STUDIES ON THE MULTIPLICITY OF MICROSOMAL MIXED-FUNCTION OXIDASE

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JEFFREY B. STEVENS
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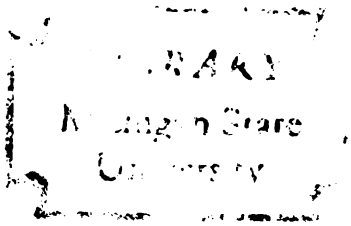

ABSTRACT

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By

Jeffrey B. Stevens

The inhibition of aminopyrine demethylase activity of rat liver microsomes by dieldrin, metopirone and DDT suggests the presence of three activities capable of demethylating aminopyrine. Two of these systems are sensitive to inhibition by DDT, however, not equally. Only one is sensitive to dieldrin and metopirone inhibition. One component is extremely sensitive to DDT inhibition and is induced by pretreatment of animals with phenobarbital. The activity of the component which is insensitive to inhibition by either of these drugs is increased preferentially by 3-methylcholanthrene pretreatment. Further data suggested where in the electron transport system that these drugs were interacting in the mixed-function oxidase system. Dieldrin and metopirone inhibition of aminopyrine demethylase has been correlated to binding the heme of the cytochrome P-450 to prevent the hydroxylation reaction occurring. DDT was found to cause inhibition by two completely

different mechanisms. One of the types of inhibition can be overcome by the addition of NADH to the reaction mixture. Both CO- and substrate-difference spectra were used to calculate an extinction coefficient for the metopirone-heme chromophore. An extinction coefficient of 146 $cm^{-1}mM^{-1}$ was found.

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Jeffrey B. Stevens

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LIST OF ABBREVIATIONS

AP	Aminopyrine
DDT	1,1,1-trichloro-2,2-tris(p-chlorophenyl)ethane
DPEA	2,4-dichloro-6-phenylphenoxyethylamine
i.p.	intraperitoneally
Lilly 18947	2,4-dichloro-6-phenylphenoxyethyldiethylamine
3-MC	3-methylcholanthrene
PB	Phenobarbital
SKF-525-A	2-ethylaminoethyl-2,2-diphenylvalerate HCl
SKF-8742-A	2-ethylaminodiethyl-2,2-diphenylvalerate HCl

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INTRODUCTION

Most of the foreign compounds which find their way into the body undergo some type of biotransformation.[1] This process in general has been termed xenobiotic (or drug) metabolism. Speculations as to why such a system had developed are based upon the detoxification and/or elimination of these foreign compounds.

The primary organ responsible for the metabolism of most xenobiotics is the liver. Within the liver cell, the enzyme system or group of systems, exist in the endoplasmic reticulum.[2,3,4] In vitro preparations of the endoplasmic reticulum are obtained by differential centrifugation of a liver homogenate and are termed microsomes. Studies have shown that the quantitative levels of activities for these enzymes are greatly dependent of the species, sex, health, and other factors generally dependent on the overall physiological state of the animal. It has also been shown that, in general, these activities are distributed equally throughout the microsomal fraction.[5]

The most unusual characteristic of this system (or systems) is its ability to transform such an extremely wide variety of compounds. This apparent lack of specificity is

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contrary to the classical principle of substrate specificity for enzymes. The large variety of substrates can also undergo a wide variety of chemical transformations. Aromatic and aliphatic hydroxylation, N- and O-dealkylation, deamination, sulfoxidation, and N-oxidation are but a few. However, in general all of these can be thought of as variations of hydroxylations.[6,7]

Another physiological phenomenon characteristic of this system is the inducability of these enzymes by a number of compounds.[8] The phenomenon of the enhancement of liver microsomal drug metabolizing activities by pretreatment of animals in vivo with lipid-soluble foreign compounds was first described by Brown, Miller and Miller[9] who administered polycyclic hydrocarbons to rats and mice. These studies were developed further by Conney and collaborators[10, 11, 12] in showing the activation was most probably due to an increased synthesis of the drug metabolizing enzymes. Therefore, this increase in activity appeared to represent an increased concentration of enzyme protein and is referred to as "enzyme induction." Pharmacologically this induction is very important since it leads to an accelerated biotransformation of drugs in vivo and then alters the duration and intensity of drug action in animals and in man. The enhancement of enzyme activity is also considered important because of its association with drug and pesticide synergism and tolerance.

All of these hydroxylation reactions require a reduced co-enzyme (usually NADPH) and molecular oxygen. The incorporation of molecular oxygen and not oxygen from water has been demonstrated to occur in the conversion of acetanilide to p-hydroxyacetanilide and of trimethylamine to trimethylamine oxide.[13,14] Other substrates used to demonstrate this phenomenon have not yielded positive results due to the rapid rate at which the incorporated oxygen exchanges with water. By the terminology of Mason, this system (s) of enzymes is classified as a mixed-function oxidase.[15]

These requirements have led to the belief that microsomal hydroxylations occur by a coupled redox reaction in which an "activated oxygen complex" capable of oxidizing various substrates is formed as an intermediate. Both the discovery of a CO-binding pigment by Klingenberg[16] and Garfinkel[17] and the partial characterization by Omura and Sato[18] showing it to be a cytochrome, greatly enhanced the belief that this theory is correct. The cytochrome was called cytochrome P-450 or simply P-450 because of its absorption maximum at 450 nm when a reduced microsomal CO-difference spectrum was taken.

The role of this unusual cytochrome was illustrated by Ryan and Engel[19] by the observation that the C_{21}-hydroxylation of 17-hydroxyprogesterone was inhibited by CO and that the inhibition was reversed by light. Later,

Estabrook, et al.,[20] showed the maximum reversal of the CO inhibition was obtained when the sample was illuminated at 450 nm.

Omura and Sato[21,22] also demonstrated that cytochrome P-450, when treated with a detergent such as deoxycholate or incubated anaerobically with phospholipase, was transformed into a derivative pigment, P-420, which had a normal hemoprotein spectrum, but was no longer enzymatically active.

Further evidence for cytochrome P-450's role in drug metabolism was presented by Orrenius and Ernster[23] who associated an increase in liver microsomal cytochrome P-450 with an increase in hydroxylase activity.

One problem receiving considerable attention in this system is multiplicity of enzymes. In order for this system to carry out its vast number of chemical reactions on its numerous substrates, the system either must be totally devoid of any specificity or have within itself some form of multiplicity. The first alternative is not feasible from a biological standpoint, therefore multiplicity must lie somewhere in this system.

One major group of investigators believes that more than one P-450 exists. Their argument rests primarily on data obtained from induction studies. Inducers of this enzyme system can be classified into two major categories: (1) compounds such as phenobarbital which induce the metabolism of a large number of drugs, and (2) compounds that

exert considerable specificity as enzyme inducers and do not stimulate many of the reactions that are stimulated by phenobarbital. This second group of inducers included polycyclic hydrocarbons such as 3-methylcholanthrene and 3,4-benzpyrene.

Drugs and other xenobiotics which are substrates or inhibitors interact with cytochrome P-450, even in the absence of NADPH to give two types of spectral change.[24,25,26,27] The "type I" spectral change is characterized by the appearance of a trough 420 nm and a peak at 385 nm in the difference spectrum when compounds such as hexobarbital or aminopyrine are added to the sample cuvette. The "type II" spectral change is produced by compounds such as aniline and pyridine and is characterized by the appearance of a peak at about 430 nm and a trough at 390 nm. The intensities of this spectral change are related to the concentration of the substrate used.

Mannering, et al.,[28] have shown that pretreatment with phenobarbital (PB) increased the intensity of both "type I" and "type II" spectra, but 3-methylcholanthrene (3-MC) pretreatment caused an increase in "type II" spectra only. These results were related to the hydroxylating activities of aniline (type II) and hexobarbital (type I). 3-MC pretreatment enhanced aniline hydroxylation activity only, while a decrease in activity for hexobarbital was observed.

There exists also a significant amount of evidence for more than one cytochrome P-450 based solely on

CO-difference spectra with the three types of microsomes. Induction studies have shown that the absorbance maximum for the CO-difference spectra can be shifted from 450 to 448 nm, depending upon the extent and type of inducer used.[29]

Levin and Kuntzman[30] have shown a biphasic decrease of radio-active hemoprotein in liver microsomal CO binding particles isolated from animals given ^{3}H-levulinic acid. They have shown that previous administration of various inducing drugs produce remarkably different amounts of heme proteins with either fast or slow turnover rates. For example, when pretreatment was done with PB, there was much more of the 'fast' turnover particles than in control, and when 3-MC was administered, the 'slower' turnover type was more prevalent.

Induction studies have provided impressive evidence for the presence of some type of multiple enzyme system. Alvares, et al.,[31] showed a difference in K_m's for the hydroxylation of benzpyrene by microsomes obtained from animals induced with PB and 3-MC. Conney, et al.,[32] showed that 3,4-benzpyrene pretreatment did not induce the metabolism of aminopyrine or hexobarbital, but did induce the metabolism of zoxazolamine.

Some other selective inducers, such as DDT and chlordane, had different effects on the induction of different activities.[33] DDT acted as a more selective stimulator of hepatic drug metabolizing enzymes than both PB and 3-MC.

Rubin, et al.,[34] found that a number of compounds which inhibited N-demethylation of ethylmorphine by liver microsomes were themselves metabolized by this system. This inhibition is believed to be a result of the alternative substrate hypothesis, which states that if two substrates are competing for a common site on an enzyme, they must be competitive inhibitors of each other. For competitive inhibition, the K_i value of a particular compound should relate to the K_m for the metabolism of that compound.

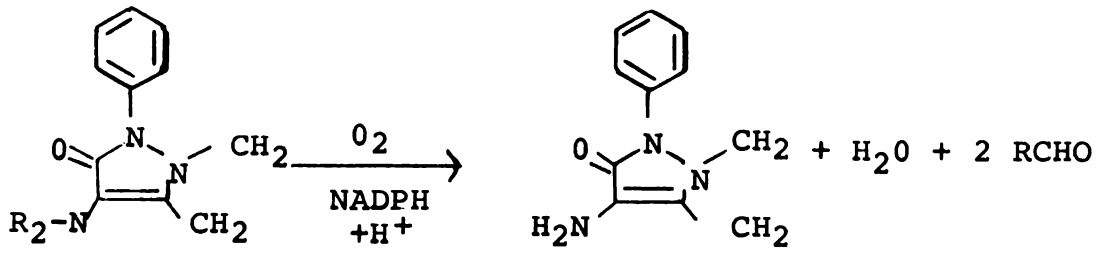
However, other evidence has shown that certain inhibiting chlorinated hydrocarbons were not metabolized.[35] One possible interpretation for this latter result is that the inhibition was not competitive. Other studies have shown slight disagreement for the correspondence of K_i's with respective K_m's,[34,36] leading to the belief that the alternative substrate hypothesis may not be completely applicable in this system.

Inhibition of drug metabolism by steroids[37] led to the belief that these steroids are in fact the natural substrates for these enzymes, and that in the presence of xenobiotics become alternative substrates for a common mixed-function oxidase system.

All of the above evidence has pointed overwhelmingly to the possibility of multiple enzyme oxidation systems, however, no direct evidence has been submitted. The

ultimate goal of this thesis would be to directly demonstrate multiplicity in some fraction of the microsomal mixed-function oxidases.

Aminopyrine (4-dimethylamino-1,5-dimethyl-2-phenyl-3-pyrazolone) was the drug substrate of choice because of the ease of enzymatic analysis. It is an analgesic and an antipyretic which was used to treat symptoms of a variety of diseases including rheumatic fever, but its occasional toxicity led to its disuse. The major metabolite is 4-aminopyrine, (4-amino-1,5-dimethyl-2-phenyl-3-pyrazolone). Brodie and Axelrod[38] first located the biotransformation in liver slices and homogenates. The ethyl and butyl analogs were also shown to be metabolized to 4-aminoantipyrine and the corresponding aldehydes[39] by the following reaction:



The metabolism of aminopyrine by liver microsomes was further characterized by Ernster and Orrenius,[40] who showed the equivalent amounts of NADPH, O_2, and substrate were used during the course of the reaction, and that the demethylase activity was stimulated, along with the

induction of the metabolism of other drugs, by PB. The role of P-450 in the reaction was confirmed by Cooper, et al.,[41] who showed that CO inhibition was reversed by monochromatic light at 450 nm. Studies by Gram, Wilson, and Fouts[42] have suggested that the removal of one of the methyl groups occurs at a fast rate followed by a slower reaction to form 4-aminoantipyrine.

Initial work in this area from this laboratory involved determining an explanation for the biphasic Lineweaver-Burk plot obtained when assaying for aminopyrine N-demethylase activity. It had been proposed that the biphasic nature of the plot was the result of the dimethyl and monomethyl derivatives being metabolized at different rates. However, these ideas were refuted by showing the biphasic Lineweaver-Burk plot still occurred when only the monomethylaminoantipyrine was used as a substrate.[28] Since the plots were also characteristic of a reaction catalyzed by two enzymes, the next step was to determine the point of multiplicity. Induction by PB stimulated the demethylase activity and produced a linear Lineweaver-Burk plot with an apparent K_m for aminopyrine at 7×10^{-4} M. Pretreatment with 3-MC caused little or no stimulation of activity, but it did increase the apparent K_m for aminopyrine by more than an order of magnitude.

It was also found that the inhibitor SKF-525-A at a concentration of 4×10^{-5} M differentiated between the

demethylase activities present in the two types of induced animals, inhibiting the activity found in microsomes of PB induced rats but having little effect on the activity in microsomes from 3-MC treated rats.

The working hypothesis for this research rests on the belief that multiplicity must exist somewhere in this system. It will be the endeavor of this research to further establish the possibility of a multiple enzyme system. Three possibilities exist for explaining the type of non-specificity present in this enzyme system. As previous evidence has tried to show, there may be the possibility of multiple cytochrome P-450's each of which has an electron transport system or they may share a common electron-transport system. Secondly, there may exist multiple binding sites on a single P-450, or lastly, a combination of these possibilities may also occur.

The first part of this thesis presents indirect evidence for multiple mixed-function oxidase activities for a single substrate. With aminopyrine as a model substrate for this system, an attempt was made to kinetically show multiple demethylase activities. Based on the idea of specific induction and/or inhibition of these various enzymes, multiple aminopyrine demethylase activities were shown. It has been shown that the use of specific inducers and/or inhibitors provide extremely valuable tools in further work with these oxidases.

In the second part, an attempt was made to produce direct evidence for this multiplicity. The solubilization and partial purification of the cytochrome P-420 provided some evidence concerning the question of multiple cytochrome P-450's.

MATERIALS AND METHODS

Chemicals

Aminopyrine (4-dimethylamino-1,5-dimethyl-2-phenyl-3-pyrazolone) and hexobarbitol (Sodium 5-(1-cyclohexen-1-yl) -1,5-dimethylbarbiturate) were purchased from K and K Laboratories, Inc., Plainview, N.Y. and used as received as substrates in the assays.

The two inhibitors, DPEA (2,4-dichloro-6-phenylphenoxyethylamine HBr) and Compound 18947 (2,4-dichloro-6-phenylphenoxyethyldiethylamine HBr) were received from the Eli Lilly and Co., Indianapolis, Indiana. Dieldrin (1,2,3,4, 10,10-hexachloro-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-1, 4,5,8-dimethanonaphthalene) was obtained from the Shell Chemical Company, New York, N.Y. and was recrystallized to a purity of 99% before use. Metopirone (2-methyl-1,2-bis (3-pyridyl)-propanone) was received from the CIBA Pharmaceutical Company, Summit, N.J. Compounds 525-A (2-ethylaminodiethyl-2,2-diphenylvalerate HCl) and 8742-A (2-ethylaminoethyl-2,2-diphenylvalerate HCl) were received as gifts from the Smith, Kline, and French Laboratories, Philadelphia, Pennsylvania. Benzo-α-pyrene (3,4-Benzpyrene), 20-methylcholanthrene (3-methylcholanthrene), testosterone, androsterone, β-estradiol, progesterone, and hydrocortisone as

well as cholestrol were all purchased from the Sigma Chemical Company, St. Louis, Mo. D,L-isocitrate, $NADP^{+}$, NADPH, NADH, and $NADP^{+}$isocitrate dehydrogenase were also purchased from the Sigma Chemical Company, along with Cytochrome C, steapsin, Bee Venom, and the following Snake Venoms: Naja Naja (Hooded Cobra) and Crotalus Atrox (Western Diamondback Rattlesnake).

