AMINOPYRINE DEMETHYLASE: KINETIC EVIDENCE FOR MULTIPLE MICROSOMAL ENZYMES

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ABSTRACT

AMINOPYRINE DEMETHYLASE: KINETIC EVIDENCE FOR MULTIPLE MICROSOMAL ENZYMES

By

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The Lineweaver-Burk plots of rat liver microsomal aminopyrine demethylase activity are non-linear. The curve is characteristic of a reaction catalyzed by two enzymes. Pretreating animals with phenobarbital stimulates the demethylase activity and produces a linear reciprocal plot with an apparent Km for aminopyrine of 7×10^{-4}M. Pretreatment with 3-methylcholanthrene causes no stimulation but increases the apparent Km for aminopyrine by an order of magnitude or more. 3-Methylcholanthrene *in vitro* has no effect on the aminopyrine demethylase activity and the changes in kinetic behavior following 3-methylcholanthrene treatment are prevented by administration of ethionine. The inhibitor, SKF-525A, at a concentration of 4×10^{-5}M, differentiates between the demethylase activities present in the two types of induced

animals, inhibiting the activity found in microsomes of phenobarbital induced rats but having little effect on the activity in microsomes from 3-methylcholanthrene treated rats. These results and the results of other investigators which suggest the existence of multiple drug metabolizing activities are discussed.

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Thomas C. Pederson

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LIST OF ABBREVIATIONS

AP	aminopyrine
i.p.	intraperitoneally
MAP	4-monomethylaminoantipyrine
3-MC	3-methylcholanthrene
NAOH	nicotinamide adenine dinucleotide reduced
NAD (H)	nicotinamide adenine dinucleotide phosphate (reduced)
OD	optical density
PB	phenobarbital
SKF-525A	2-diethyaminoethyl-2,2-diphenylvalerate

INTRODUCTION

The endoplasmic reticulum of liver contains an enzyme system or group of enzyme systems which, in the presence of NADPH and O_2 will metabolize a large number of drugs, steroids, and carcinogenic compounds.[1-3] The variety of transformations: aromatic and aliphatic hydroxylation, N- and O- dealkylation, deamination, sulfoxidation, and N-oxidation, can all be visualized as variations of hydroxylation.[3,4] The requirement for NADPH

$$R\text{-}H + NADPH + H^+ + O_2 \rightarrow R\text{-}OH + NADP^+ + H_2O$$

and molecular oxygen suggests that this enzyme system may be classified as a mixed function oxidase by the terminology of Mason.[5] The incorporation of atmospheric oxygen and not water oxygen has been demonstrated to occur in the conversion of acetanilide to p-hydroxy-acetanilide and of trimethylamine to trimethylamine oxide.[6,7] The demonstration of atmospheric oxygen incorporation into most other substrates has not been possible because of the rapid rate at which the incorporated oxygen exchanges with water.

The identification of the oxygen activating component involved in this system began with the discovery of an unique CO binding hemoprotein in liver microsomes by Klingenberg[8] and Garfinkel[9] which was partially characterized by Omura and Sato.[10] The cytochrome was called cyt. P-450, or P-450, because of its absorbance at 450 mμ when reduced and saturated with CO. The role of this cytochrome in liver microsomal drug metabolism was demonstrated by Cooper et al.[11] The microsomal flavo-protein NADPH-cyt C reductase has been implicated as part of the electron transport chain transferring electrons from NADPH to P-450.[12] A similar multienzyme oxidase system in the mitochondria of the adrenal cortex responsible for steroid hydroxylations has been separated into three components: a flavo-protein, a non-heme iron protein, and a P-450 complex.[13] Attempts to identify other components involved in hepatic drug metabolism have not been successful. Most attempts at dissociating the microsomal components destroy all activity and P-450 is converted to an inactive form called P-420.[10] A method which partially solubilizes the drug metabolizing enzymes, involving the use of salts and detergents in the presence of protecting

agents, has been developed by Lu and Coon[14] and modified by Rikaus and VanDyke.[15]

In addition to the broad substrate specificity, another important property of the liver drug metabolizing system is its inducibility. The enhancement of liver microsomal drug metabolizing activity after treatment of the animals with polycyclic hydrocarbons such as benzpyrene and 3-methylcholanthrene was first described in 1954 by Brown, Miller and Miller.[16] Subsequent investigation indicated that the activation was the result of increased synthesis of drug metabolizing enzymes.[12,17] In 1959 it was reported that phenobarbital and other drugs acted as inducers of drug metabolizing activity.[18,19] Since then, over two-hundred drugs, insecticides, carcinogens and other chemicals have been reported to stimulate the drug metabolizing activity in liver microsomes.[20]

One of the more unusual properties of the microsomal drug metabolizing system is its ability to carry out transformations of an extremely wide variety of structurally unrelated substrates which is difficult to understand in view of the common concept of substrate specificity as found in other enzyme systems. The large number of compounds which are metabolized could mean at one

extreme that there is a specific oxidase for each type of compound or at the other extreme, a single oxidase of remarkable nonspecificity. The first concept is difficult to accept for teleological reasons. The second concept, while supported by many general characteristics of the system, is becoming increasingly untenable.

The suggestion that liver microsomes contain more than one enzyme system for the oxidation of drugs and other foreign compounds was first proposed to explain the differential induction by phenobarbital and polycyclic hydrocarbons. Phenobarbital stimulates the metabolism of many compounds whereas, induction by 3-methylcholanthrene stimulates the metabolism of relatively few compounds.[20] There are also marked species and sex differences in the metabolism of various drugs.[21-23] Investigations of substrate competition by Rubin et al.,[24] have shown that for several compounds their K_i as an inhibitor was not the same as their K_m for metabolism, and some substrates were unable to inhibit the metabolism of other compounds.

The multiple enzyme hypothesis has been supported by spectral studies which suggest that liver microsomes contain more than one form of P-450. This was first proposed by Imai and Sato[25] who found that two spectral species

are detectable when the reduced cytochrome interacts with ethylisocyanide. It has also been found that the spectral properties of P-450 from 3-methylcholanthrene induced rats differs from that obtained with microsomes from non-induced rats. The absorption maximum of the CO difference spectrum of reduced P-450 shifts from 450 mμ to 446 mμ and the ratio of the two absorption bands (455 and 430 mμ) of the ethylisocyanide difference spectrum shifts in favor of the absorption at 455 mμ.[26,27] However, attributing these results to the existence of more than one P-450 has been questioned by experiments which indicate that the various spectral forms are interconvertible.[28]

Difference spectra characteristic of heme proteins are also produced by the addition of substrates of the drug metabolism system to the oxidized form of P-450. There are two types of substrate difference spectra: type one has a peak at 420-430 mμ and a trough at about 390 mμ, type two spectra have a trough at about 420 mμ and a peak at 385 mμ.[29,30] Most substrates examined, including aniline, phenobarbital and monomethylaminoantipyrine, have a type one difference spectra. Substrates which have a type two difference spectra include phenacetin, dihydrosafrole and aminopyrine. It is likely that these

spectral changes occur as a result of the substrate binding to a non-heme moiety of P-450. Similar spectra can be produced by adding organic solvents such alcohols having short carbon chains.[31]

Aminopyrine (4-dimethylamino-1, 5-dimethyl-2-phenyl-3-pyrazolone) is a drug which is frequently used as a substrate to measure N-demethylase activity in liver microsomes. It is an analgesic and antipyretic drug which at one time was used to treat the symptoms of a variety of diseases including rheumatic fever, but its occasional toxicity led to its gradual disuse.[32] When the drug was administered to humans, almost all of it was altered in the body before excretion, the major metabolite being 4-aminoantipyrine (4-amino-1,5-dimethyl-2-phenyl-3-pyrazolone). The location of this transformation was first indicated by Brodie and Axelrod[33] who reported that rabbit liver slices and homogenates convert aminopyrine to 4-aminoantipyrine. It was subsequently demonstrated by LaDu et al.,[34] that liver homogenates of rabbit, rat, and guinea pig would dealkylate aminopyrine, monomethylaminoantipyrine, and their ethyl and butyl analogs to form 4-aminoantipyrine and the corresponding aldehyde. Both O_2 and NADPH were required and the activity was

located in the microsomal fraction. They also reported that the N-dealkylation activity could be inhibited by SKF-525A. The metabolism of aminopyrine by liver microsomes was further characterized by Ernster and Orrenius[12] who showed that equivalent amounts of NADPH, O_2, and substrate were used during the reaction and that the demethylase activity was stimulated following induction of the metabolism of other drugs by phenobarbital. Studies by Gram, Wilson and Fouts[35] have suggested that the removal of one methyl group to form monomethylaminoantipyrine occurs as a fast reaction followed by a slower reaction to form 4--aminoantipyrine. The role of P-450 in these reactions was confirmed by Cooper et al.,[11] who showed that the CO inhibition of demethylation could be reversed by monochromatic light at 450 mμ. The aminopyrine demethylase activity present in rat liver during different stages of growth has been studied by Soyka[36] who found that newborn rats had very little activity, however, during the first 30 days after birth, the activity increased 3-fold. A similar increase in aminopyrine demethylase activity occurred again in male rats at the age of puberty. This is apparently the result of induction by the sex hormones, testosterone and androsterone which are also

metabolized by the microsomal system.[37,38]

This thesis presents evidence suggesting that liver microsomes contain more than one oxidase system capable of demethylating aminopyrine. Attempts to determine saturating concentrations of aminopyrine for demethylation consistently showed that the activity continued to increase as the substrate concentration became very high. Attempts to explain this non-enzymatically were without success. The subsequent kinetic analysis and study of the effects of induction and inhibition on aminopyrine demethylase activity in rat liver microsomes support the thesis that liver microsomes contain multiple drug metabolizing systems with varying substrate specificity.

