_{max} increased to an insignificantly higher value compared to control. The possible reasons for this effect on V_{max} are probably related to the properties of the enzyme and the B_1.