DDT (1,1,1-trichloro-2,2-tris(p-chlorophenyl)ethane) was obtained from Dr. Bieber who had previously recrystallized it to purity. Phenobarbitol (Sodium 5-ethyl-5-phenylbarbiturate) was purchased from Merck and Company, Inc., Rahway, N.J. Oxygen and Carbon Monoxide were obtained from the Matheson Company, Inc., Joliet, Illinois.

KCN was purchased from Mallinckrodt Chemical Works, St. Louis, Mo. Nitroblue tetrazolium was purchased from Aldrich Chemical Company. Phospholipase A was received from C. F. Boehringer and Soehne GmbH Mannheim.

Animals

All experiments were done with microsomes isolated from the livers of male rats of the Holtzman Strain weighing between 200 and 250 grams. Animals induced with phenobarbital were given 0.1% phenobarbital in their drinking water at least one week prior to the isolation. Animals induced with 3-methylcholanthrene were given an i.p. injection, of 20 mg/kg in corn oil, 24 hrs. prior to isolation.

Preparation of Microsomes

The animals were killed by decapitation with an animal guillotine and the livers were immediately perfused *in situ* with approximately 10 mls. of ice cold 1.15% KCl containing 0.2% nicotinamide by injection into the portal vein. The livers were then removed, weighed, and minced by chopping with a pair of scissors. The tissue was then homogenized in three to four volumes of 1.15% KCl containing 0.2% nicotinamide,[43] added to inhibit any $NADP^{\pm}$ase that may be present, in a Potter-Elvehjem homogenizer equipped with a teflon pestle. The homogenate was then centrifuged at 10,000×g (8,500 rpm, SGA rotor) for twenty minutes in a Sorvall RC2-B automatic refrigerated centrifuge. The supernatant was decanted and saved, while the pellet containing the nuclear, mitochondrial, and other subcellular fractions was discarded. The microsomal fraction was then isolated by centrifuging the previous supernatant in a Spinco centrifuge at 105,000×g (30,000 rpm, 30 rotor) for 90 minutes. The pellet was resuspended in Tris-HCl buffer (0,05 *M*, pH 7.5) containing 50% glycerol. A protein determination was done on the resuspended microsomes by the Lowry Method[44] and usually ranged from 30 to 60 mg/ml. All the above operations were done at 0-5°C. The microsomes were stored at -15°C under N_2 until they were to be used. Providing they were kept anaerobic, full N-demethylase activity was retained for a number of weeks.

Aminopyrine Demethylase Assay

The N-demethylase activity was assayed by measuring spectrophotometrically the rate of formaldehyde production using the Nash method.[45] All assays contained: 7m$\underline{M}$ $MgCl_2$, Tris-HCl (0.05$\underline{M}$, pH 7.5), 0.5m$\underline{M}$ NADPH or a NADPH generating system made up of $NADP^+$ (0.1m$\underline{M}$), D,L-isocitrate (2m$\underline{M}$), and isocitrate dehydrogenase (0.05 units/ml). The desired levels of aminopyrine and inhibitors were also added. The reaction mixtures were incubated at 37°C under air in a Dubnoff metabolic shaker. The reaction was stopped by addition of an equal volume of 10% trichloroacetic acid. All tubes were then allowed to stand at room temperature for 20 minutes to allow for total protein precipitation. An equal volume of Nash reagent (2$\underline{M}$ $NH_4C_2H_3O_2$, 0.5$\underline{M}$ CH_3COOH, and 0.02$\underline{M}$ 2,4-pentanedione) was then added to each tube. The reaction product, diacetyl dihydrolutidine (DDL) has a yellow color and was read spectrophotometrically at 412 nm in a Coleman Jr. Spectrophotometer equipped with a flow cell. The extinction coefficient used was 7.08 cm^{-1}, $\mu\underline{M}^{-1}$ HCHO.

The inhibitors: dieldrin, DDT, metopirone, benzpyrene, hexobarbitol, Lilly 18947, SKF-525-A, and SKF-8742-A were all added to the incubation mixture in a minimum amount of acetone, prior to the addition of any of the enzymes. The acetone was then subsequently removed by blowing N_2 gas into and over the incubation mixture. All of the steroids were also added in acetone.

Cytochrome C Reductase Assay

Incubations were in 0.05M KPO_4 buffer, pH 7.3 containing 10^{-4}M EDTA. Protein was mixed with 0.2 ml of cytochrome C stock solution (36nM) and the above buffer to a total volume of 1.0 ml. The reaction was initiated with 10 µl NADPH (10mM). The activity was followed by reading the absorbance increase at 550 nm. Reduced cytochrome C has an extinction coefficient of 27.7 cm^{-1}, mM^{-1}.

Nitroblue tetrazolium (NBT) Reductase Assay

Protein (0.1 ml) was added to 0.5 ml of NBT stock solution (1.0 mM) and enough 0.05M KPO_4 buffer, pH 7.3 to make a final volume of 1.0 ml. The reaction was initiated by addition of 10 µl of NADPH (10 mM) and followed at a wavelength of 580 nm. One unit of activity corresponded to absorbance change of 1.0 per minute.

CO-difference Spectroscopy

The carbon monoxide difference spectra were obtained by resuspending the protein in 0.05M Tris-HCl buffer, pH 7.5. The solutions in the reference and sample cuvettes were reduced by the addition of dithionite (<1 mg). To obtain the CO difference spectra, the sample cuvette was bubbled with deoxygenated CO until it was saturated. The CO gas was deoxygenated by Fieser's Deoxygenating Solution which contains: concentrated KOH (2.7M), Na-anthraquinone-β-sulfonate (0.068M), and Na-dithionite (0.81M). Spectra

were recorded with either a Beckman DB or a Coleman 124 spectrophotometer and a Sargent SRL recorder. CO difference spectra were used to analyze for both cytochrome P-450 and cytochrome P-420. The extinction coefficient used for P-450 was 91 cm^{-1}, $m\underline{M}^{-1}$ and for P-420 111 cm^{-1}, $m\underline{M}^{-1}$ as described by Omura and Sato.[46]

Cytochrome b_5 Assay

Cytochrome b_5 assays were also accomplished by difference spectroscopy. Equal concentrations of protein was added to the reference and sample cuvettes just as in the CO-difference spectra procedure, with dithionite (<1 mg) only being added to the sample cuvette. A reduced vs. oxidized difference spectrum was taken between 440 nm and 400 nm. The absorbance difference between 424 nm and 409 nm was measured. The extinction coefficient between these two wavelengths is 163 cm^{-1}, $m\underline{M}^{-1}$.

Substrate Difference Spectroscopy

Substrate difference spectroscopy was a technique used to determine Type I or Type II binding curves for various microsomal preparations (see Introduction). DDT (100 $\mu\underline{M}$), aminopyrine (25 $m\underline{M}$), Lilly 18747 (100 $\mu\underline{M}$), DPEA (100 $\mu\underline{M}$), dieldrin (100 $\mu\underline{M}$), and metopirone (33 $\mu\underline{M}$) were used as ligands. Substrates were added to 1.0 mg/ml microsomes, diluted with Tris-HCl buffer, pH 7.5, with or without dithionite for reduction of P-450. The spectra were recorded on a Cary Spectrophotometer.

$CO-O_2$ Binding Equilibrium Experiments for P-450

Microsomes were diluted to a final concentration of 1.0 mg/ml with 0.05 M Tris-HCl buffer, pH 7.5. Both cuvettes were then reduced with dithionite. CO was added by addition of a known volume of Tris-HCl buffer saturated by CO (assumed to be 930 μM), and the absorbance change between 450 nm and 485 nm was taken.

Metopirone (33 μM) and DDT (100 μM) were added to both cuvettes to determine their effects upon to amount of P-450 reduced.

Polyacrylamide Gel Electrophoresis

The disc gel electrophoresis system which was used was a modification of the method by Takayama.[47] The gels were prepared by mixing stock solutions A and B with tetramethylethylenediamine in proportions, 3:1:0.02 ($\frac{V}{V}$). Stock solution A consisted of 6 grams of acrylamide, 0.16 grams N,N'-methylene bisacrylamide, 12 grams urea, 28 ml glacial acetic acid and water to make 60 ml final volume. Stock solution B contained 12 grams urea, and 0.3 grams ammonium persulfate in 20 ml water. Stock solution A could be stored up to 6 months if kept refrigerated, however, solution B was made fresh before each experiment. The buffer system used was 10% acetic acid, at both the anode and cathode. Polymerization of the gels was carried out in a water bath at 47°C for 15 minutes. A thin layer of water was placed on each gel prior to incubation to insure a flat surface

on the top of each gel. The gels were then covered with a solution containing 5M urea in 75% acetic acid and pre-electrophoresed for one hour at 5 milliamps per tube to remove the ammonium persulfate.

All protein samples were dissolved in a mixture containing phenol, acetic acid, and water (2:1:1) to a final concentration of 1.0 mg/ml and from 0.1 to 0.5 mg applied to each gel. Only running gels of 7.5% acrylamide, 5.5 cm in length were used. Electrophoresis was routinely performed at room temperature for one hour with a constant current of 5 milliamps per tube.

The gels were stained for a minimum of one hour in Coomassie Blue (0.05% in 12.5% TCA) and destained by diffusion in distilled water for at least 30 minutes.

Partial Purification of P-420 from Microsomes

Microsomes, isolated from phenobarbital treated animals, were diluted to a final protein concentration of 10 mg/ml with 0.05M KPO_4 buffer, pH 7.5. 0.072% ($\frac{W}{V}$) powdered steapsin was dissolved into the solution. It was then transferred to Thunberg tubes and made anaerobic under N_2.

The tubes were placed in a Dubnoff metabolic shaker-waterbath at 37°C and allowed to incubate for 60 minutes with occasional shaking.

The incubated solutions were then pooled and centrifuged at 105,000g (40,000 rpm, 40.2 rotor) for 60 minutes.

Both the supernatant and the pellet were assayed for cytochrome P-420 and cytochrome b_5. The supernatant contained 75-100% of the cytochrome b_5 with only 25% of the cytochrome P-420.

The pellet was resuspended in 0.05M Tris-HCl buffer, pH 9.5 to a protein concentration of 0.5 mg/ml. Powdered snake venom (Naja Naja) was dissolved into the suspension at 0.5 mg/ml. The suspension was then incubated with continuous shaking in air at 37°C for 20 minutes.

The incubated solution was then centrifuged at 196,000g (50,000 rpm, 50 rotor) for 30 minutes. The supernatant contained 90-100% of the P-420. It was cooled in an ice bath and fractionated by ammonium sulfate precipitation.

The fraction precipitating between 60-75% saturation contained essentially all the P-420 present in solution. Upon centrifuging at 3,800g (5,000 rpm, SGA rotor) in a Sorvall centrifuge, a yellow, floating pellet was obtained.

It was removed by the use of a spatula and redissolved in 0.05M Tris-HCl, pH 9.5. If the P-420 solution was kept refrigerated for at least 48 hours, a precipitate resulted along with high turbidity. This precipitate has also been reported by another investigator[48] as microtubules.

3-MC microsomes were treated exactly by the same procedure, except 0.01% ($\frac{w}{v}$) steapsin was used. Both solutions gave typical reduced CO-difference spectra for P-420 with a maximum absorption at 420 nm.

Optimizing Conditions for P-450 Reductase Assay

Whole microsomes were diluted with 0.1 M KPO_4 buffer, pH 7.5 until a change in absorbance (450-485 nm) was equivalent to 0.2 when a P-450 CO-difference spectra was taken. Glycerol concentrations, varying from 0 to 25%, in buffer, were used to dilute whole microsomes and then the rate of reduction under anaerobic conditions was followed. Excessive levels of NADPH were added to start the reaction which was followed at 450 nm on a Hitachi spectrophotometer.

Glycerol concentration was then held at 15% and the pH of the enzyme solutions was varied from 6.0 to 9.0 using 0.5 increments. The pH optimum for this particular reductase was found to be 7.0.

EXPERIMENTAL

Lineweaver-Burk plots of aminopyrine demethylase activity from microsomes isolated from untreated male rats (control microsomes) were found to be non-linear (Figure 1). Non-linear Lineweaver-Burk plots are characteristic of multiple enzymes with varying K_m values for a single substrate.[48] However, similar plots of aminopyrine demethylase activity from microsomes isolated from animals pretreated with phenobarbital (PB microsomes) were linear (Figure 1). Phenobarbital pretreatment resulted in an induction of an activity with an apparent K_m for aminopyrine of 5×10^{-4} M. 3-methylcholanthrene (3-MC microsomes) pretreatment of animals resulted in greater activity at high substrate concentrations (>2mM) resulting in an apparent K_m for aminopyrine of 2×10^{-3} M.

Similar experiments were performed using ethylmorphine as the substrate. No differences in K_m's were found for the different types of microsomes when values were extrapolated from 2.5 mM substrate concentrations or lower and concentrations above 20 mM resulted in substrate inhibition of activity in all three types of microsomes (Figure 2).

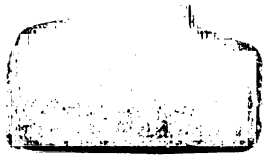


Fig. 1.--Lineweaver-Burk plots of aminopyrine demethylase activity in microsomes from control (Control), phenobarbital (PB) and 3-methylcholanthrene (3-MC) pretreated animals. Velocity is in mμM of formaldehyde min^{-1}, mg^{-1} protein. Substrate concentrations are molar.