MATERIALS AND METHODS

Chemicals

Aminopyrine was purchased from K and K Laboratories, Inc., Plainview, N.Y. and either recrystallized or used as received since identical results were obtained. Phenobarbital was purchased from Merck and Co., Inc., Rahway, N.J. Benzpyrene was purchased from Aldrich Chem. Co., Milwaukee, Wisc. SKF-525A was a gift of the Smith, Kline and French Laboratory, Philadelphia, P. 3-Methylcholanthreme, D, L-ethionine, D, L-isocitrate, $NADP^+$, NADPH, NADH, and NADP-isocitrate dehydrogenase were all purchased from Sigma Chem. Co., St. Louis, Mo. 4-Monomethylaminoantipyrine was received as a gift from the Sterling Winthrop Drug Co., New York, N.Y. and purified by column-chromatography on silica gel-G. Carbon monoxide was obtained from the Matheson Co., Inc., Joliet, Illinois. Ethyl isocyanide was synthesized in our lab.

Animals

Initial experiments were done with microsomes isolated from the livers of male goats because of their high P-450 content and N-demethylase activity. The majority of experiments have been done using male rats of the Holtzman strain weighing between 200 and 250 g. Animals induced with PB were given daily i.p. injections of 50 mg/kg in water for 5 days prior to sacrificing. Animals treated with 3-MC were given a single injection, i.p., of 20 mg/kg in corn oil 24 hours prior to being sacrificed. Rats treated with ethionine were given injections i.p. of 500 mg/kg 60 and 30 minutes before injection of inducer, according to the method of Alvares et al.[26] The ethionine was dissolved in water by raising the pH to about 10, which did cause pain when injected into the rats but did not appear to cause any lasting effects.

Preparation of Microsomes

The animals were exsanguished and the livers perfused in situ by injection of 10 ml of cold 1.15% KCl into the portal vein within 1 or 2 cm of the liver. The liver was then removed, blotted, weighed, and minced by chopping

with a scissors. The minced tissue was homogenized in four volumes of 1.15% KCl containing 0.2% nicotinamide, added to inhibit $NADP^+$-ase, with about 5 strokes in a Potter-Elvehjem homogenizer equipped with a teflon pestle. The homogenate was centrifuged at 15,000g for 20 minutes and the precipitate containing the nuclear and mitochondrial fractions discarded. The microsomal fraction was isolated as a pellet by centrifuging the 15,000g supernatant at 105,000g for 90 minutes. The supernatant was discarded and the microsomes were resuspended in Tris-HCl buffer (0.05M; pH 7.5) containing 50% glycerol. In experiments in which the rats were not starved prior to being sacrificed, the microsomal pellet was carefully separated from the glycogen on the bottom of the tube by loosening the pellet in a small volume of buffer with a swirling action. The protein concentration of the resuspended microsomes varied between 30 and 50 mg/ml. Protein was assayed by the Lowry method.[39] All operations were performed at 0-5°. The microsomes were either used immediately or stored at -15° under N_2. These microsomes retained their full aminopyrine demethylase activity for several weeks providing they were kept anaerobic.

Aminopyrine Demethylase Assay

The N-demethylase activity was assayed by measuring the rate at which formaldehyde was produced using the Nash method.[40] In most experiments, to obtain valid expressions for the rate of formaldehyde production, fixed point assays were made at two or three-minute intervals over a ten-minute period. One ml aliquots were removed from the incubation mixtures and diluted into 1 ml of 10% trichloroacetic acid. After allowing time for protein precipitation (about 5 minutes) two ml of Nash reagent (2M $NH_4C_2H_3O_2$; 0.05 M CH_3COOH; 0.02 M 2,4-pentanedione) were added and the mixtures were heated at 50° for ten minutes. The assay mixtures were centrifuged at 1000g to remove precipitated protein and the O.D. of the supernatant at 412 mμ was determined using a Coleman Jr. Spectrophotometer equipped with a flow cell. The extinction coefficient used was 7.08 OD ml^{-1} of assay μM^{-1} of HCOH.

Reaction mixtures were incubated at 37° under air in a Dubnoff metabolic shaker, and unless otherwise stated contained microsomes (0.8 mg/ml), $MgCl_2$ (7mM), NADPH (0.5mM), Tris HCl (0.05M; pH7.5), and the desired levels of substrates and inhibitors. 3-MC was added in 25 μl

of acetone to 5 ml incubation mixtures. In several experiments, the NADPH was provided by a generating system containing: D,L-isocitrate (2mM), $NADP^+$ (0.1mM), and NADP-isocitrate dehydrogenase (0.05 units/ml).

Benzpyrene Hydroxylase Assay

The concentration of hydroxylated metabolites of benzopyrene was determined using a method similar to that of Nebert and Gelbain.[41] One ml aliquots were removed from the incubation mixtures and diluted into 1 ml of cold acetone. The acetone sample was extracted vigorously with 3.25 ml of hexane. Two ml of the organic layer were removed and extracted with 3 ml of 1 N NaOH. The relative concentrations of the hydroxylated metabolites of benzpyrene in the aqueous layer was determined by measuring the fluorescence at 522 mμ when excited at 396 mμ. Problems were encountered in trying to obtain accurate reproducable fluoresence values from the aqueous layers.

Difference Spectroscopy

The carbon monoxide and ethylisocyanide difference spectra of reduced P-450 were obtained with microsomes resuspended at a concentration of 2 mg/ml in 1.0M phosphate buffer (pH 7.5) containing 50% glycerol. The high ionic strength buffer and glycerol clarify the microsomal suspension and prevent the conversion of P-450 to P-420[10]. Microsomes in both sample and reference cuvettes were reduced by adding dithionate (~ < 1 mg). To obtain a CO difference spectra CO gas was bubbled into the sample cuvette until it was saturated. Ethylisocyanide difference spectra were obtained by adding about as much as could be dissolved in the phosphate glycerol buffer. The spectra were recorded by a Coleman-Hitachi Model 124 Spectrophotometer.

Synthesis of Ethylisocyanide

Ethylisocyanide was synthesized by the method of Jackson and McKusick.[42] One mole of silver cyanide was added with stirring to one mole of ethyl iodide in a 3 liter, 3 necked, round bottom flask fitted with a refluxing condenser and sealed stirrer. The mixture was

heated on a steam bath and stirred for about 2 hours until a viscous, homogeneous, brown liquid formed. The stirrer was raised above the liquid, the steam bath removed, 100 ml of water was added through the condenser, 2.75 moles of potassium cyanide in 85 ml of water was added and the mixture stirred for 10 minutes. The apparatus was rearranged for simple distillation, and the distillate was collected in a cooled receiver until the temperature of the solution in the distillation flash reached 115-120°. To the distillate 2.5g of NaCl was added, the aqueous layer removed and the crude product washed with two portions of water. The product was dried overnight over anhydrous sodium sulfate and then purified by fractional distillation. The distillate coming over at 76-77° was collected and saved. Reported boiling point is 79°. The yield was 24g, 44% of theoretical (reported yield 47-55%).

Caution! All operations in which ethylisocyanide is heated should be done behind a shield to protect the operator from the possibility of an explosion. Furthermore, the extremely vile odor and high volatility of ethylisocyanide require that all operations be performed in a hood.

EXPERIMENTAL

The N-demethylase activity of goat liver microsomes at various concentrations of aminopyrine is shown in the Lineweaver-Burk plot in Figure 1. The reciprocal plot deviates considerably from linearity at high substrate concentrations and the activity is increased by the addition of NADH. The requirement for both NADPH and NADH for maximal activity has been reported by other investigators.[1] No activity is observed with NADH alone, which indicates the effect of NADH is not due to transhydrogenase activity. Since the demethylation of aminopyrine involves the removal of two methyl groups, it was conceivable that the non-linear reciprocal plot could be explained by the existence of monomethylaminoantipyrine as a dissociable intermediate with altered kinetic parameters. The Lineweaver-Burk plot of MAP demethylase activity in goat liver microsomes is shown in Figure 2. The non-linear reciprocal plot, similar to that obtained with AP, indicates that the non-Michaelis-Menten kinetics must be a property of the microsomal enzymes.

Figure 1.--Lineweaver-Burk plot for the N-demethylation of aminopyrine by goat liver microsomes. Velocities are given as mμ moles of formaldehyde formed min^{-1}, mg^{-1} of microsomal protein. Substrate concentration is in moles $liter^{-1}$. The microsomal protein concentration in these assays was 0.72 mg/ml.

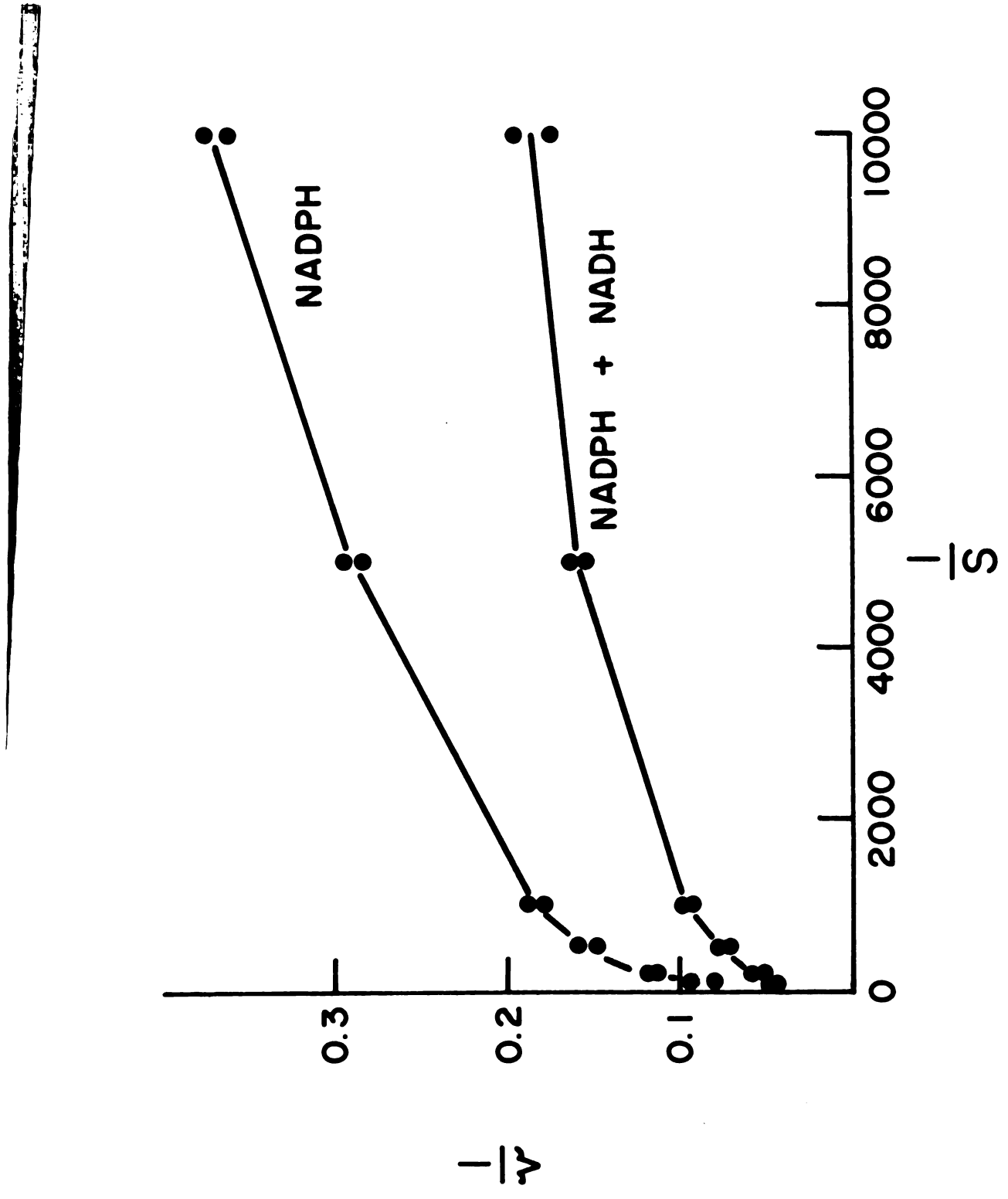

Figure 1

Figure 2.--Lineweaver-Burk plot for the N-demethylation of 4-monomethyl-aminoantipyrine by goat liver microsomes. The microsomal protein concentration in these assays were 0.75 mg/ml.

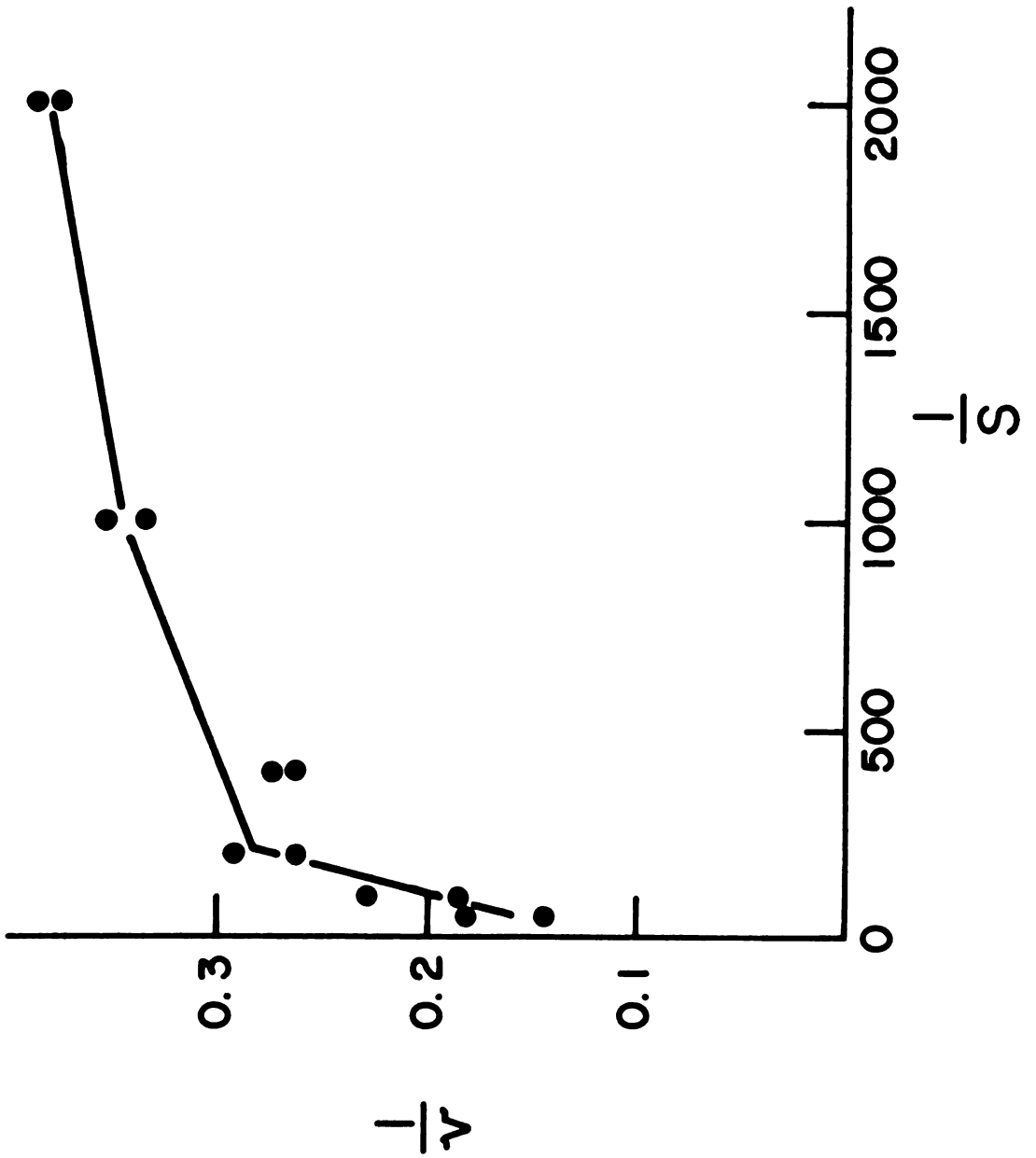

Figure 2

Aminopyrine demethylase activity in microsomes from control, PB or 3-MC treated rats, is shown in Figure 3. The curve obtained with microsomes from PB treated animals is linear and corresponds to an apparent Km of 7×10^{-4}M, in agreement with the Km of 8×10^{-4}M reported by Ernster and Orrenius.[12] The portion of the curve for control rat microsomes obtained at low substrate concentrations of AP yields a similar apparent Km, the apparent Km for aminopyrine obtained with microsomes from 3-MC treated animals is at least an order of magnitude greater. Although AP demethylase activity is not stimulated by 3-MC treatment, benzypyrene hydroxylase activity in these microsomes is stimulated about 5-fold. The effect of 3-MC on benzypyrene hydroxylation has previously been reported by Alvares et al.[43]

The CO difference spectra of the reduced cytochrome P-450 present in the microsomes of the untreated, PB and 3-MC treated rats are shown in Figure 4. As was originally reported by Alvares et al.,[26] there is an increase in the amount of P-450 present in microsomes from both PB and 3-MC treated rats and, in addition, the absorption maximum of the P-450 from the 3-MC treated animals has shifted to about 448 mμ. The ethylisocyanide

Figure 3.--Lineweaver-Burk plots for the N-demethylation of aminopyrine by male rat liver microsomes. Rats induced with 3-MC were given a single injection i.p. of 20 mg/kg in corn oil 24 hours before being sacrificed, and, rats induced with PB were given daily injections i.p. of 50 mg/kg in water 5 days prior to being sacrificed. Control rats were untreated.