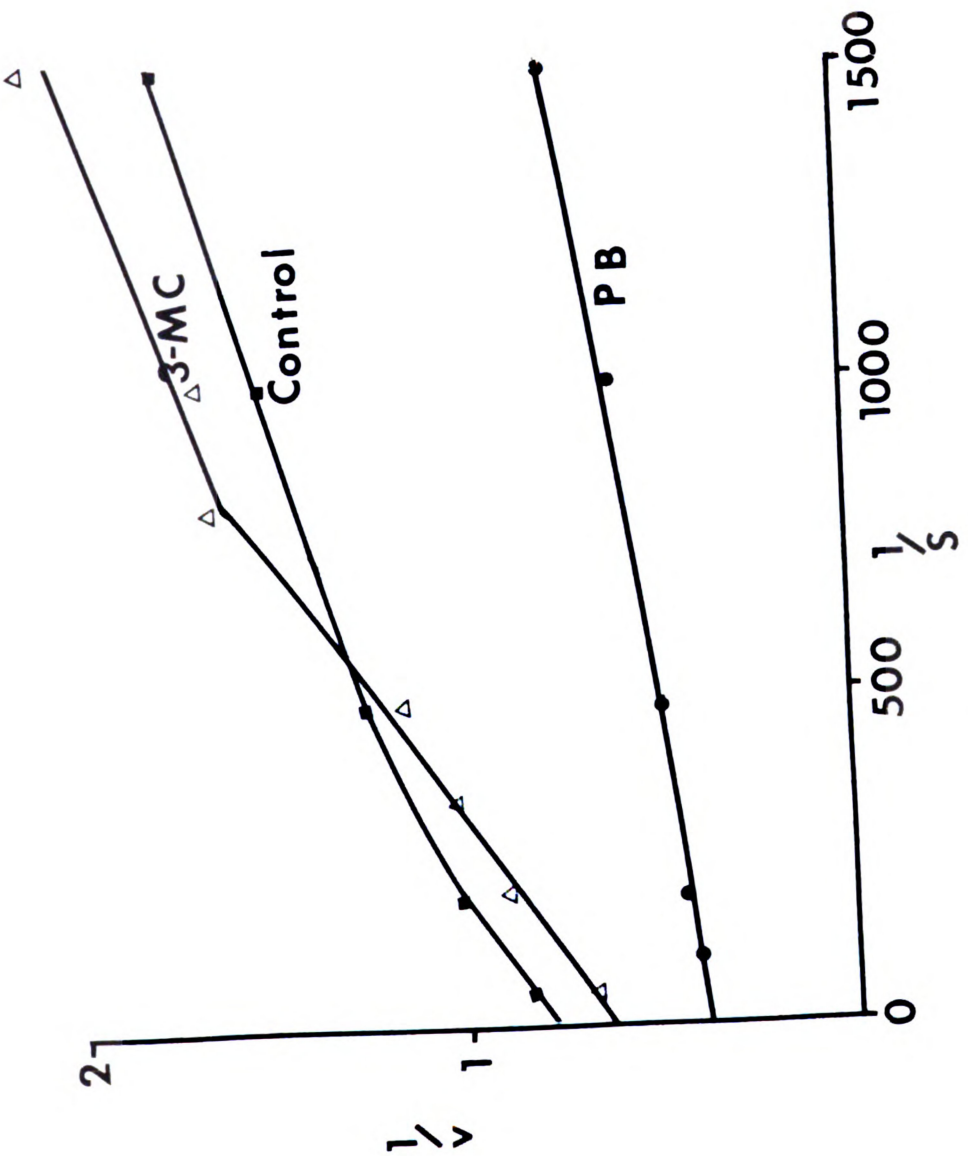
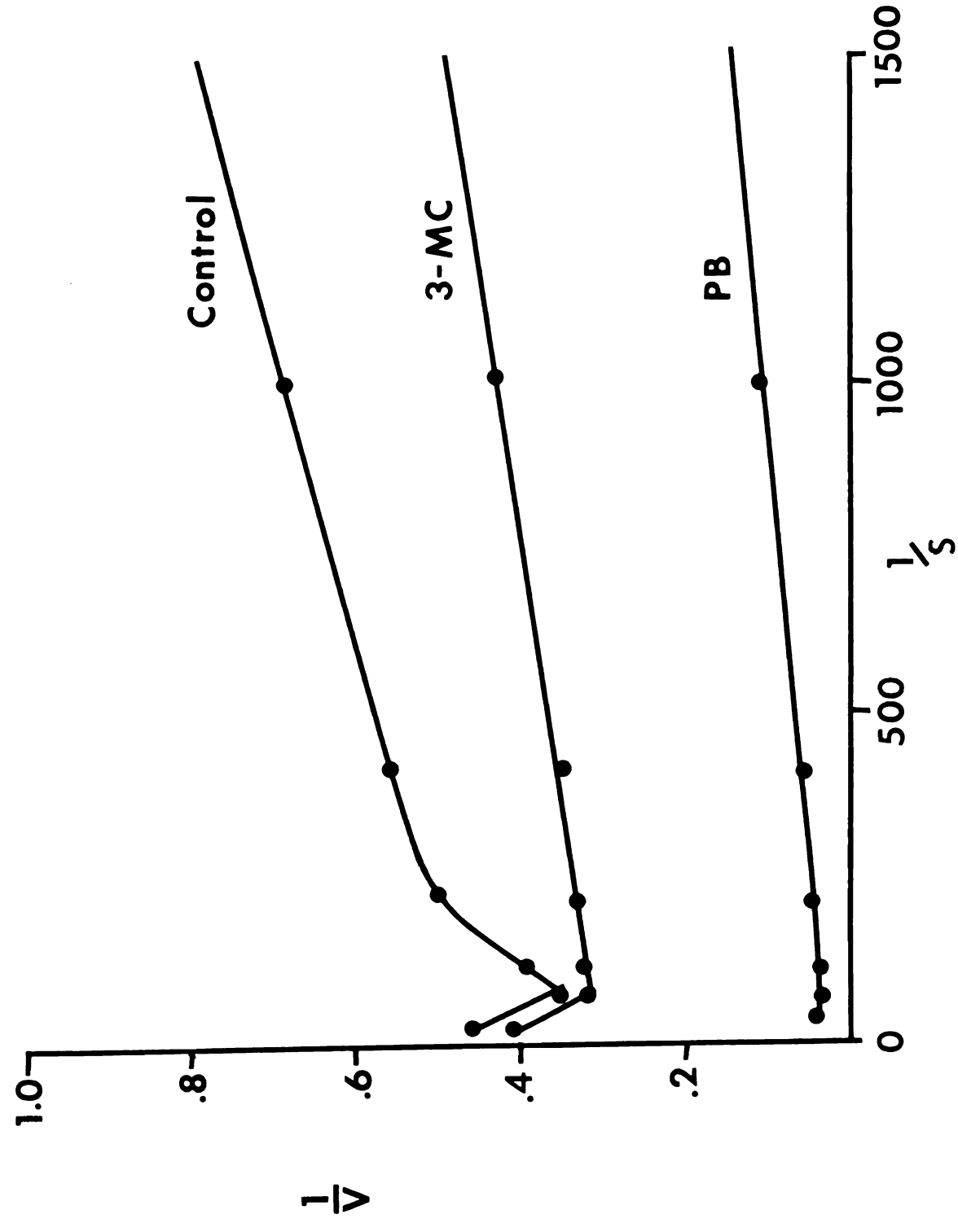

3-MC
Control
PB
2
1
0
500
1000
1500
1/v
1/s

Fig. 2.--Lineweaver-Burk plots of ethylmorphine demethylase activity in microsomes from control (Control), phenobarbital (PB) and 3-methylcholanthrene (3-MC) pretreated animals. Velocity is in mμM of formaldehyde min^{-1}, mg^{-1} protein. Substrate concentrations are molar.


Control
3-MC
PB
1.0
.8
.6
.4
.2
0
500
1000
1500
1/V
1/S

Ethylmorphine N-demethylase in both control and PB microsomes has been plotted with a constant level of aminopyrine on a double reciprocal plot. Upon analysis it had become evident that phenobarbital had induced an activity which was not common to both demethylase substrates. The two activities were additive over a wider range in PB microsomes (Figure 3).

Lineweaver-Burk plots of aminopyrine demethylase activity from microsomes isolated from untreated female animals were also found to be non-linear, but their activity was less than the activity from male animals. Female animals treated with testosterone (40mg/kg) showed non-linear activity with increase in enzymatic activity occurring only at lower levels of substrate (Figure 4). Male animals treated with testosterone showed no effects in metabolism with this substrate (Figure 4).

The following compounds were tested for the inhibition of aminopyrine in control, PB, and 3-MC microsomes: SKF-525-A, SKF-8742-A, Benzpyrene, DDT, Testosterone, Lilly 18947, Hexobarbitol, Metopirone, and Dieldrin (Figures 5-11). In control and PB induced microsomal activity Lilly 18947, SKF-525-A, and Benzpyrene gave linear Lineweaver-Burk plots when they were used as inhibitors indicating that only one activity was being inhibited. However, when 3-MC microsomes were used, the non-linearity of the reciprocal plots remained. From Figures 5 and 6 it

Fig. 3.--Lineweaver-Burk plots of ethylmorphine demethylase activity in microsomes from control and phenobarbital pretreated animals with or without 6×10^{-4} M aminopyrine in the incubation mixtures. Velocity is in mμM of formaldehyde min^{-1}, mg^{-1} protein. Substrate concentrations are molar.

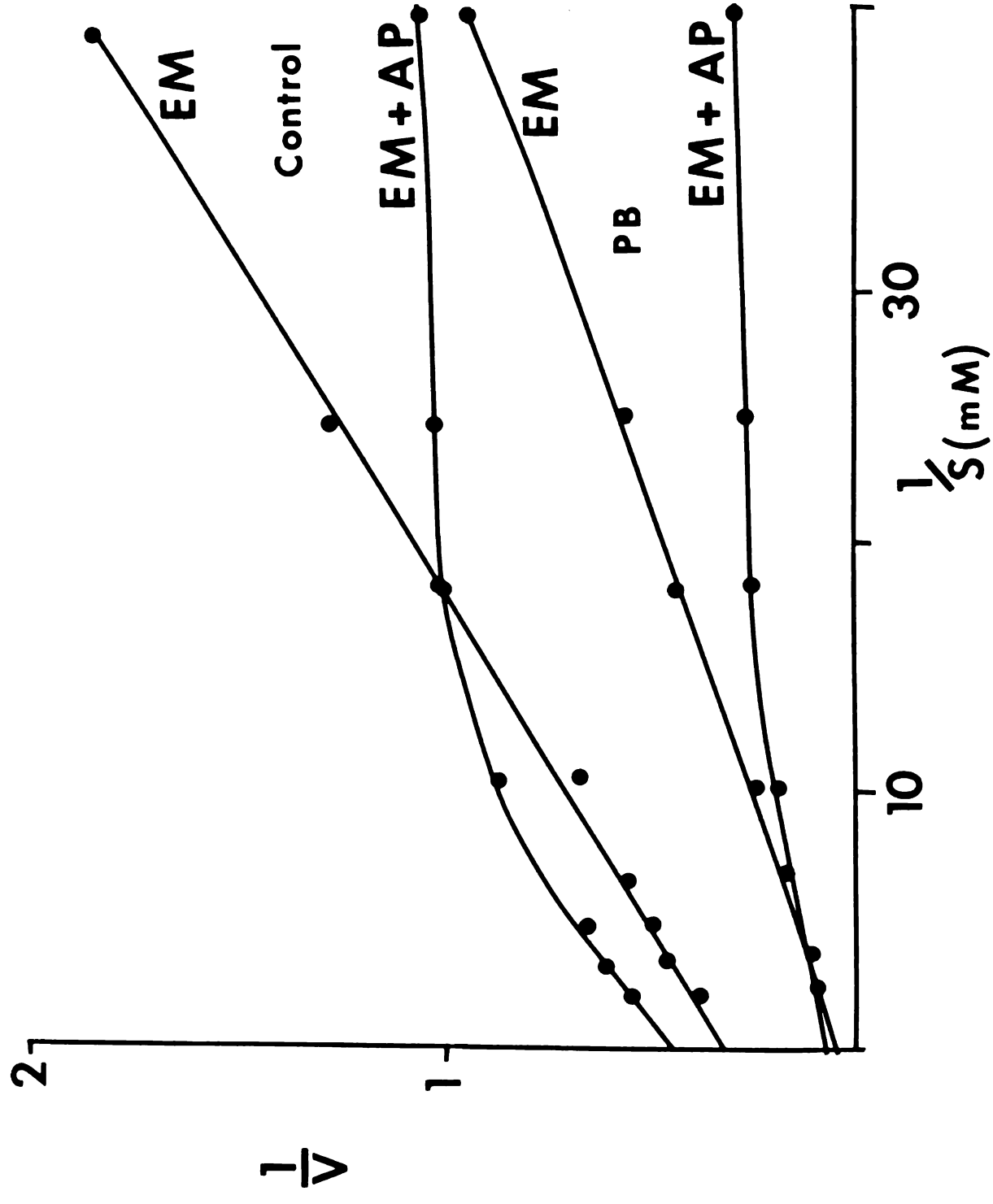

EM
Control
EM+AP
EM
PB
EM+AP
2
1
10
30
1/V
1/S(mM)

Fig. 4.--Lineweaver-Burk plots of aminopyrine demethylase activity in male and female control microsomes and animals pretreated with testosterone. Velocity is in mμM of formaldehyde min^{-1}, mg^{-1} protein. Substrate concentrations are molar.

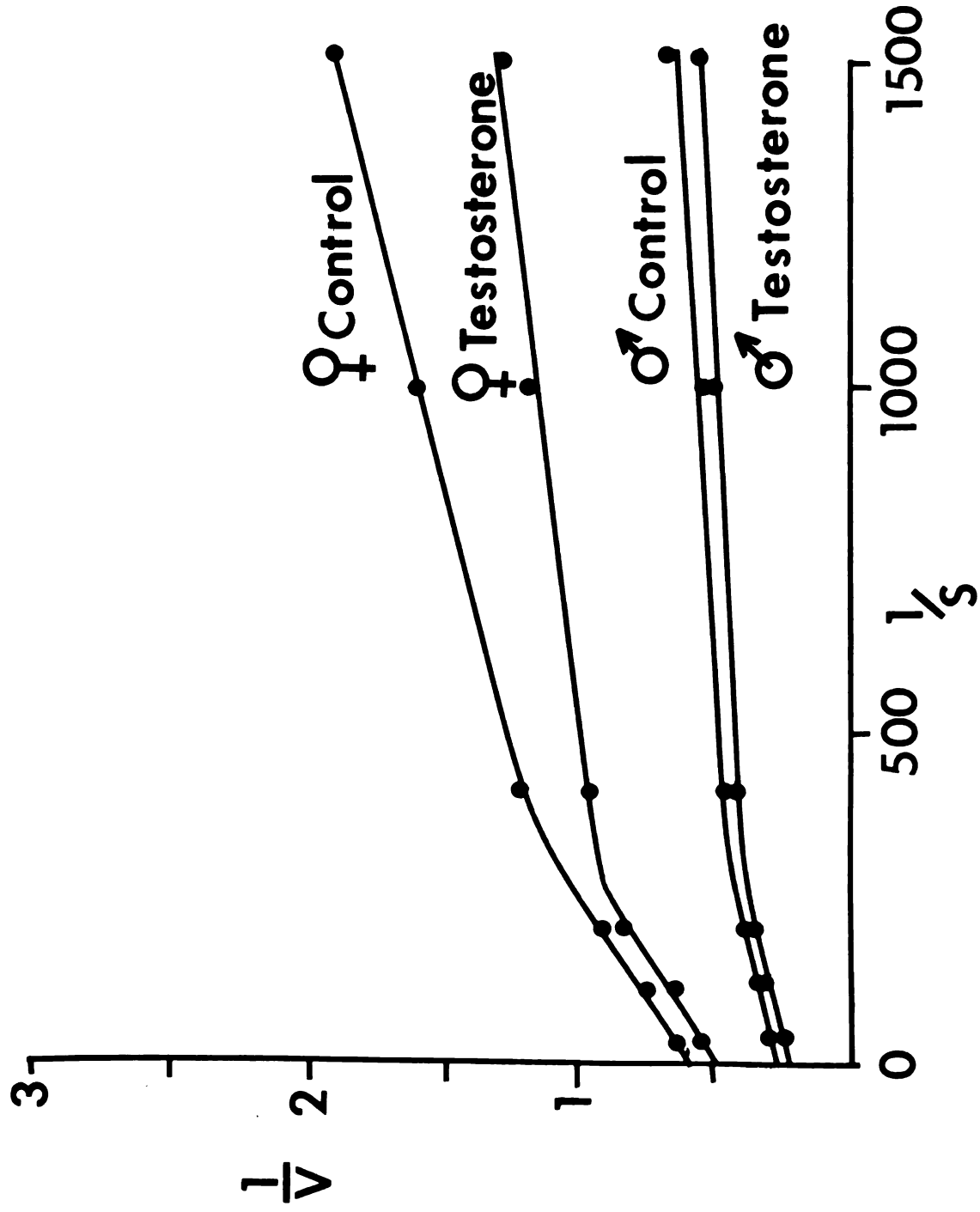

♀Control
♀Testosterone
♂Control
♂Testosterone
1/V
1/S
3
2
1
0
500
1000
1500

Fig. 5.--Lineweaver-Burk plots of aminopyrine demethylase activity in microsomes from control animals with the inhibitors dieldrin (200 μM), metopirone (10 μM), and hexobarbitol (1.2mM) in the incubation mixture. Velocity is in mμM of formaldehyde min^{-1}, mg^{-1} protein. Substrate concentrations are molar.

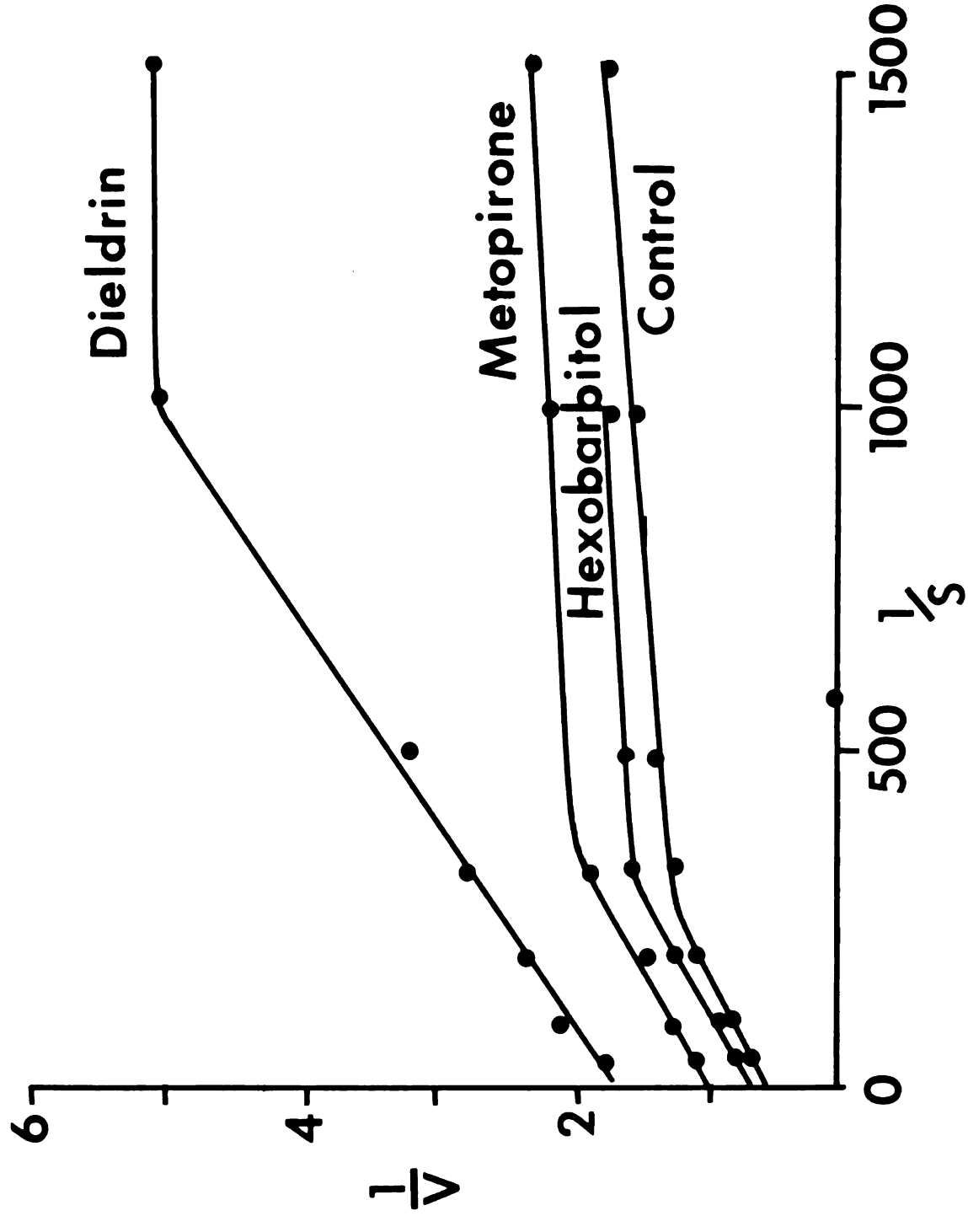

Dieldrin
Metopirone
Hexobarbitol
Control
6
4
2
0
500
1000
1500
1/V
1/S

Fig. 6.--Lineweaver-Burk plots of aminopyrine demethylase activity in microsomes from control animals with the inhibitors SKF-525-A (20 μM) and SKF-8742-A (50 μM) in the incubation mixture. Velocity is in mμM of formaldehyde min^{-1}, mg^{-1} protein. Substrate concentrations are molar.

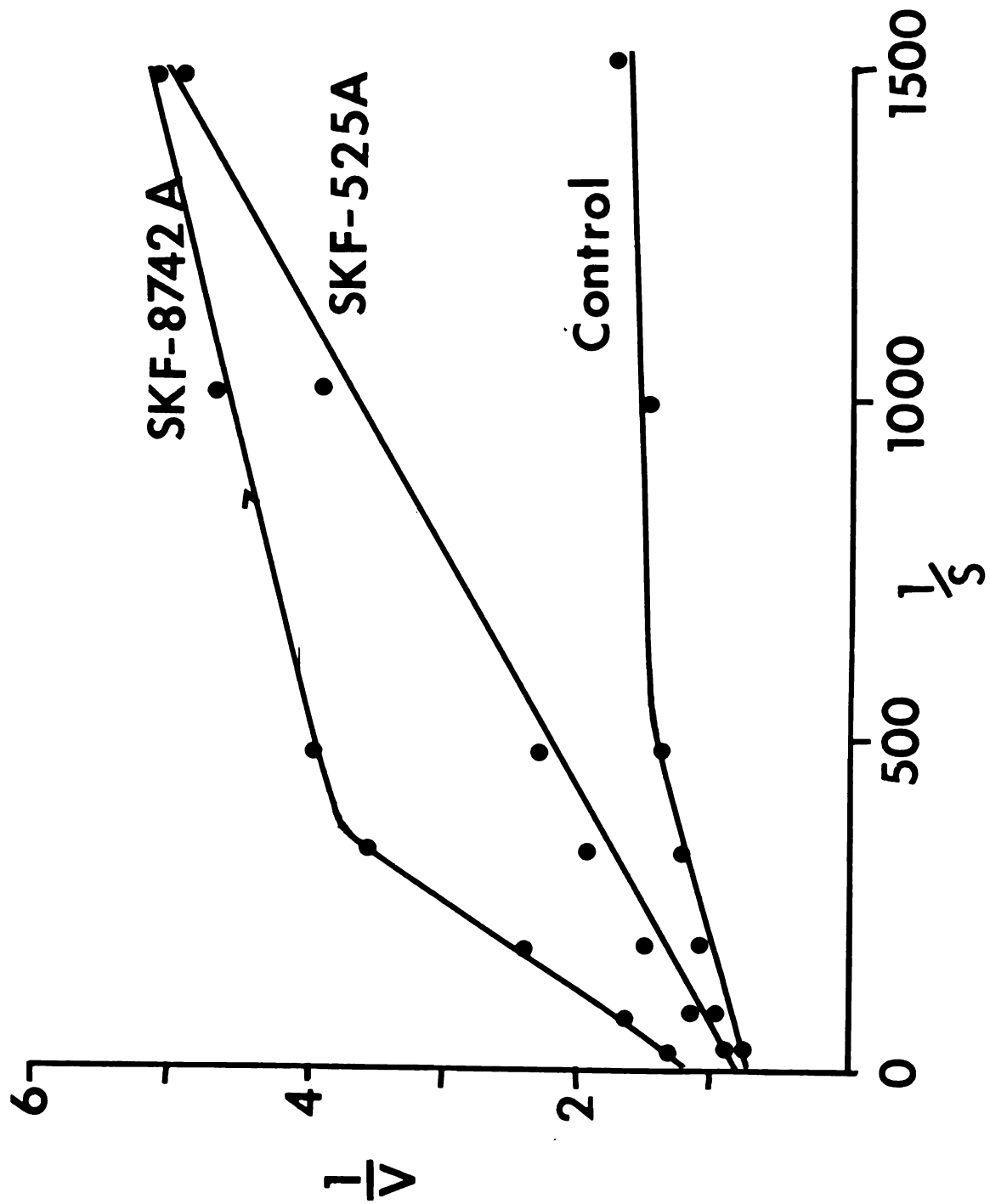

SKF-8742A
SKF-525A
Control
6
4
2
0
500
1000
1500
1/V
1/S

Fig. 7.--Lineweaver-Burk plots of aminopyrine demethylase activity in microsomes from control animals with the inhibitors benzpyrene (100 μM), Lilly 18947 (100 μM), and testosterone (1.4 mM) in the incubation mixtures. Velocity is in mμM of formaldehyde min^{-1}, mg^{-1} protein. Substrate concentrations are molar.

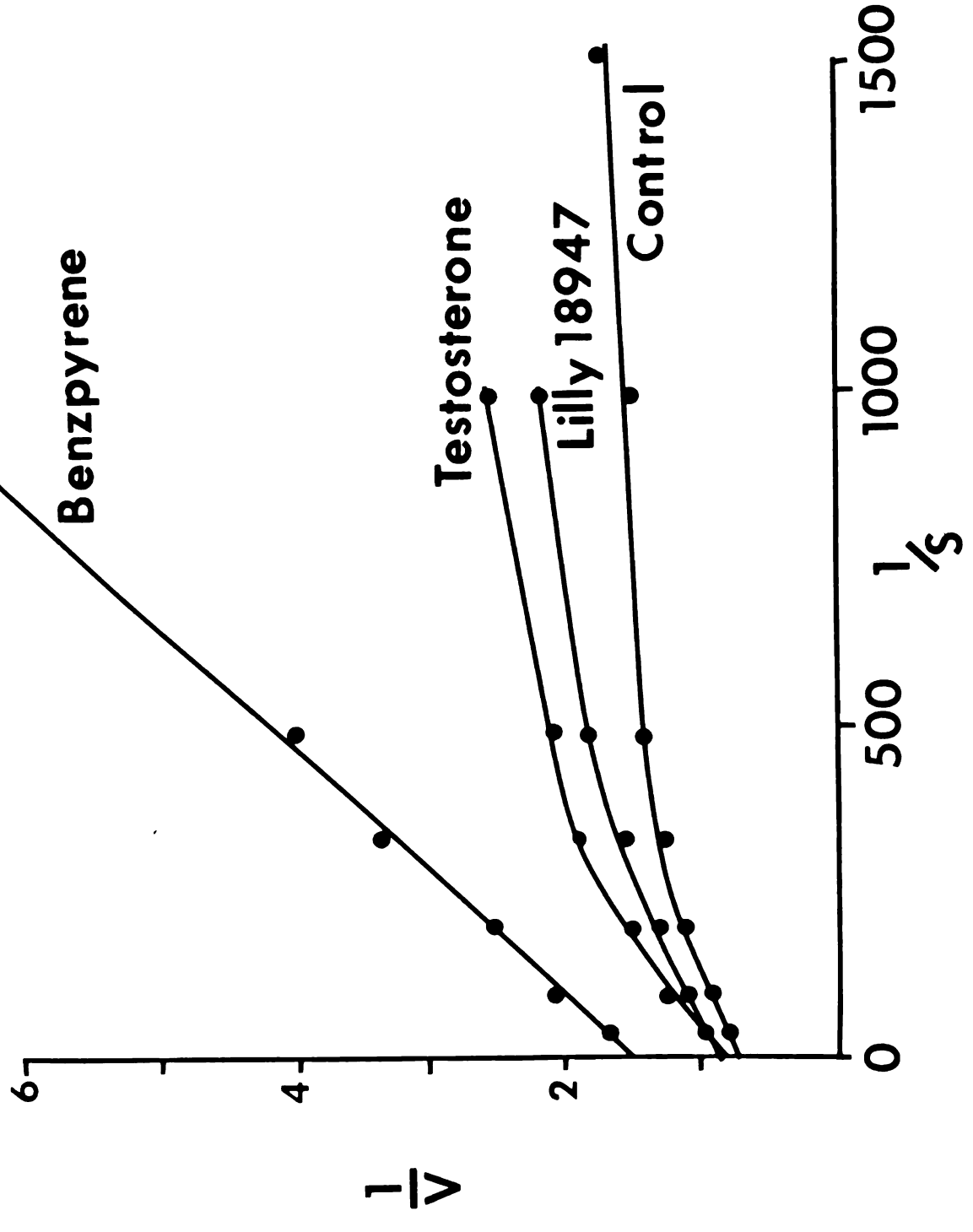
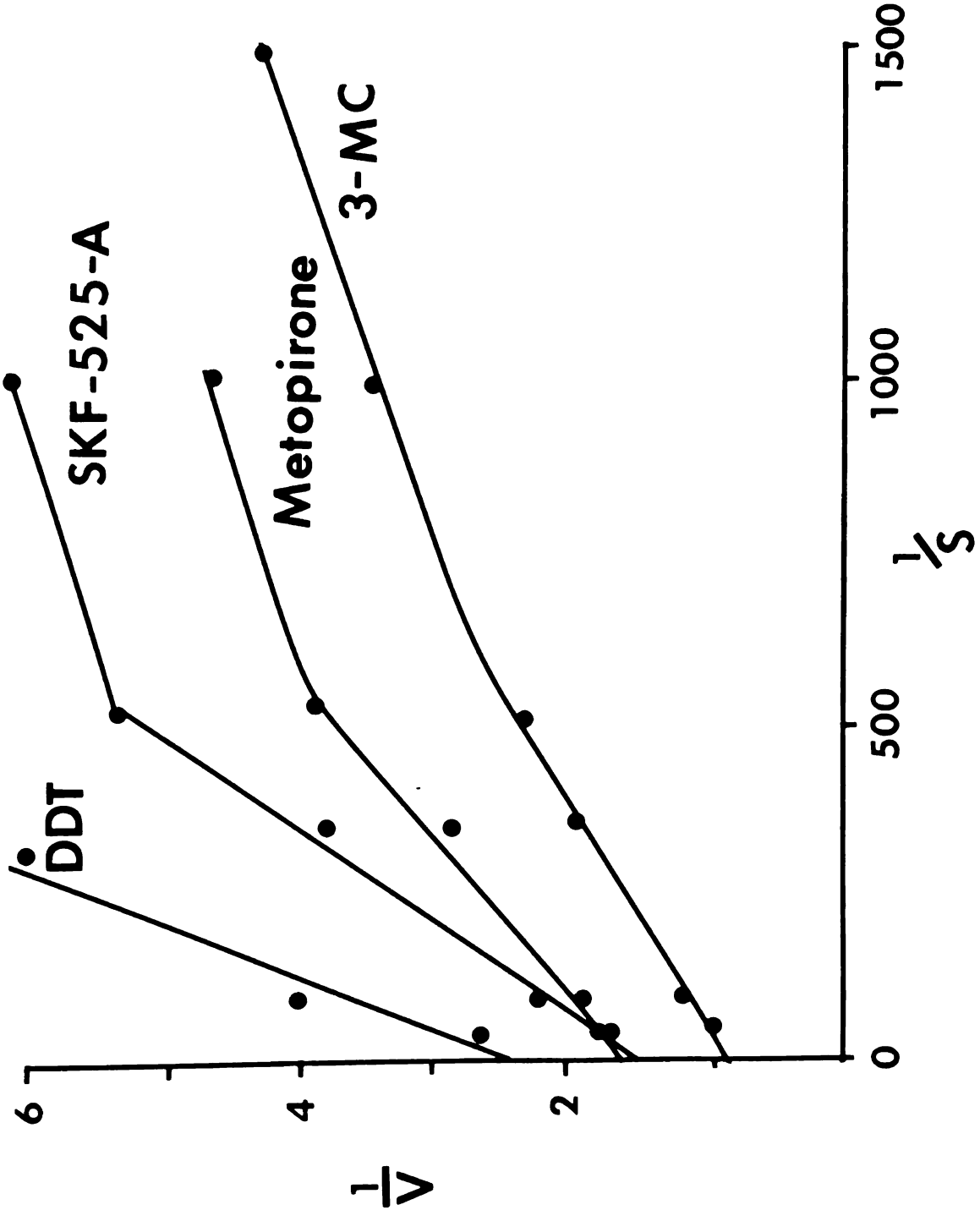

Benzpyrene
Testosterone
Lilly 18947
Control
6
4
2
0
500
1000
1500
1/V
1/S

Fig. 8.--Lineweaver-Burk plots of aminopyrine demethylase activity in microsomes from 3-methylcholanthrene (3-MC) pretreated animals with the inhibitors SKF-525-A (20 μM), DDT (200 μM), and metopirone (10 μM) in the incubation mixtures. Velocity is in mμM of formaldehyde min^{-1}, mg^{-1} protein. Substrate concentrations are molar.