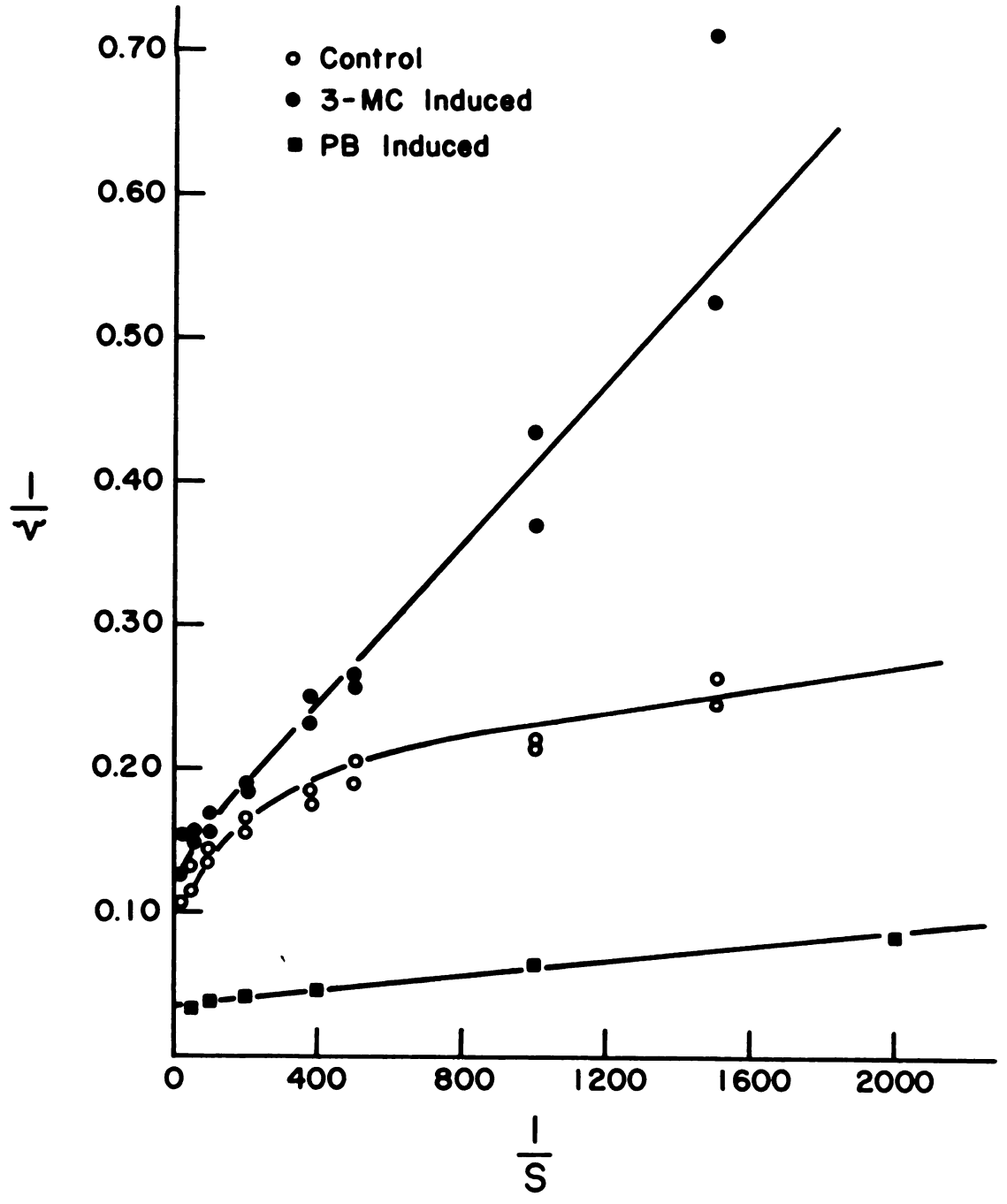
0.70
0.60
0.50
0.40
0.30
0.20
0.10
1/v
Control
3-MC Induced
PB Induced
0
400
800
1200
1600
2000
1/S

Figure 3

Figure 4.--CO difference spectra of dithionite reduced rat liver microsomes. The microsomal protein concentration in each case is 2 mg ml^{-1}. The microsomal suspension was clarified by using 1.0M phosphate buffer (pH 7.5) containing 50% glycerol.

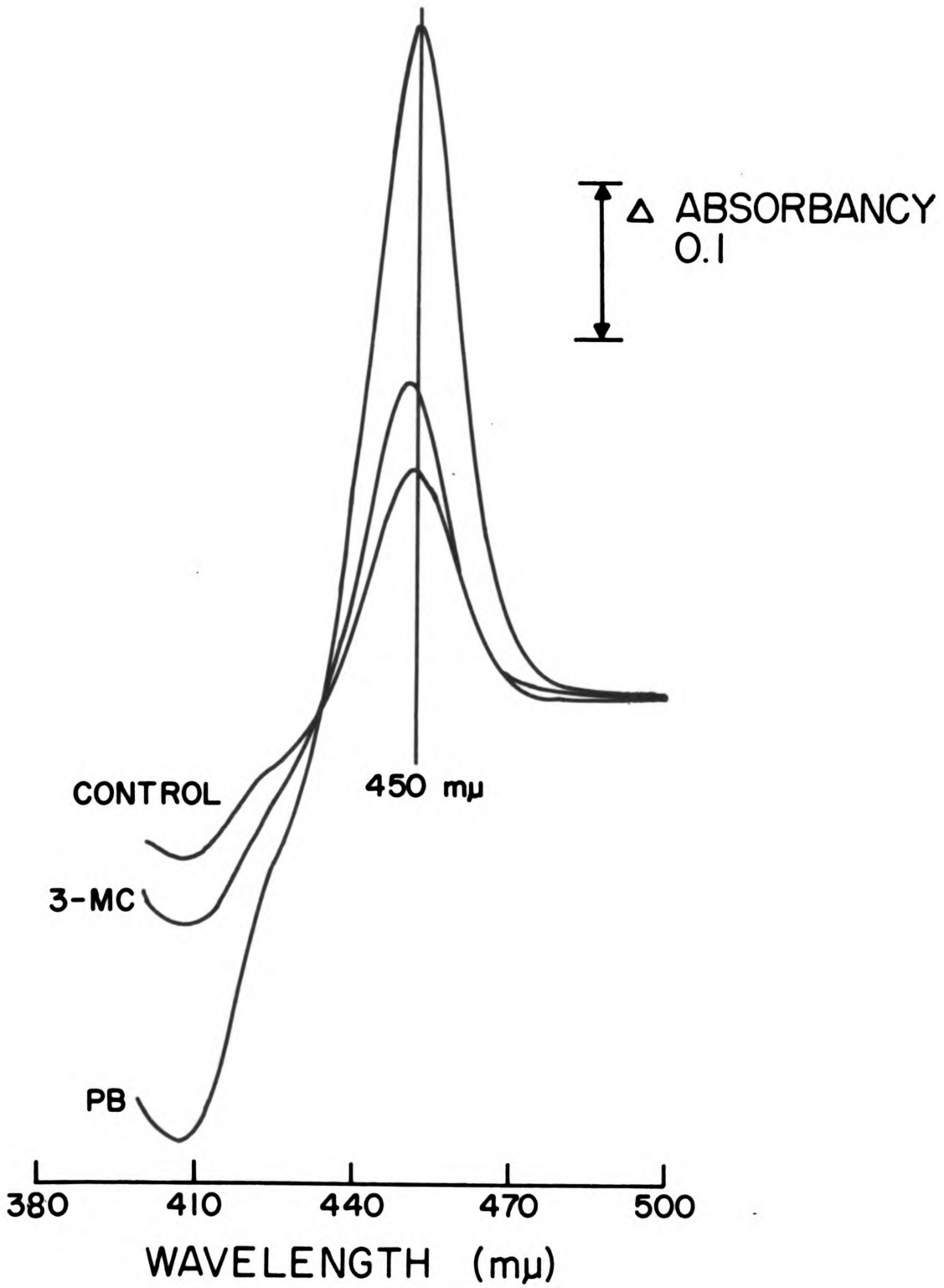
Δ ABSORBANCY
0.1
CONTROL
3-MC
PB
450 mμ
380
410
440
470
500
WAVELENGTH (mμ)

Figure 4

difference spectra of reduced P-450 are shown in Figure 5. Sladek and Mannering[27] reported that the ratio of the absorption at 455 mμ relative to 430 mμ increased in the microsomes from 3-MC treated animals. The spectra presented here have absorption maximum at 455 and 435 mμ which is apparently caused by the glycerol phosphate buffer. These spectral results, however, are of debatable value since, in addition to the dependence of the spectral properties on pH and ionic strength,[44] we were unable to saturate the P-450 with ethylisocyanide.