DDT
SKF-525-A
Metopirone
3-MC
6
4
2
0
500
1000
1500
1/V
1/S

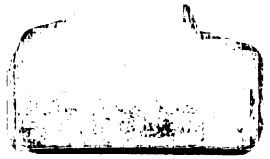


Fig. 9.--Lineweaver-Burk plots of aminopyrine demethylase activity in microsomes from 3-methylcholanthrene (3-MC) pretreated animals with the inhibitors SKF-8742-A (50 μM), hexobarbitol (1.2 mM), and benzpyrene (100 μM) in the incubation mixtures. Velocity is in mμM of formaldehyde min^{-1}, mg^{-1} protein. Substrate concentrations are molar.

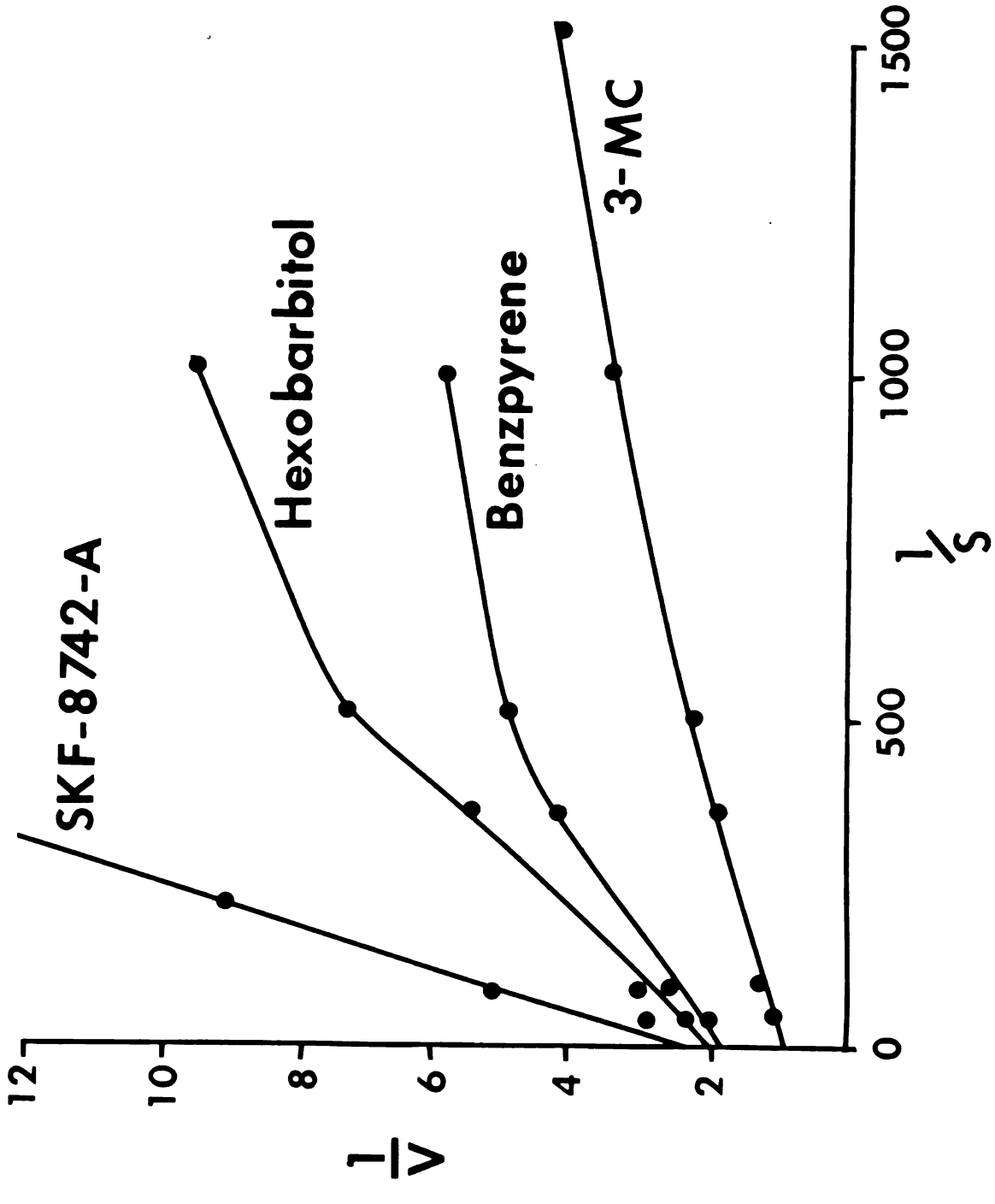
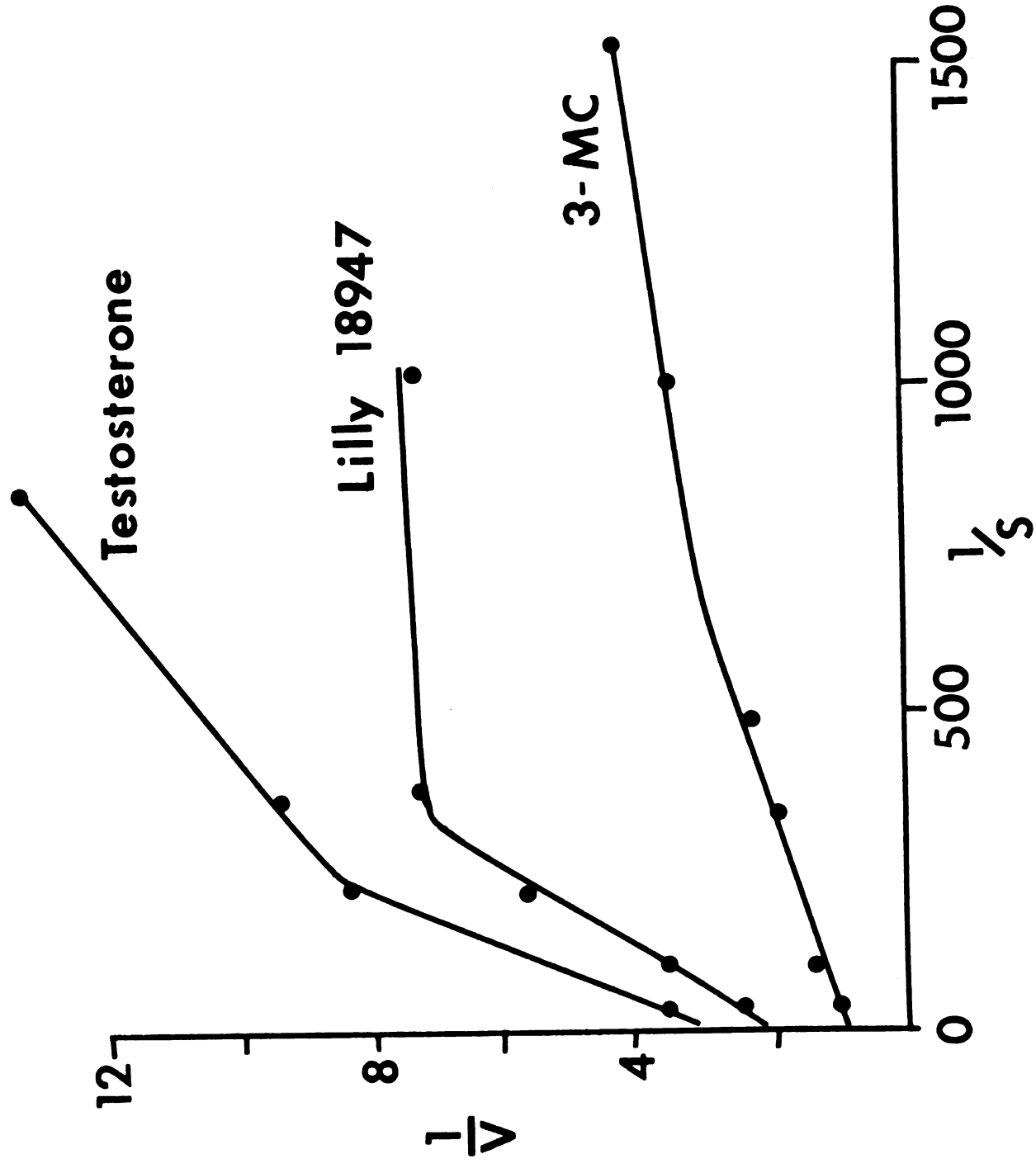

SKF-8742-A
Hexobarbitol
Benzpyrene
3-MC
12
10
8
6
4
2
0
500
1000
1500
1/V
1/S

Fig. 10.--Lineweaver-Burk plots of aminopyrine demethylase activity in microsomes from 3-methylcholanthrene (3-MC) pretreated animals with the inhibitors testosterone (1.4 mM), and Lilly 18947 (100 μM) in the incubation mixtures. Velocity is in mμM of formaldehyde min^{-1}, mg^{-1} protein. Substrate concentrations are molar.


Testosterone
Lilly 18947
3-MC
12
8
4
0
500
1000
1500
1/V
1/S

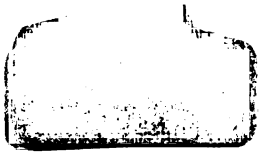
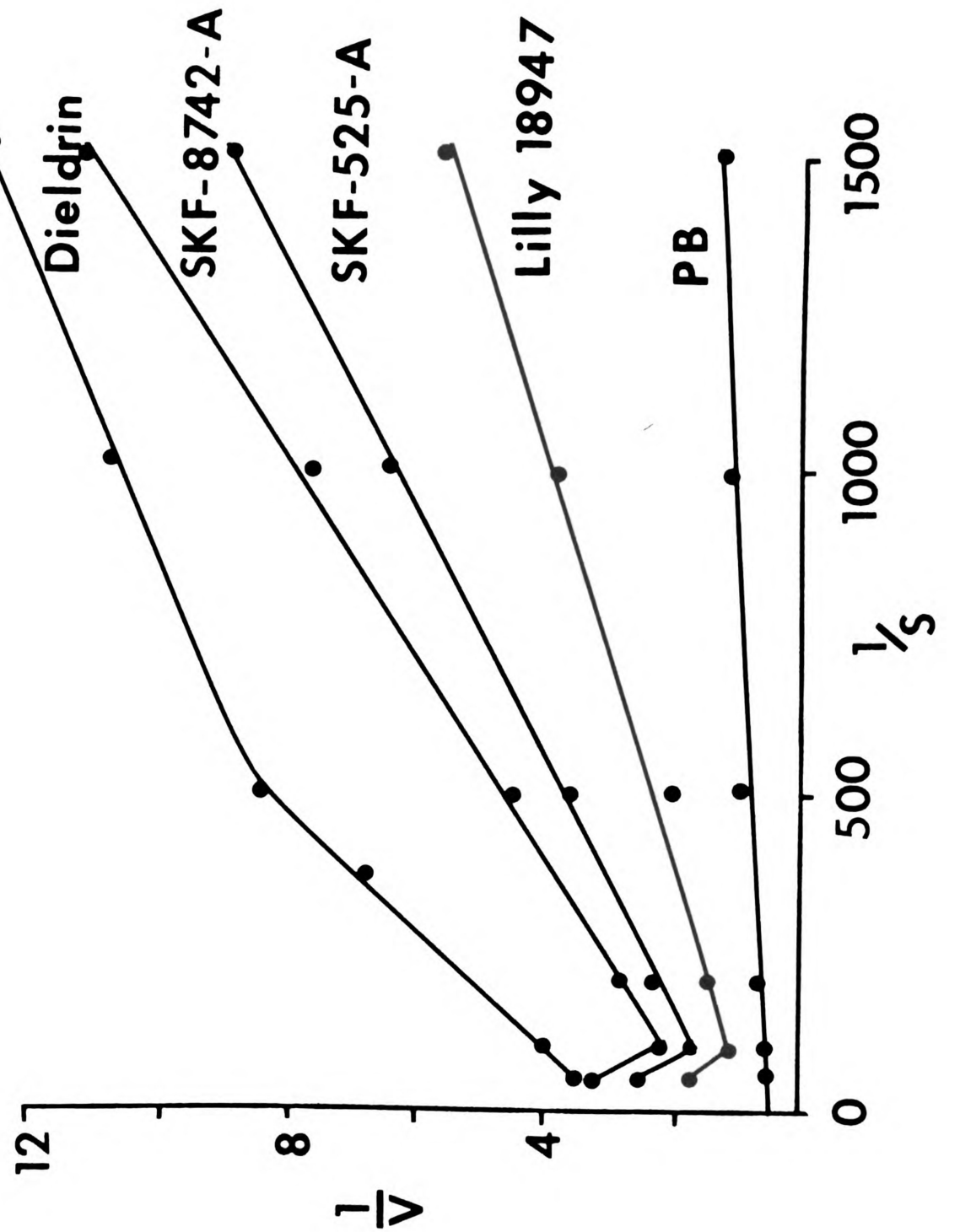


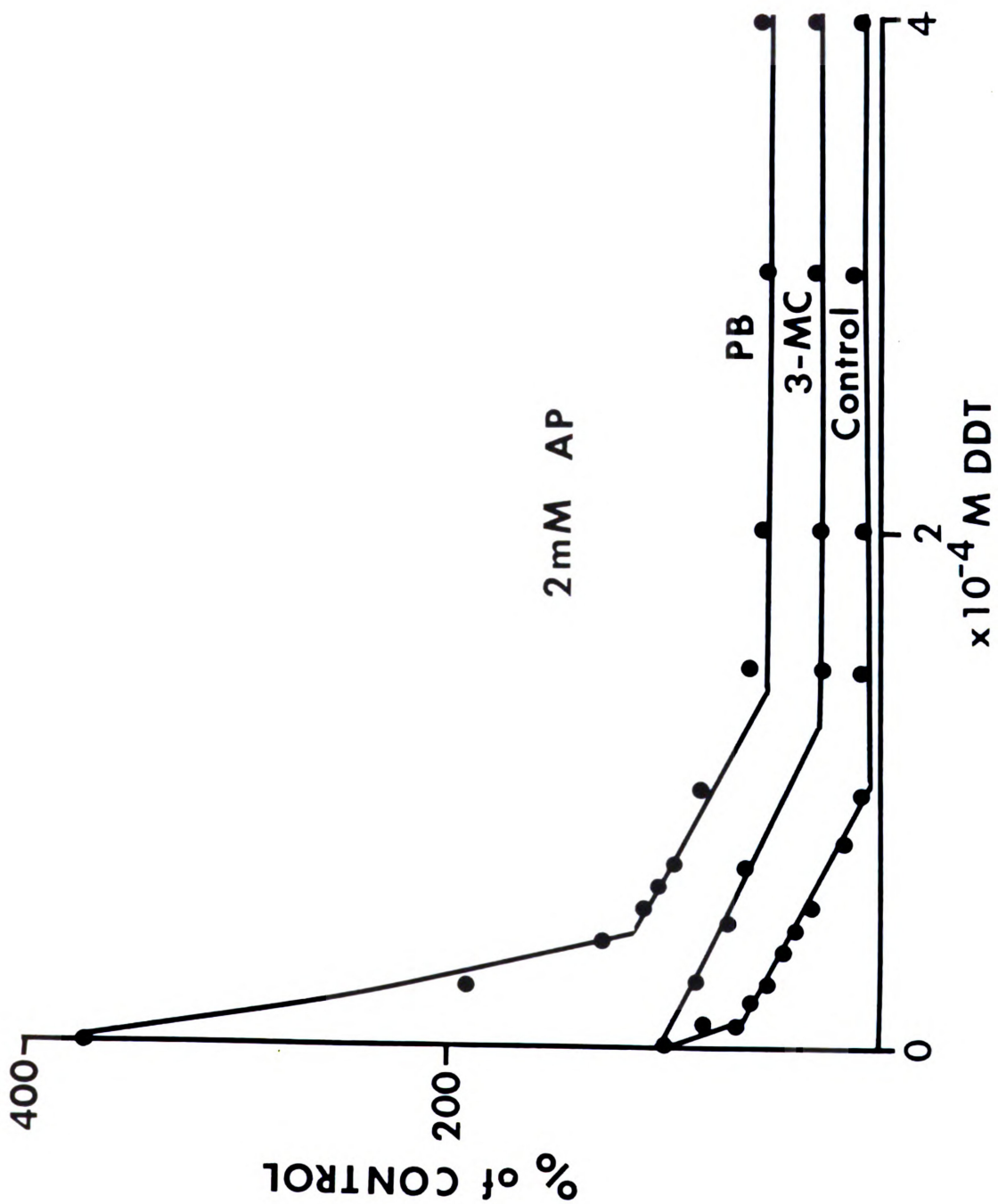
Fig. 11.--Lineweaver-Burk plots of aminopyrine demethylase activity in microsomes from phenobarbital (PB) pretreated animals with the inhibitors dieldrin (200 μM), SKF-525-A (20 μM), SKF-8742-A (50 μM), and Lilly 18947 (100 μM) in the incubation mixtures. Velocity is in mμM of formaldehyde min^{-1}, mg^{-1} protein. Substrate concentrations are molar.


Dieldrin
SKF-8742-A
SKF-525-A
Lilly 18947
PB
12
8
4
0
500
1000
1500
1/V
1/S

seemed that in control microsomes dieldrin and metopirone inhibited activity at low substrate levels. When 3-MC microsomes were used, SKF-8742-A exhibited a greater extent of inhibition than other two types of microsomes (Figures 6 and 9). However, in PB induced microsomes, dieldrin was a better inhibitor than SKF-8742-A (Figure 11).

The inhibition of aminopyrine demethylation in control, PB, and 3-MC microsomes at increasing concentrations of the inhibitor DDT at a constant substrate level is shown in Figure 12. The data was presented as percent control microsomal activity in absence of inhibitor. The curves are not hyperbolic as classical kinetics predicts[49] but rather seem to be made up of three linear segments which divided the total demethylase activity into three components, one not inhibited by DDT, one moderately inhibited by DDT, and a third which was extremely sensitive to DDT inhibition. In PB microsomes the component which was most sensitive to DDT inhibition was induced to an activity 10 times that in control microsomes. This component was essentially completely inhibited at 50 μM DDT. The second component, also sensitive to DDT, was slightly more active in 3-MC microsomes only, and it was inhibited from 50-150 μM DDT. The activity which was insensitive to DDT inhibition does not contribute significantly to the overall activity of control microsomes at this substrate level (2mM aminopyrine).

Fig. 12.--Inhibition of microsomal aminopyrine demethylase activity by DDT. Activity is expressed as percent of control activity (100%) in the absence of inhibitor. Microsomes were obtained from control animals (Control) and from animals pre-treated with phenobarbital (PB) and 3-methylcholanthrene (3-MC). Aminopyrine concentration was 2 m*M*.


% of CONTROL
400
200
0
2
4
x10⁻⁴ M DDT
2mM AP
PB
3-MC
Control

At increasing substrate levels, the contribution of the DDT-insensitive component to the total activity seemed to increase significantly relative to the other two components (Figure 13). At concentrations of DDT over 150 μM both of the DDT-sensitive components would be completely inhibited, and therefore it was possible to study the DDT-insensitive component.

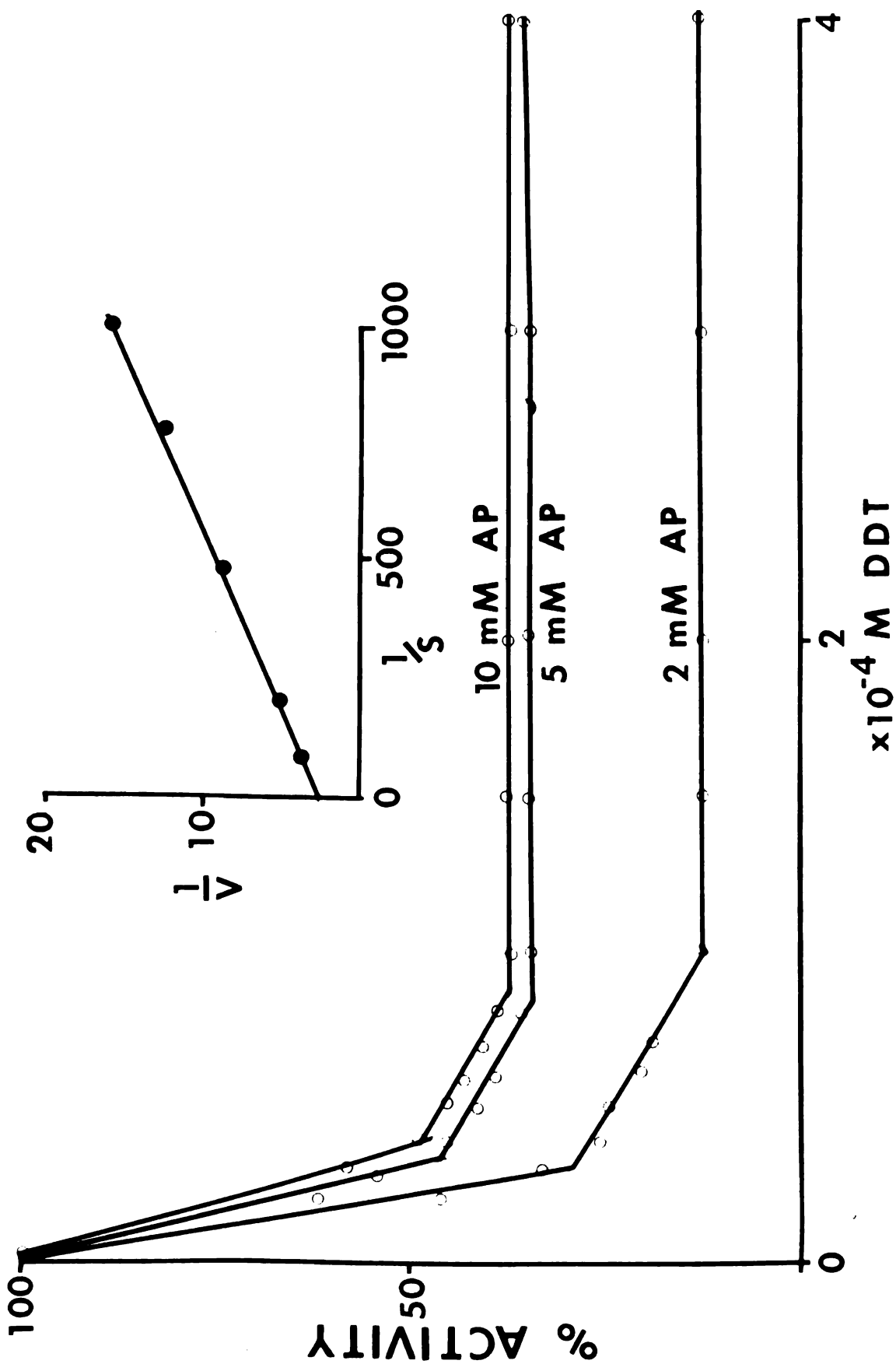
As can be seen in Figure 13 and 13a, the activity of this component was related to the substrate concentration as described by the Lineweaver-Burk plot. If the activities at various levels of substrate and 200 μM DDT are plotted in a reciprocal plot, a K_m of 1.3×10^{-2} M was obtained (13a), which was about an order of magnitude greater than the K_m obtained by extrapolation of the Lineweaver-Burk plot for 3-MC microsomes shown in Figure 1.

Figure 14 shows the effect of increasing levels of DDT on the aminopyrine demethylase activity in microsomes prepared from control rats (male and female) and rats pretreated with testosterone. Testosterone induced the DDT-insensitive component in female control microsomes to a level equivalent to that in male microsomes, yet testosterone had no effect on male control microsomal activity.

When dieldrin was used as the inhibitor of aminopyrine demethylation in PB microsomes, only two components were evident. At substrate concentrations above 2 mM, demethylase activity at increasing levels of dieldrin did

Fig. 13.--The inhibition of aminopyrine demethylase in microsomes from PB pretreated animals by DDT at various concentrations of aminopyrine (AP).

Fig. 13a.--Lineweaver-Burk plot of aminopyrine demethylase activity in microsomes from phenobarbital (PB) pretreated animals at 0.1 m$\underline{M}$ DDT. Activity is expressed as mμmoles of formaldehyde $\overline{min}^{-1}$, mg^{-1} protein. Substrate concentrations are molar.


% ACTIVITY
100
50
0
2
4
x10-4 M DDT
10 mM AP
5 mM AP
2 mM AP
1/V
20
10
0
500
1000
1/S

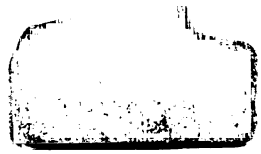
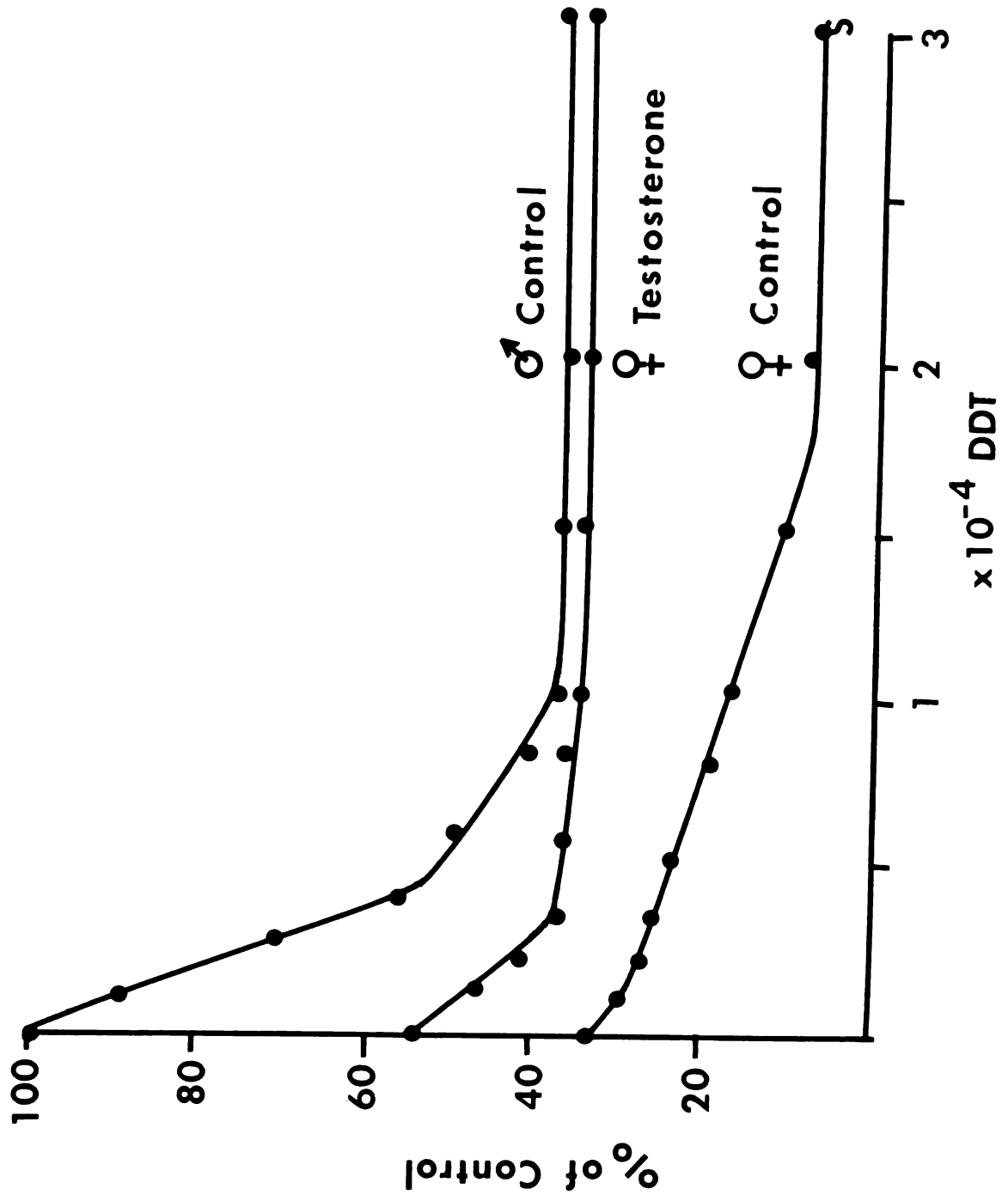


Fig. 14.--Inhibition of microsomal aminopyrine demethylase activity by DDT. Activity is expressed as percent of male control (100%) in the absence of inhibitor. Microsomes were obtained from male and female control and from females pretreated with testosterone. Aminopyrine concentration was 5 m$\underline{M}$.


100
80
60
40
20
% of Control
♂ Control
♀ Testosterone
♀ Control
1
2
3
x10⁻⁴ DDT

not give a hyperbolic curve but seemed to be divided into two linear portions representing two components (Figure 15); one having a low K_i for dieldrin and the second not inhibited by dieldrin. Repeating an experiment like that shown in Figure 13, using dieldrin as the inhibitor at concentrations greater than 100 μM, a non-linear Lineweaver-Burk plot was obtained (Figure 16 and 16a). When metopirone was used as the inhibitor, quite similar results were obtained.

Comparison of the relative amounts of each component in control, PB, and 3-MC microsomes suggest that the dieldrin and metopirone sensitive component was that which was induced by PB (Figure 15). The dieldrin and metopirone sensitive activity therefore must be made up of two components, that which was moderately sensitive to DDT inhibition and that which was DDT-insensitive. Thus the PB induced component was not the same as the low K_m component in control microsomes. The K_m of the PB induced component could only be estimated assuming that the low K_m component of control microsomes would not contribute significantly to the activity in "highly" induced PB microsomes. Using this assumption a K_m for aminopyrine of 5×10^{-4} M was obtained.