Since the increase in apparent Km following treatment with 3-MC could be the result of inhibition by traces of 3-MC or its metabolites remaining in the isolated microsomes, 3-MC was added to incubation mixtures containing microsomes from untreated animals. The results, shown in Figure 6, demonstrate that 3-MC at a concentrating of 5×10^{-5}M, failed to inhibit aminopyrine demethylation. The same results were obtained when the microsomes were preincubated with the 3-MC for five minutes. If all the 3-MC injected into the animals were still present in the isolated microsomes, the concentration in such incubation mixtures would be about 7×10^{-5}M.

Figure 5.--Ethylisocyanide difference spectra of dithionite reduced rat liver microsomes. The protein concentration in each case is 2 mg ml^{-1}. The microsomal suspension was clarified by suspension in 1.0M phosphate buffer (pH 7.5) containing 50% glycerol. The concentration of ethylisocyanide, which is not very soluble in this buffer, is about 0.35 mM.

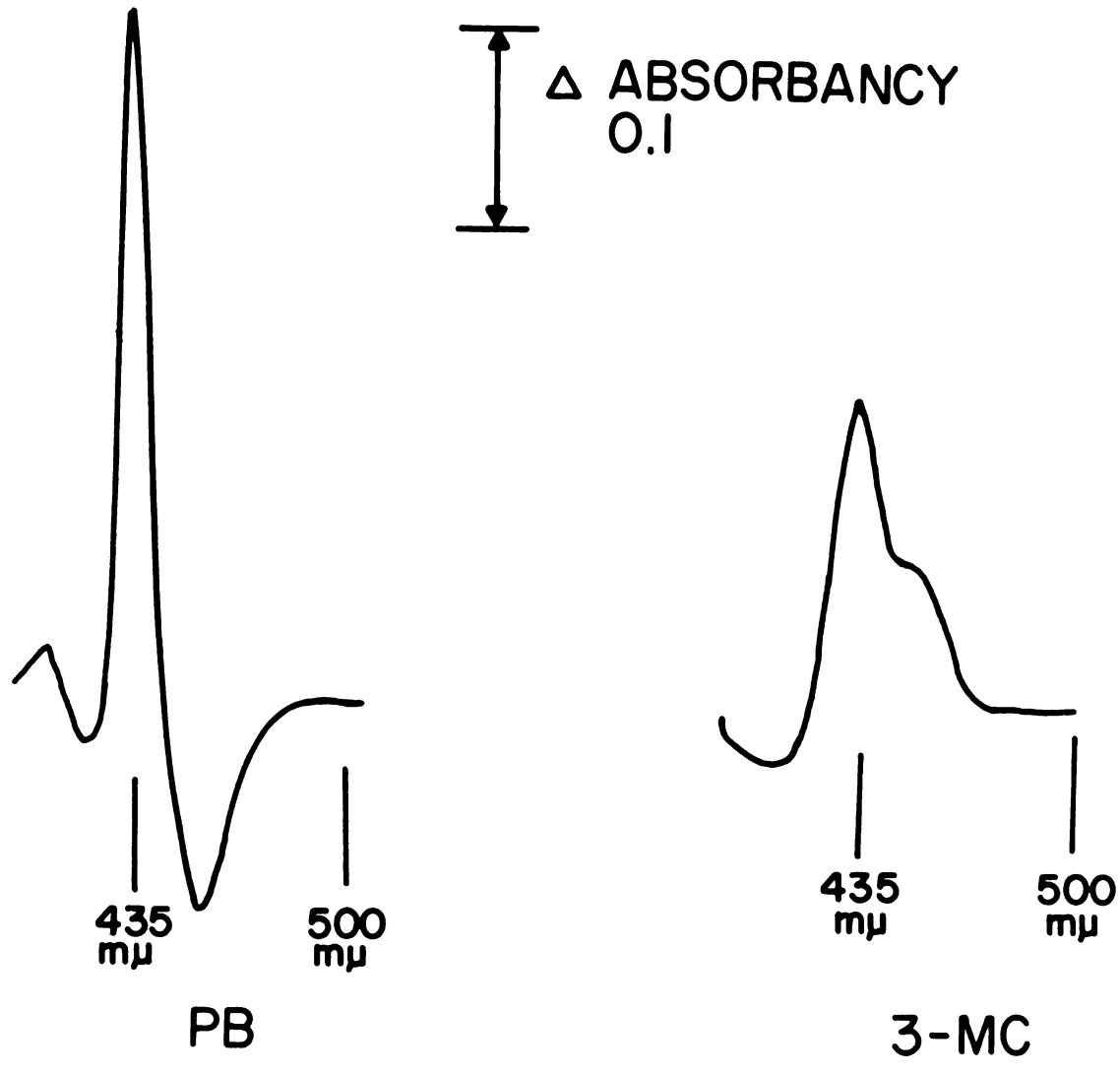

Figure 5

Figure 6.--Lineweaver-Burk plot for N-demethylation of aminopyrine by rat liver microsomes demonstrating the effect of 3-MC *in vivo* and *in vitro*. The 3-MC was added to the incubation mixtures in 25 µl of acetone. The same experiment was performed adding the 3-MC to the microsomes and incubating at 32° for 5 min before being added to the assay incubation mixtures. Identical results were obtained.

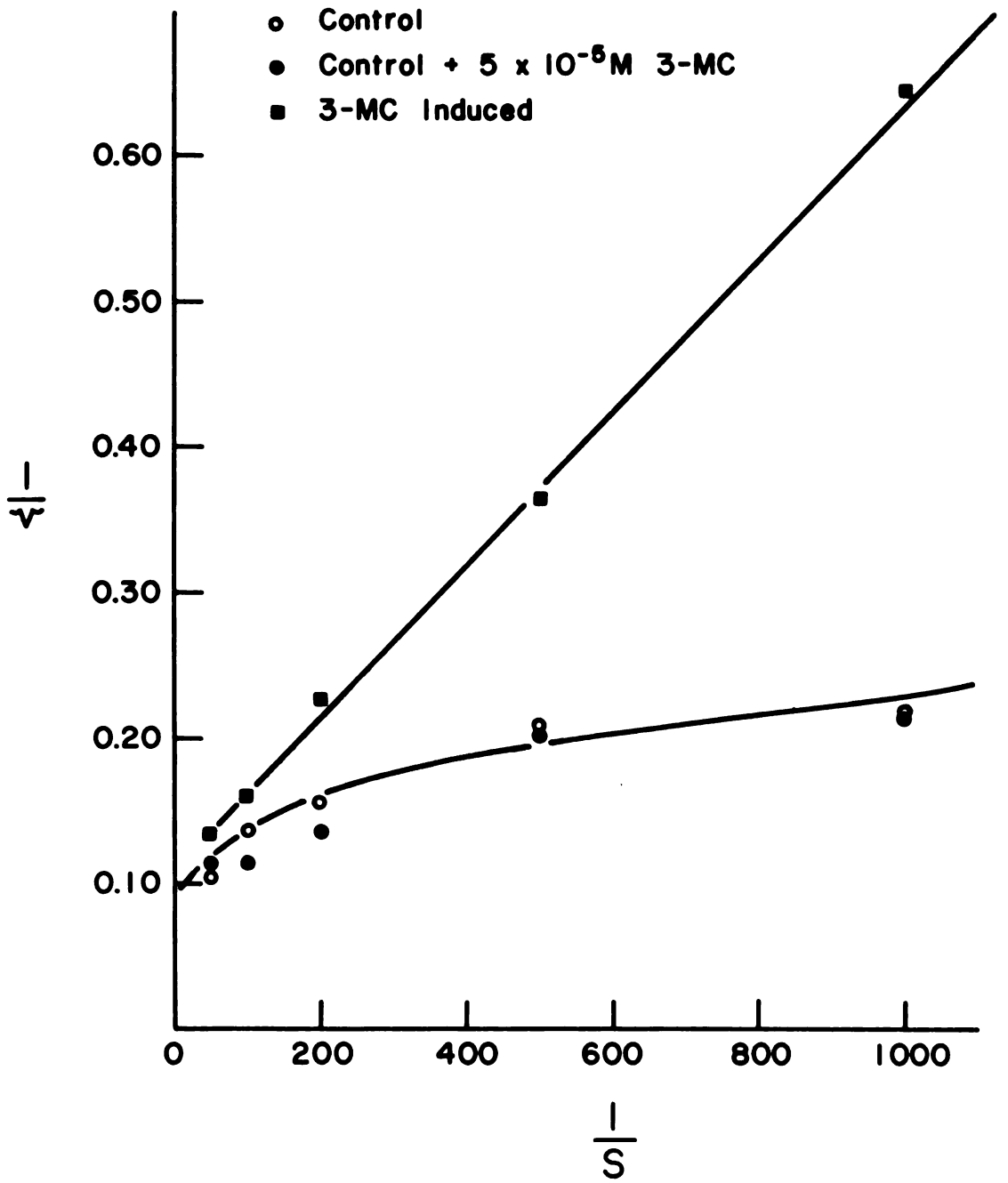
Control
Control + 5 x 10⁻⁵M 3-MC
3-MC Induced
0.60
0.50
0.40
0.30
0.20
0.10
1/V
0
200
400
600
800
1000
1/S