Pretreatment of animals with 3-MC consistently decreased the maximum degree of inhibition obtainable by either DDT or dieldrin (Figures 12 and 15). These results

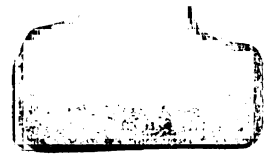
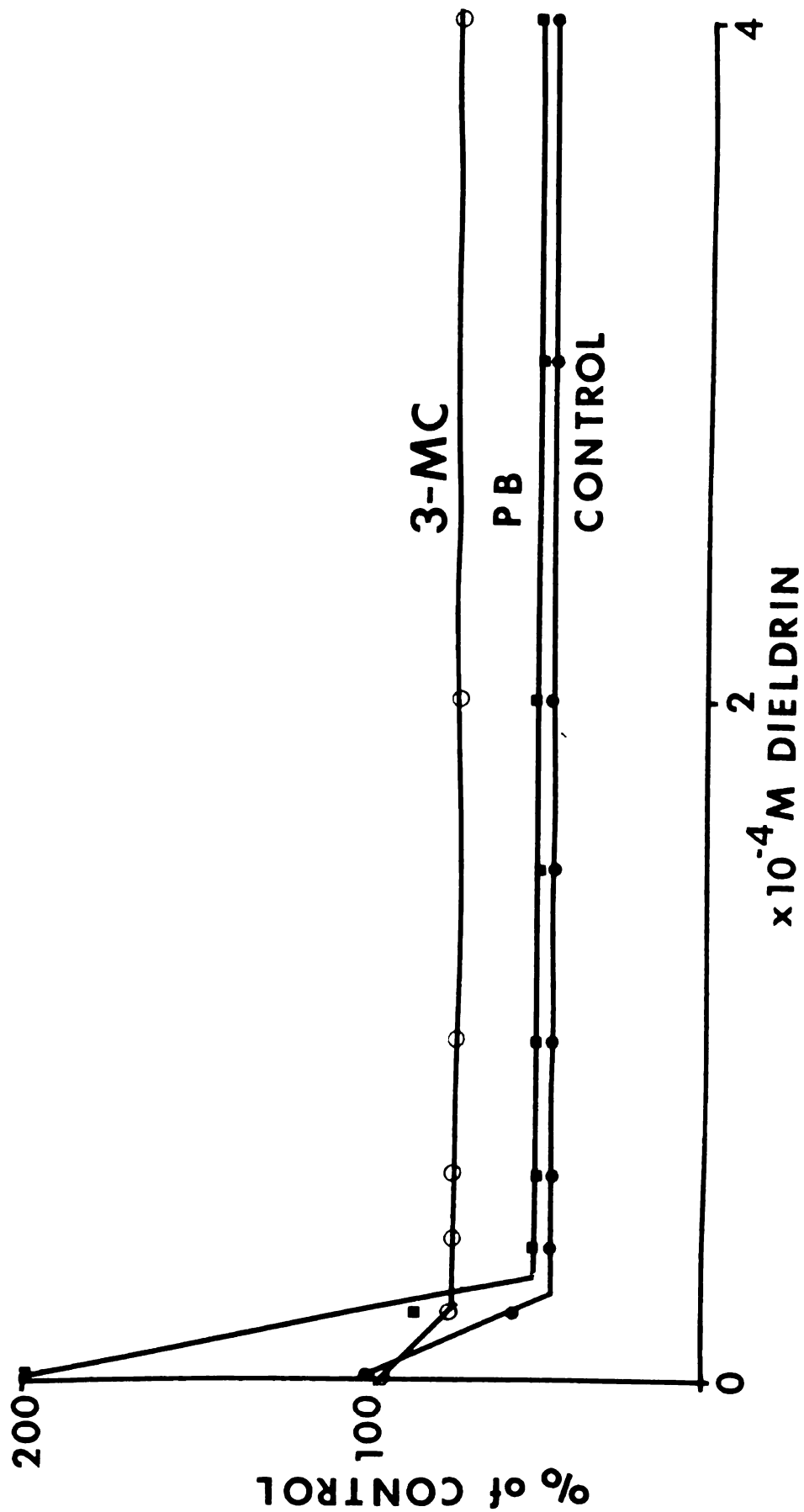


Fig. 15.--Inhibition of aminopyrine demethylase activity in microsomes from control (Control), 3-methylcholanthrene (3-MC) and Phenobarbital (PB) pretreated animals by dieldrin. Activity is expressed as percent of control activity in the absence of inhibitor. Aminopyrine concentration was 5 m<u>M</u>.


% of CONTROL
200
100
0
2
4
x10^-4M DIELDRIN
3-MC
PB
CONTROL

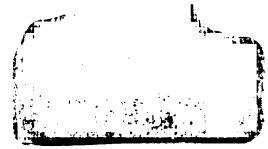
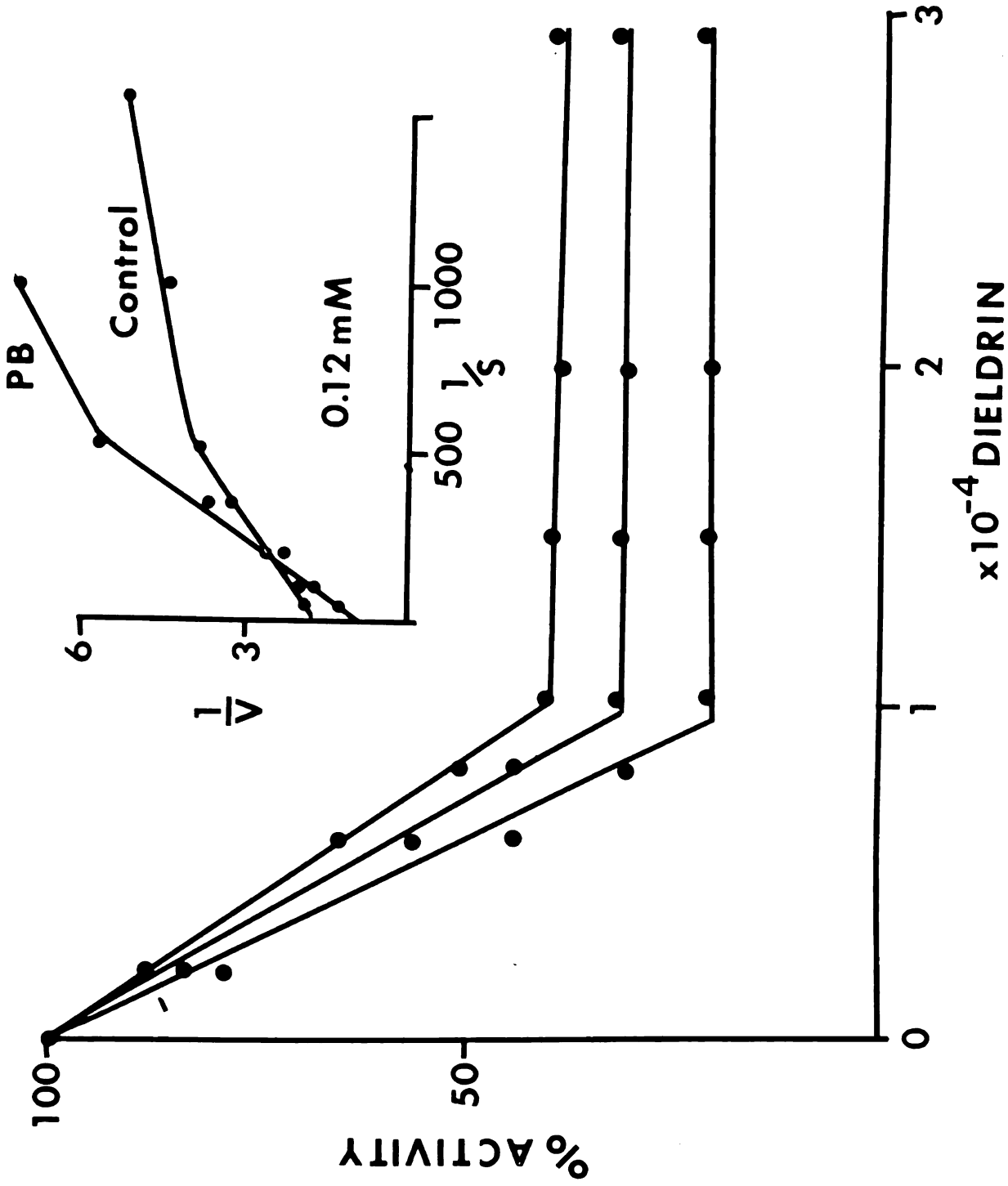


Fig. 16.--The inhibition of aminopyrine demethylase in microsomes from phenobarbital pretreated animals by dieldrin at various concentrations of amino-pyrine (AP).

Fig. 16a.--Lineweaver-Burk plots of aminopyrine demethylase activity in microsomes from control (Control) and phenobarbital (PB) pretreated animals at 0.12 mM dieldrin. Activity is expressed as mμMoles of formaldehyde min^{-1}, mg^{-1} protein. Substrate concentrations are molar.


% ACTIVITY
100
50
0
1
2
3
x10^{-4} DIELDRIN
PB
Control
0.12 mM
500
1000
1/S
1/V
6
3

could be explained if 3-MC pretreatment induced the synthesis of the DDT-insensitive component. This was investigated by comparing the aminopyrine demethylase activity in control and 3-MC microsomes in the presence of 200 μM DDT. The results (Figure 17) showed a two-fold increase in maximum velocity with no change in the K_m.

Inhibition of the high K_m component for aminopyrine demethylase was seen using SKF-525-A, DPEA, and Lilly 18947. DDT (200 μM) was present in all assays to insure complete inhibition of the two DDT-sensitive components. All three types of classical inhibition: competitive, non-competitive, and uncompetitive were seen using these three inhibitors, respectively (Figure 18).

Experiments were then designed to study the mechanism of inhibition of some of these inhibitors, in hopes of learning the nature of the multiplicity of aminopyrine demethylase.

Inhibition studies such as those shown in Figure 12 and Figure 14 were performed in a pure oxygen atmosphere to determine if any reversal of inhibition could be obtained. A pure oxygen atmosphere resulted in a slight additional inhibition of all activities rather than a reversal of inhibition. Increasing NADPH concentrations resulted in only a slight reversal of inhibition at extremely high concentrations (28 mM). However, a K_m for NADPH for this particular reaction was found to be 9.2×10^{-5} M.

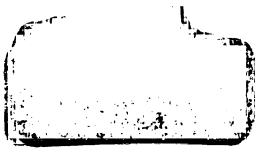
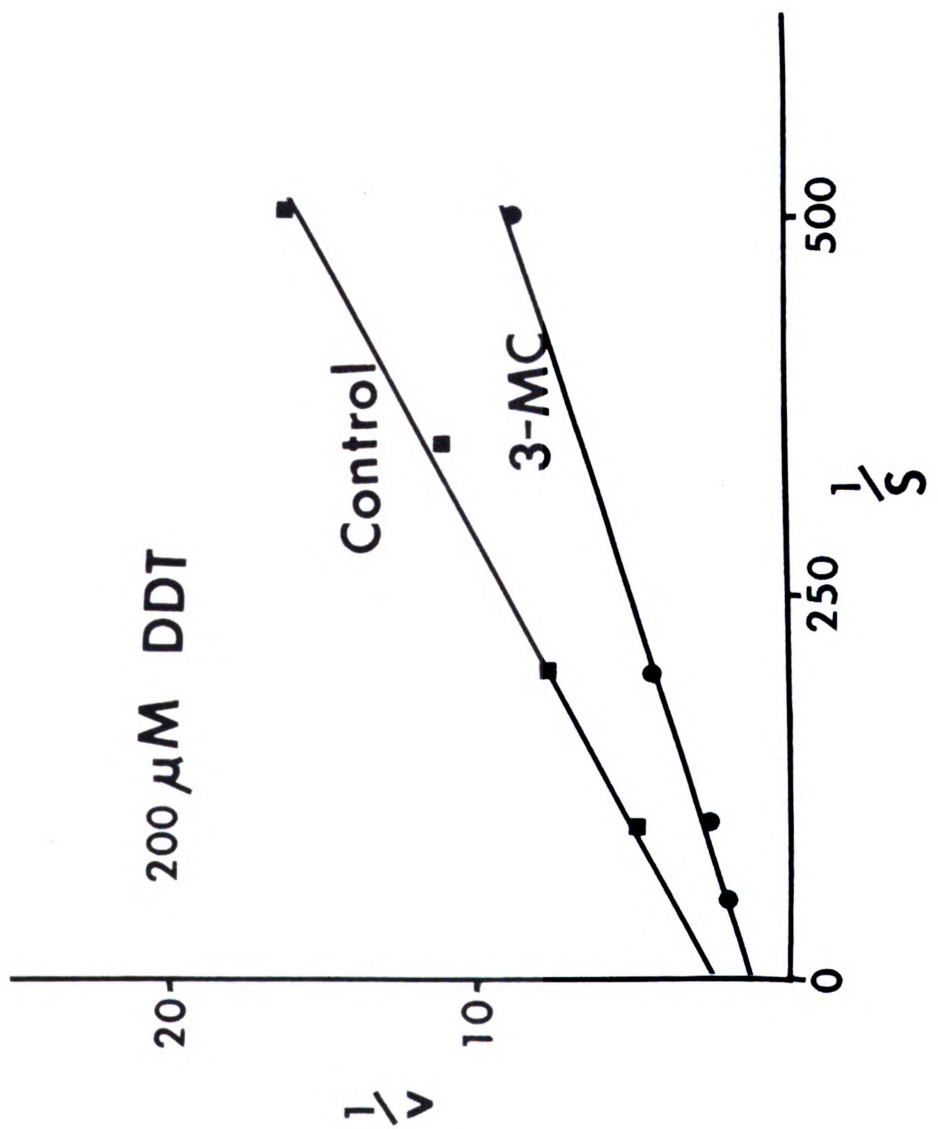


Fig. 17.--Lineweaver-Burk plot of aminopyrine demethylase activity in microsomes from control (Control) and 3-methylcholanthrene (3-MC) pretreated animals in the presence of 200 μM DDT. Velocity is in mμMoles of formaldehyde min^{-1}, mg^{-1} protein. Substrate concentrations are molar.


200 μM DDT
Control
3-MC
20
10
0
250
500
1/v
1/S

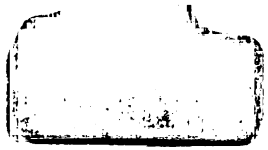
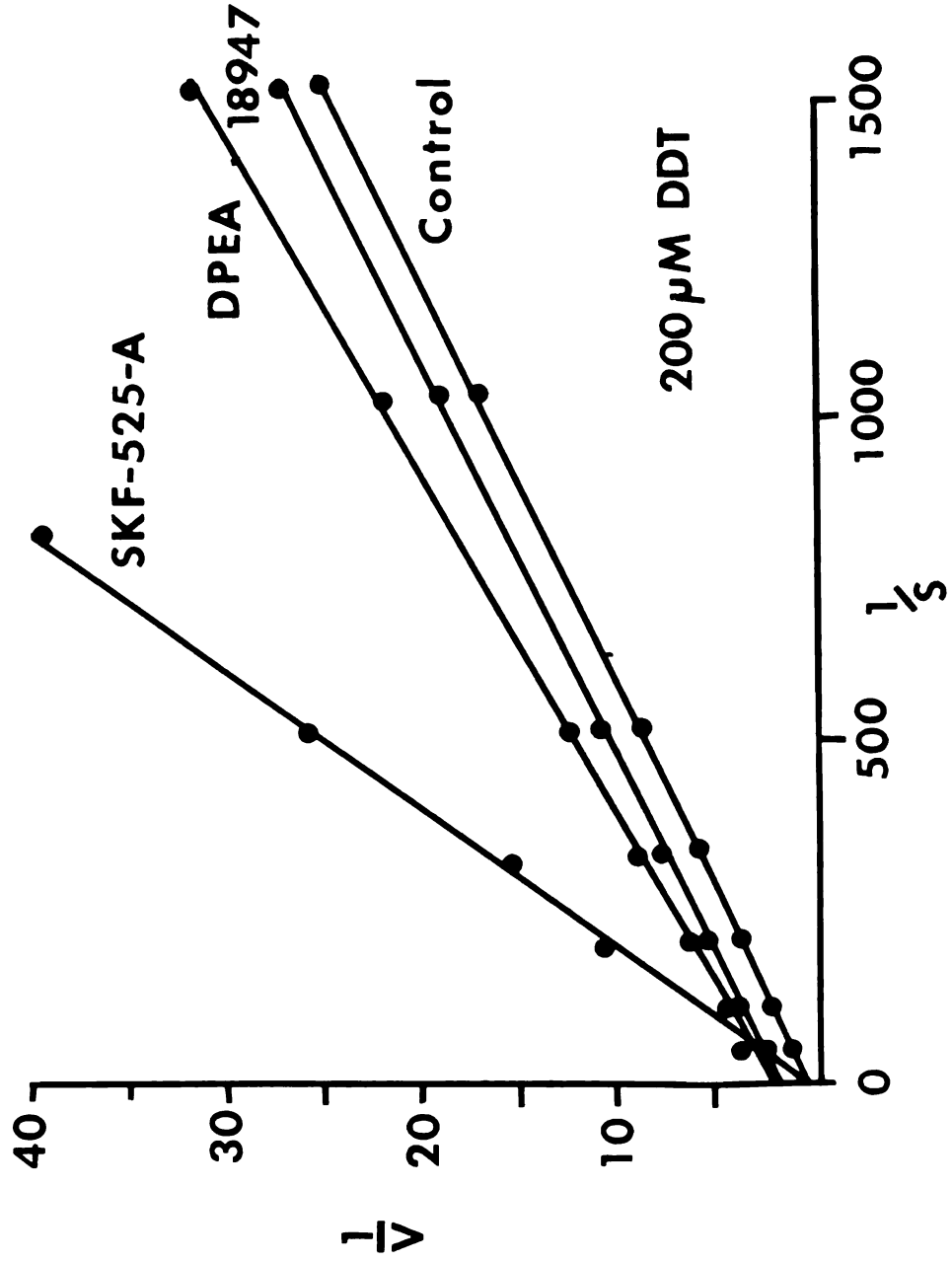


Fig. 18.--Inhibition of microsomal aminopyrine demethylase activity in microsomes from animals pretreated with 3-methylcholanthrene in the presence of 200 μM DDT by SKF-525-A (1.0 mM), DPEA (0.5 mM), and Lilly 18947 (1.0 mM). Velocity is in mμM of formaldehyde min^{-1}, mg^{-1} protein. Substrate concentrations are molar.