Figure 6

Attempts to demonstrate the presence of 3-MC or its metabolites in microsomes from 3-MC treated rats were not successful. The thin layer chromatogram of microsomal extracts viewed under UV light is shown in Figure 7. It can be seen that the extract from the microsomes from the incubation mixtures to which 3-MC was added produced, in addition to a fluorescent spot migrating like 3-MC, another spot near the origin. The spot at the origin could also be visualized by spraying the plate with $FeCl_3$ suggesting that it contains hydroxylated metabolites of 3-MC. Extracts from the microsomes of untreated and 3-MC treated animals produced no visible spots indicating that 3-MC is not present at levels detectable by this method.

The association between the change in apparent Km and induction was investigated by using ethionine to block induction. Alvares et al.,[45] had previously shown that ethionine and actinomycin D prevent the changes in spectral properties of P-450 following 3-MC treatment. The results of this experiment, shown in Figure 8, indicate that induction is closely associated with the change in kinetic properties.

Figure 7.--Thin layer chromatograms of microsome extracts viewed under U.V. light. Microsomes were extracted with benzene and benzene-hexane 1:1 and the amounts indicated were spotted from concentrated solutions containing the extracted material from 10 mg of microsomes ml^{-1}. The chromatographic solvent used was benzene-hexane 1:1 and the plates were layered with silica gel G containing a fluorescent indicator.

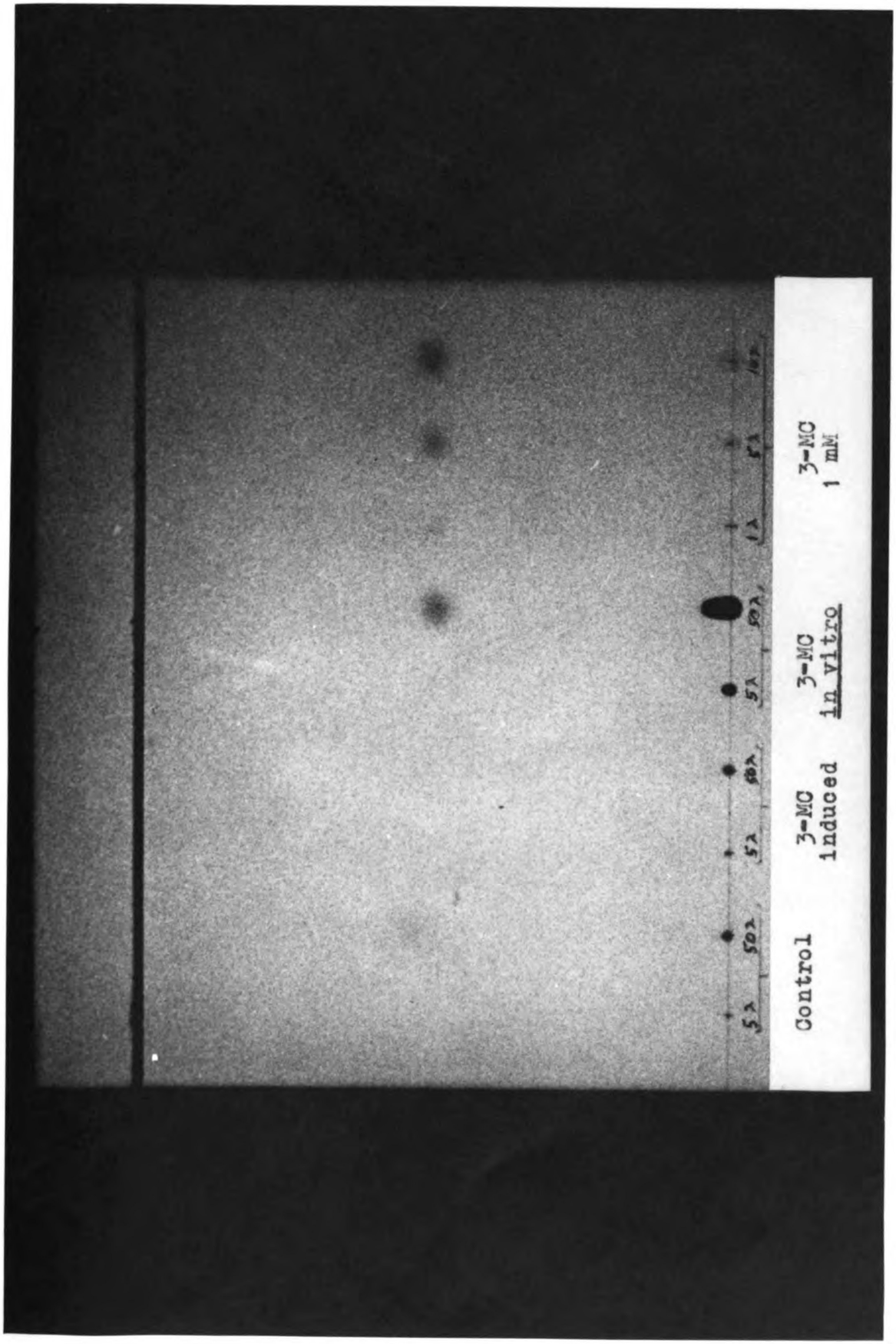

Figure 7

Figure 8.--Lineweaver-Burk plots for the N-demethylation of aminopyrine by rat liver microsomes showing the effect of ethionine on the changes produced by treatment with 3-MC. The animals were injected i.p. with ethionine, 500 mg/kg, 60 and 30 minutes before being injected with 3-MC, 20 mg/kg in corn oil, or corn oil and sacrificed 24 hours after the last injection.

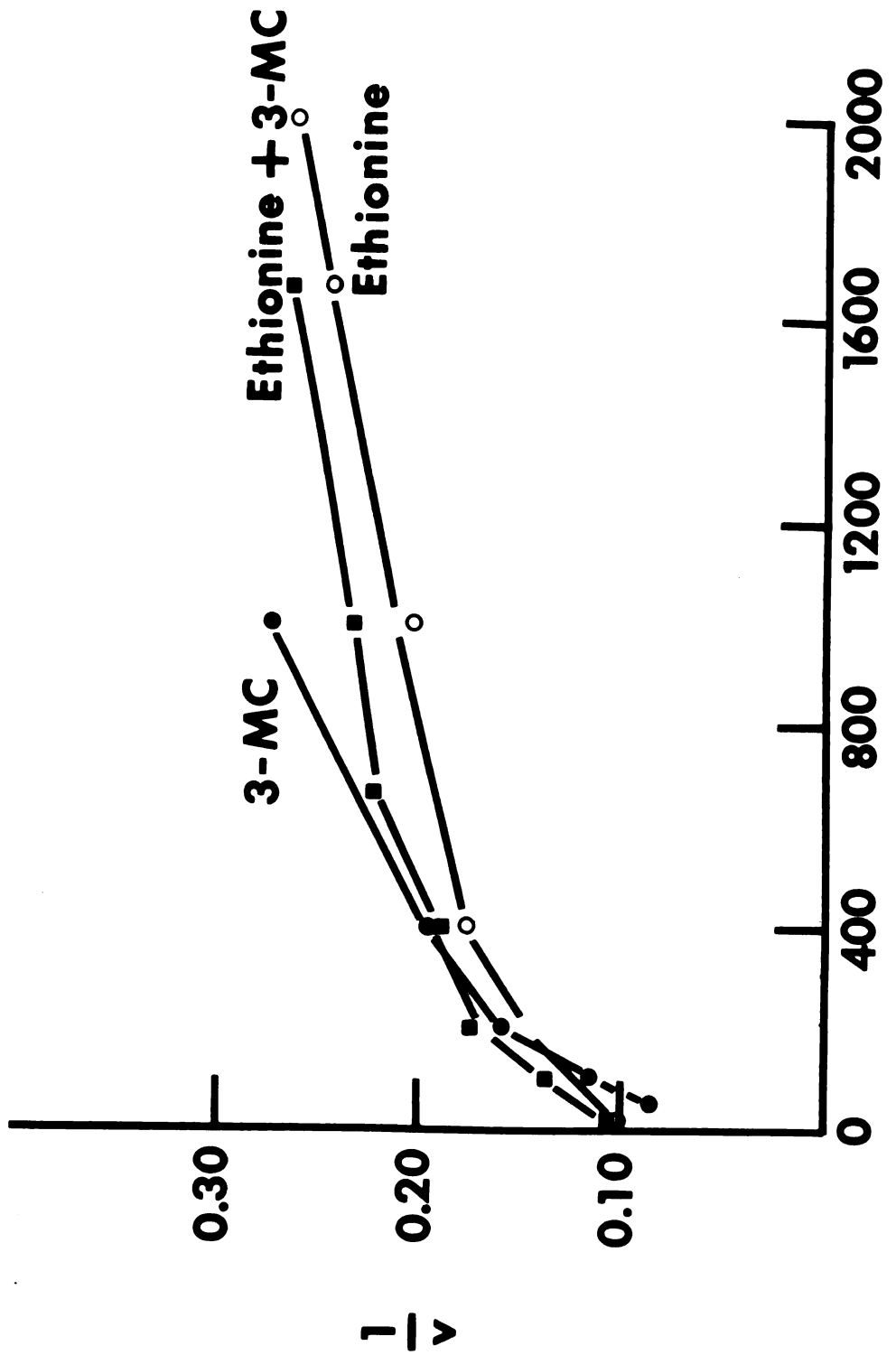
Ethionine + 3-MC
Ethionine
3-MC
0.30
0.20
0.10
1/v
0
400
800
1200
1600
2000
1/S

Figure 8

Sladek and Mannering[46] reported that the inhibitor SKF-525A at a concentration of 4×10^{-5}M inhibited the demethylation of 3-methyl-4-monomethyl-aminoazobenzene in liver microsomes from PB induced or untreated rats but not 3-MC induced rats. We similarly found (Figure 9), that SKF-525A strongly inhibits AP demethylase activity in microsomes from untreated or PB induced animals, but not in microsomes from 3-MC induced animals.

Figure 9.--Inhibition of aminopyrine demethylation in rat liver microsomes by SKF-525A. The concentration of aminopyrine used, 1 mM and 5 mM, are approximately the respective concentrations at half maximal velocity in PB type and 3-MC microsomes.

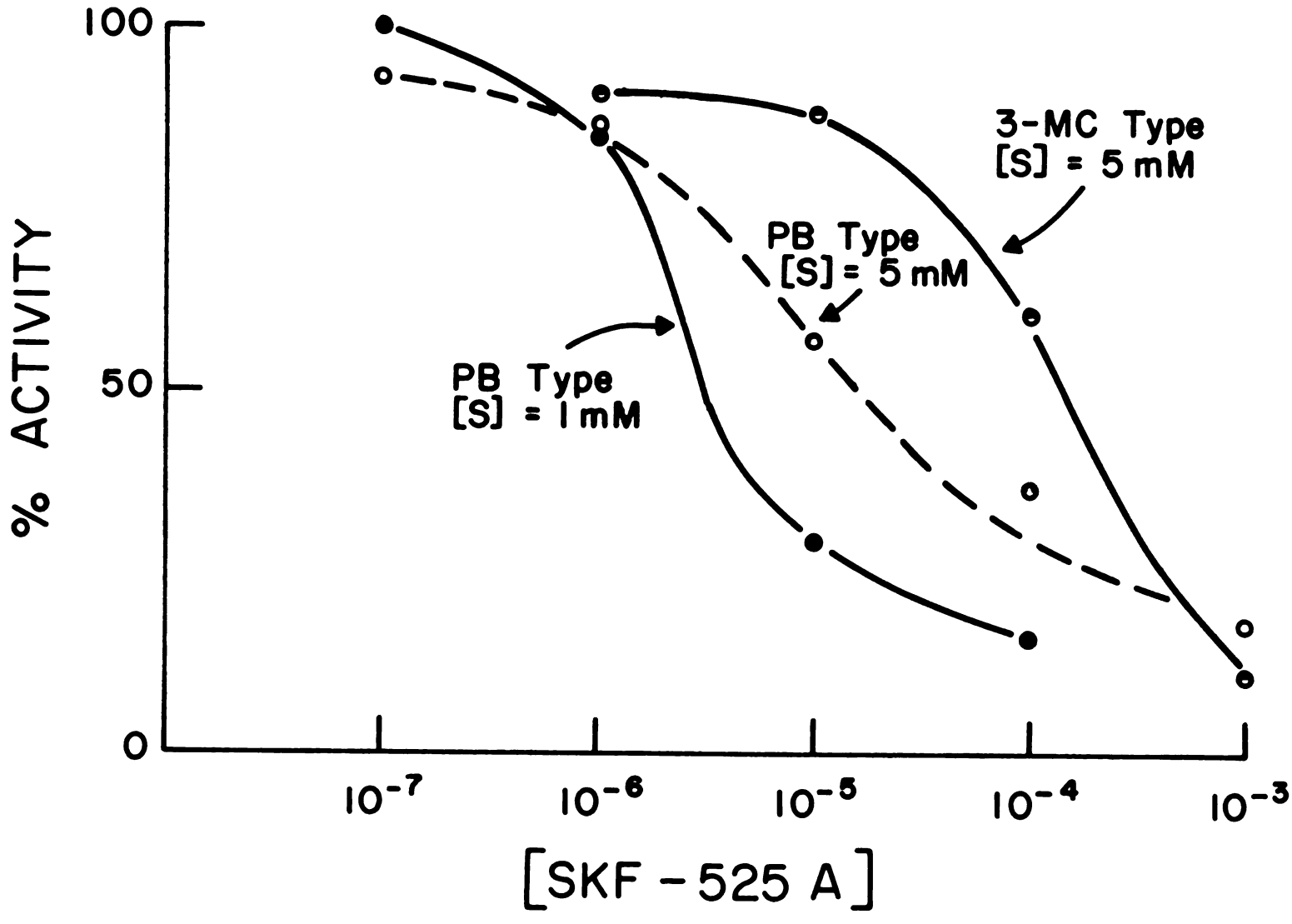
100
50
0
% ACTIVITY
3-MC Type
[S] = 5 mM
PB Type
[S] = 5 mM
PB Type
[S] = 1 mM
10-7
10-6
10-5
10-4
10-3
[SKF - 525 A]

Figure 9

DISCUSSION

The results of the experiments presented in this thesis are consistent with the proposal that liver microsomes contain more than one enzyme capable of oxidatively demethylating aminopyrine. The curvature of the Lineweaver-Burk plots is characteristic of a reaction catalyzed by two enzymes with different Km values.[47] Figure 10 shows the correlation between the observed values and a theoretical Lineweaver-Burk plot for two enzymes with Km values of 4×10^{-4} and 2×10^{-2}M and respective values for Vmax of 5.5 and 6.5. Similar kinetic evidence for multiple drug metabolizing enzymes has also been reported by other investigators. Wada and coworkers[48] observed nonlinear reciprocal plots of aniline hydroxylase activity in mouse and rat liver microsomes and furthermore found that the curvature was increased in the presence of the inhibitor predonisolone. Likewise, Lewis et al.,[49] reported that the reciprocal plots of aldrin epoxidation by pig liver microsomes showed similar departures from linearity at high substrate concentrations in the presence of inhibition

Figure 10.--Theoretical Lineweaver-Burk plot for aminopyrine demethylation catalyzed by two enzymes with Km values of 4×10^{-4} and 2×10^{-2} M. ———— theoretical, • observed values in rat liver microsomes.

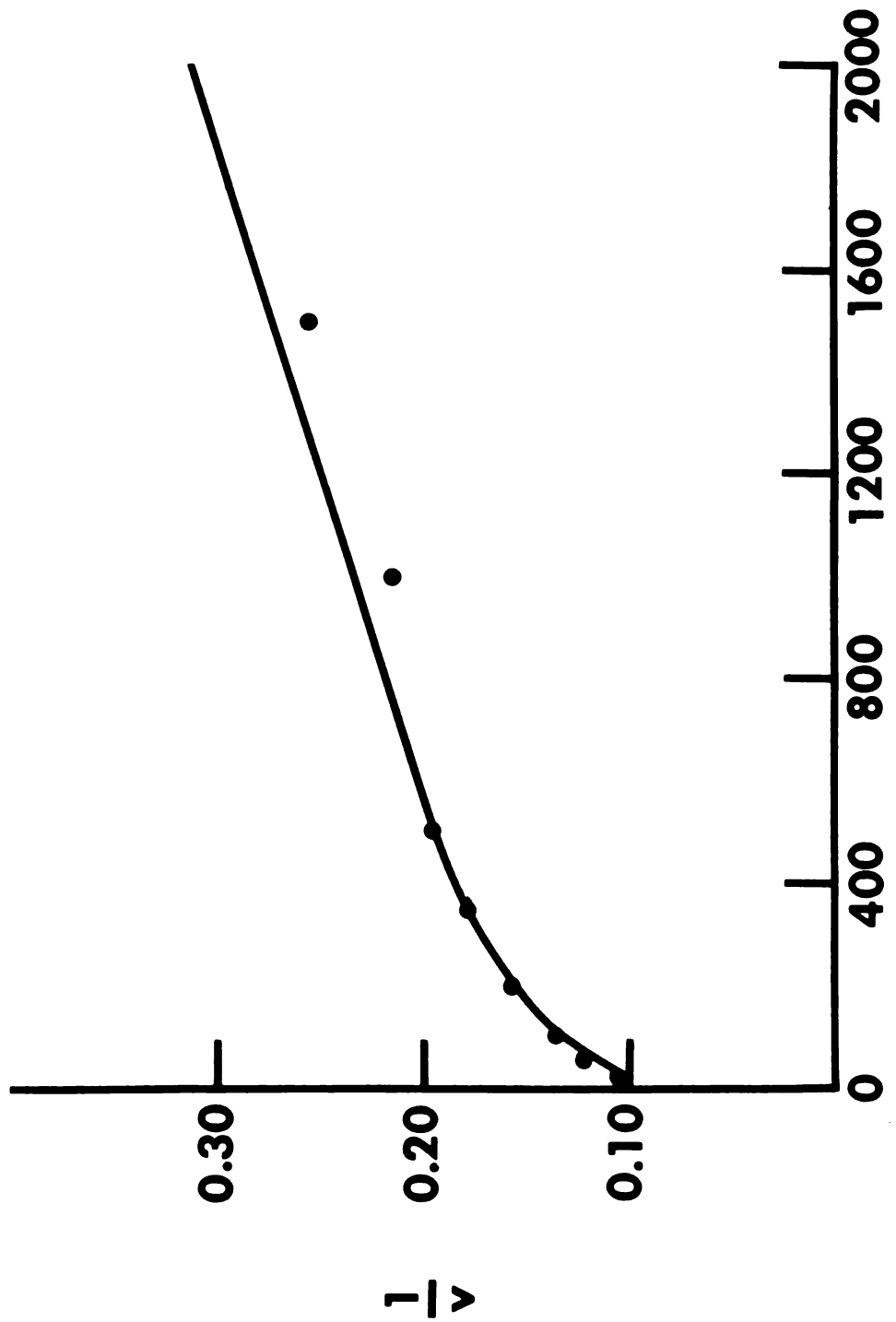

Figure 10

by 1,3-benzodioxoles. This type of kinetic analysis has led to the discovery of other multiple enzyme catalyzed reactions such as the formation of glucose-6-phosphate from glucose in rat liver, which was shown by Walker[56] to be catalyzed by two ATP: hexose-6-phosphotransferases, one having high affinity and the other low affinity for glucose.

The kinetic behavior of AP demethylase following pretreatment with PB or 3-MC reflects the established difference in induction by barbituates and polycyclic hydrocarbons; that is, PB will induce AP demethylase but 3-MC does not.[20] However, a quantitative change in the enzyme does occur following 3-MC pretreatment and this change must involve protein synthesis or turnover. The ability of ethionine to prevent the effects of 3-MC suggests that 3-MC induces the synthesis of enzyme(s) with altered substrate specificity which can demethylate AP if AP is present at high concentrations. The reciprocal plot for AP demethylase activity in control rat microsomes indicates that both PB and 3-MC type enzymes are present but at lower levels.

Similar changes in hepatic drug metabolizing activity following induction by polycyclic hydrocarbons

have been reported by several investigators. Kuntzman et al.,[51] have shown in the 6β-, 7α-, and 16α- hydroxylation of testosterone by rat liver microsomes, PB induction stimulates the hydroxylation of all three positions but causes the largest increase in 16α-hydroxylation. Alvares et al.,[43] have demonstrated that the stimulation of benzpyrene hydroxylation by 3-MC is accompanied by a decrease in apparent Km. The stimulation by PB shows no such decrease in Km. Studies by Nebert and Gelbain[41] of arylhydroxylase activity in hamster fetal cell cultures show that only polycyclic hydrocarbons are capable of inducing the hydroxylase activity.

The inhibition by SKF-525A in further evidence for the difference in substrate specificity in PB and 3-MC induced animals. It should be noted that the differences in sensitivity to inhibition by SKF-525A represent a difference in affinity for the inhibitor and not a consequence of difference in affinity for AP which would have produced an opposite result. Anders and Mannering[52] have shown that pretreatment of rats with SKF-525A will induce the microsomal enzyme activity towards hexobarbital and its own metabolism. The N-dealkylated metabolites of SKF-525A were found to be as effective as

SKF-525A at inhibiting hexobarbital metabolism *in vitro*. George and Tephly[53] reported that the hexobarbital inhibition of ethylmorphine N-dealkylation is competitive whereas the hexobarbital inhibition of norcodeine O-dealkylation is non-competitive in rat liver microsomes. Pretreatment with PB stimulates the metabolism of ethylmorphine greatly but has only a small effect on norcodeine metabolism. They were also able to show a difference in heat lability between ethylmorphine and norcodeine dealkylation. Attempts to show differences in heat lability of AP demethylase activity between the PB and 3-MC type microsomes have been unsuccessful. Preliminary results, however, indicate that benzypyrene is able to inhibit AP demethylase activity in PB induced microsomes but not in 3-MC induced microsomes.

The existence of multiple drug metabolizing enzymes is also supported by evidence based on species differences in the metabolism of drugs. Liver microsomes from rabbits hydroxylate aniline primarily in the para position, but those of rats hydroxylate mainly the ortho position of aniline.[54] Treatment of rats with phenylbutazone stimulates the 6β, 7α-, and 16α-hydroxylation of testosterone whereas the treatment of dogs with

phenylbutazone has no effect on the 7α-hydroxylation by the liver microsomes, but does stimulate the 6β- and 16α-hydroxylation reactions.[55] Similar differences in metabolic activity have been found between animals from different strains and opposite sex.[56,57]

It must be stated that the experimental results presented in this thesis do not constitute conclusive proof for the existence of multiple AP demethylating enzymes in liver microsomes. The possibility still exists that 3-MC or its metabolites interact with the enzyme system to alter kinetic and spectral properties by some method which has not been reproduced *in vitro*. The 3-MC may alter only the newly synthesized enzyme which would still explain the effect of ethionine. The data in Figure 7 shows that microsomes from 3-MC treated animals do not contain large amounts of the 3-MC which was injected into the animal. However, Bresnick *et al.*,[58] using labeled 3-MC observed some labeling of the microsomal fractions in which the label appeared to be bound to a protein component of the microsome. If the 3-MC were bound to the P-450, the complex could have altered spectral and kinetic properties. Another possibility is that two populations of microsomes exist with either different enzymes or

enzymes in different environments. It is well known that PB treatment produces proliferation of the endoplasmic reticulum and size of the liver whereas 3-MC causes little or no increase in microsomal protein.[1] It has also been observed that smooth surfaced microsomes metabolize substrates more rapidly than do rough microsomes.[59] However, Ernster and Orrenius observed that during induction the rough endoplasmic reticulum shows the increase in drug metabolizing activity first and the smooth later suggesting that the smooth membranes are merely older membranes from which the ribosomes have dissociated.[12] The key to understanding the qualitative differences in inducing agents lies perhaps in the work reported by Wold and Steele[60] who observed that PB primarily effects the transcription of ribosomal RNA whereas 3-MC produces an increase in the transcription of RNA resembling messenger RNA.

The nature of the changes in the microsomes effected by polycyclic hydrocarbon induction may be indicated by the following observations. Daly et al.,[61] have recently reported that the retention of deuterium in p-deuteroacetanalide following p-hydroxylation by liver microsomes varies with species, sex, and type of induction.

Retention of p-deuterium occurs when the deuterium atom migrates during hydroxylation to the meta position. The degree of retention increases after the animals are pretreated with PB and decreases after pretreatment with 3-MC suggestiong that there has been a change in its environment in which the acetanilide is located during hydroxylation. There is also evidence in addition to the spectral studies which suggests the existence of more than one type of P-450. Conney et al.,[62] have reported that there is considerable difference in degree of inhibition of the 6β-, 7α-, and 16α- hydroxylations of testosterone by carbon monoxide. Levine and Kuntzman[63] have presented evidence for two P-450's or two pools of P-450 by showing a biphasic decay of pulse labeled P-450. Treatment with 3-MC increased the ratio of the slow decaying component to the fast decaying component.

This thesis presents kinetic evidence for multiple enzymes capable of demethylating AP in liver microsomes. This evidence, together with that presented elsewhere, strongly supports the existence of multiple drug metabolizing enzymes in mammalian liver. However, the identification of the components that convey substrate specificity and are altered by induction will require the separation

and identification of the cytochromes and other components which constitute the drug metabolizing system.

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