SKF-525-A
DPEA
18947
Control
200 μM DDT
40
30
20
10
0
500
1000
1500
1/V
1/S

KCN was also tested as an inhibitor of the demethylase system. However, the results were almost identical to the effect of DDT when the KCN concentration was varied from 0 to 4.0 m$\underline{M}$. Since the DDT and KCN inhibition studies seemed related, the next set of experiments were done to determine if DDT had affected the reductase activity, a logical choice since it took so high a KCN concentration that it would seem reasonable to assume that it wasn't binding to the heme group of P-450. The effect of metopirone and dieldrin was also investigated. To test this hypothesis, cytochrome C- and nitroblue tetrazolium-reductases were chosen as reductases since they might be involved in microsomal electron transport for aminopyrine demethylase. However, none of the above compounds were effective inhibitors of either reductase.

The requirement for both NADPH and NADH for maximal aminopyrine demethylase activity has been reported by other investigators.[50] NADH alone supported no aminopyrine demethylase activity, however, in the presence of NADPH, NADH does seem to stimulate activity suggesting that it supplies reducing equivalents. Therefore, it was reasoned that if a relationship between an inhibitor and NADH could be shown, the mechanism of inhibition might involve the electron transport system. NADH was found to reduce P-450 but at a slower rate than NADPH (Figure 19). DDT was found to stimulate the reduction of P-450 by either NADH

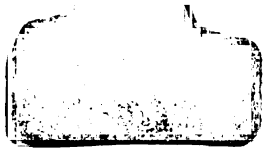
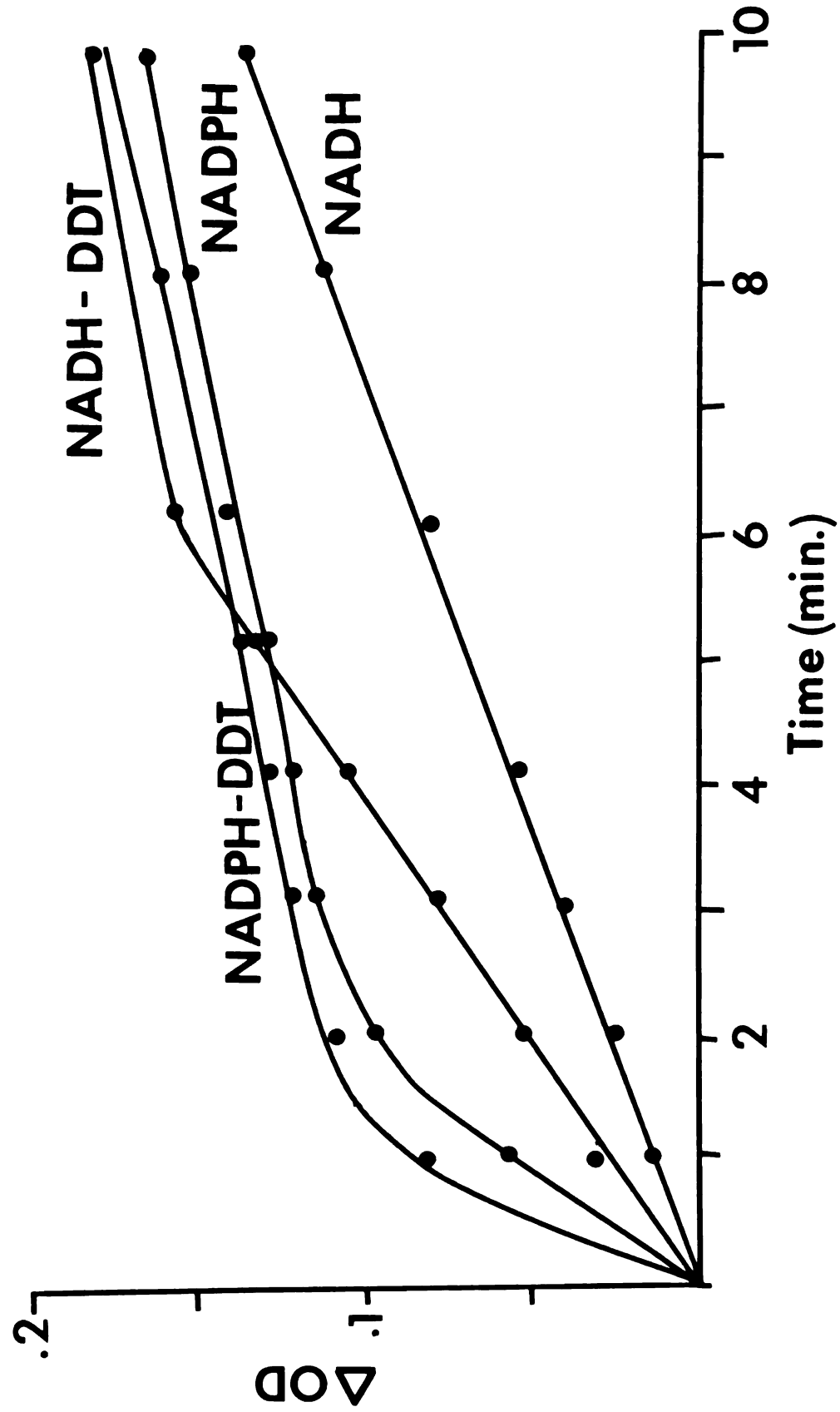


Fig. 19.--Reduction of cytochrome P-450 measured at 450 nm by NADPH and NADH with and without DDT (500 μM) present in the solution.


NADH - DDT
NADPH
NADH
NADPH-DDT
ΔOD
.2
.1
2
4
6
8
10
Time (min.)

or NADPH, however not to the same extent. A greater stimulation was observed when NADH was used. The reduction of P-450 was biphasic[51] and DDT enhanced both the rate and extent of the fast phase of P-450 reduction when NADPH was used (Figure 19). The two rate curves were not additive suggesting a common electron transport system. Thus, the effect of NADH on aminopyrine demethylase activity must not involve reduction of P-450.

Since DDT affected the NADH reduction of P-450, experiments were then designed to observe the effect of NADH or DDT inhibition of aminopyrine demethylase. Typical DDT inhibition curves (Figure 12) varying the NADH concentrations were plotted on Figure 20, and it was found that NADH reversed the inhibition of the moderately sensitive DDT component.

To determine if any of these compounds were inhibiting by binding to the P-450 heme group, substrate difference spectra were obtained using various inhibitors and substrates in both the oxidized and reduced states of the microsomal P-450 particle. Typical type I and type II difference spectra[24] were seen with most inhibitors. DDT failed to show any spectral differences, however, both metopirone and dieldrin showed unusual spectral changes. Metopirone produced a typical type II spectrum with oxidized P-450; however, in the reduced state, metopirone gave a difference spectrum with a large maximum at 446 nm

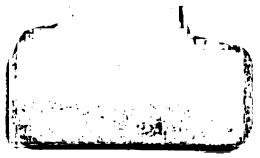
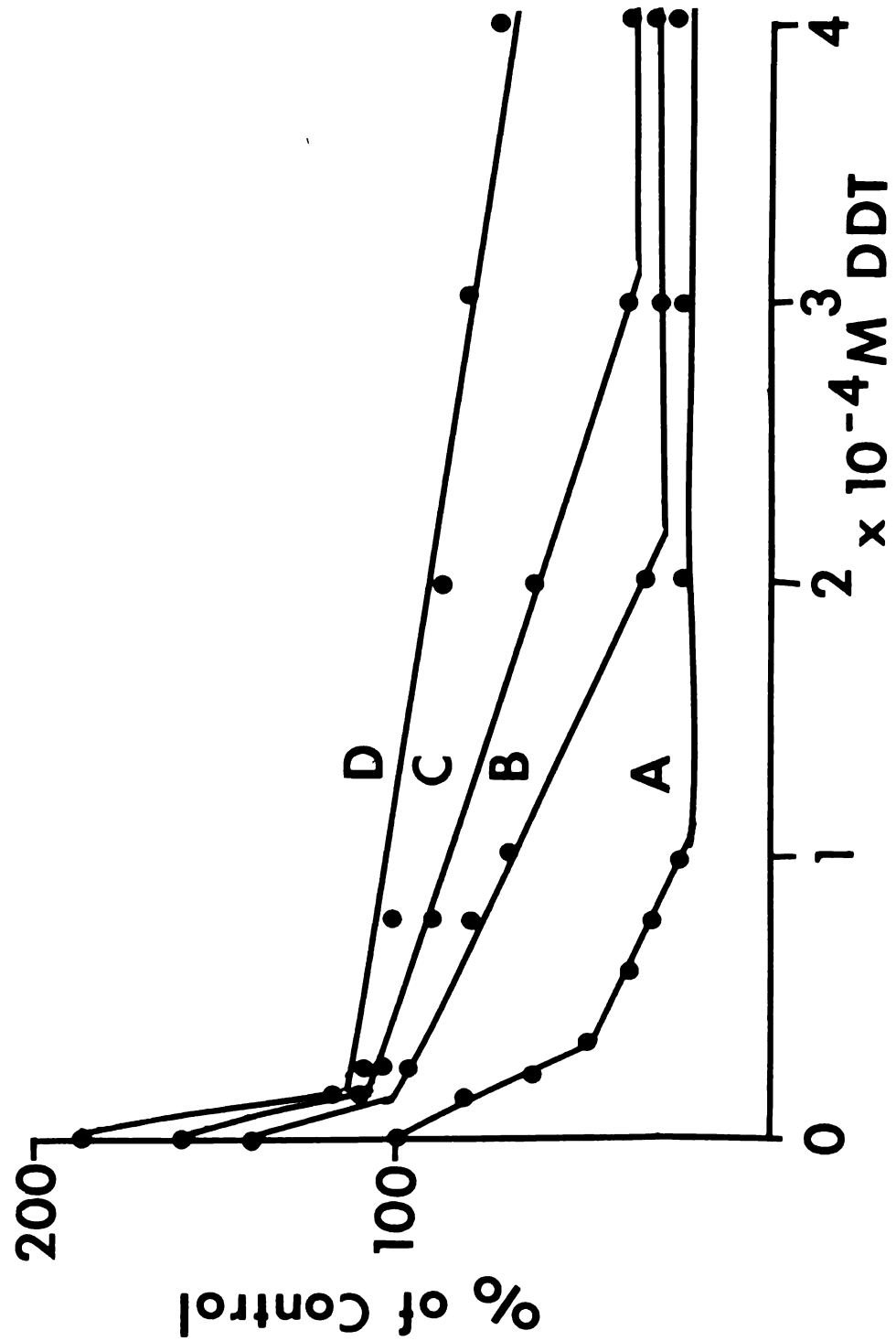


Fig. 20.--Inhibition of microsomal aminopyrine demethylase activity by DDT in the presence of various levels of NADH. Plot A represents 0.00 NADH concentration, B represents 0.1 M, C represents 0.3 M, and D represents 0.5 M. Activity is expressed as percent of control activity (100%) in the absence of NADH.


% of Control
200
100
0
1
2
3
4
x 10⁻⁴M DDT
A
B
C
D

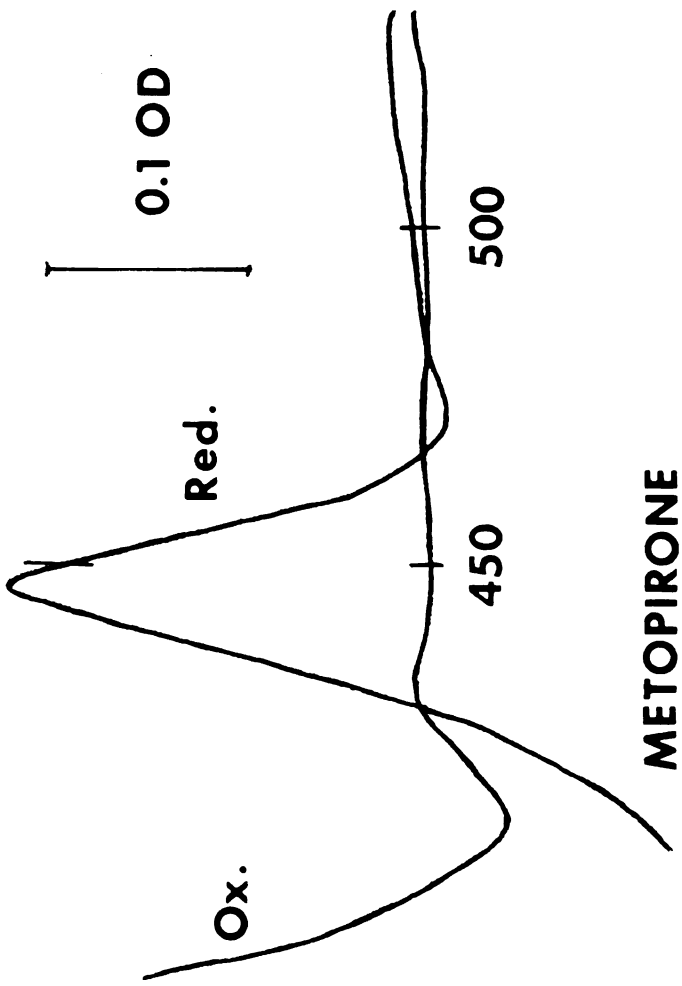
(Figure 21). Dieldrin gave an atypical type I difference spectrum in the oxidized state and a maximum at 450 nm with reduced microsomes (Figure 21).

It was then reasoned that these inhibitors could be effecting the affinity of P-450 for 0_2 thus causing inhibition of activity. CO binding experiments were therefore performed on P-450 using a CO saturated buffer to introduce a specified concentration of CO to compete with the metopirone already present.

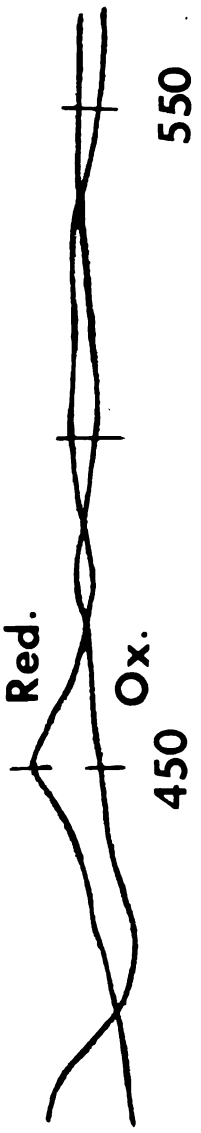
It was found that DDT had no effect upon the binding of CO to microsomal P-450. However, when the inhibitors metopirone and dieldrin were added, a general decrease in the amount of P-450 available for binding CO was found (Figure 22). It had been previously found that metopirone and dieldrin had strong P-450 binding spectra (Figure 21) indicating some type of interaction with the heme group of P-450. The extent of this inhibition of CO binding by metopirone or dieldrin varied depending upon the type of microsomes used. PB microsomes showed the greatest interaction between metopirone and CO while 3-MC pretreated microsomes resulted in the least interaction (Figure 23).

When difference spectra with metopirone and dieldrin were obtained using all three types of microsomes at equal P-450 concentration, it was found that PB microsomes had exhibited the largest metopirone (446 nm) peak and 3-MC microsomes the smallest peak. Dieldrin gave only a slight

Fig. 21.--Oxidized and reduced substrate difference spectra with PB microsomes using metopirone (top) and dieldrin (bottom) as ligands.



METOPIRONE



DIELDRIN

Fig. 22.--CO binding curves for microsomes of control animals (middle) and animals pretreated with PB (top), and 3-MC (bottom) with and without metopirone present in solution. O.D. was measured at 450 nm.

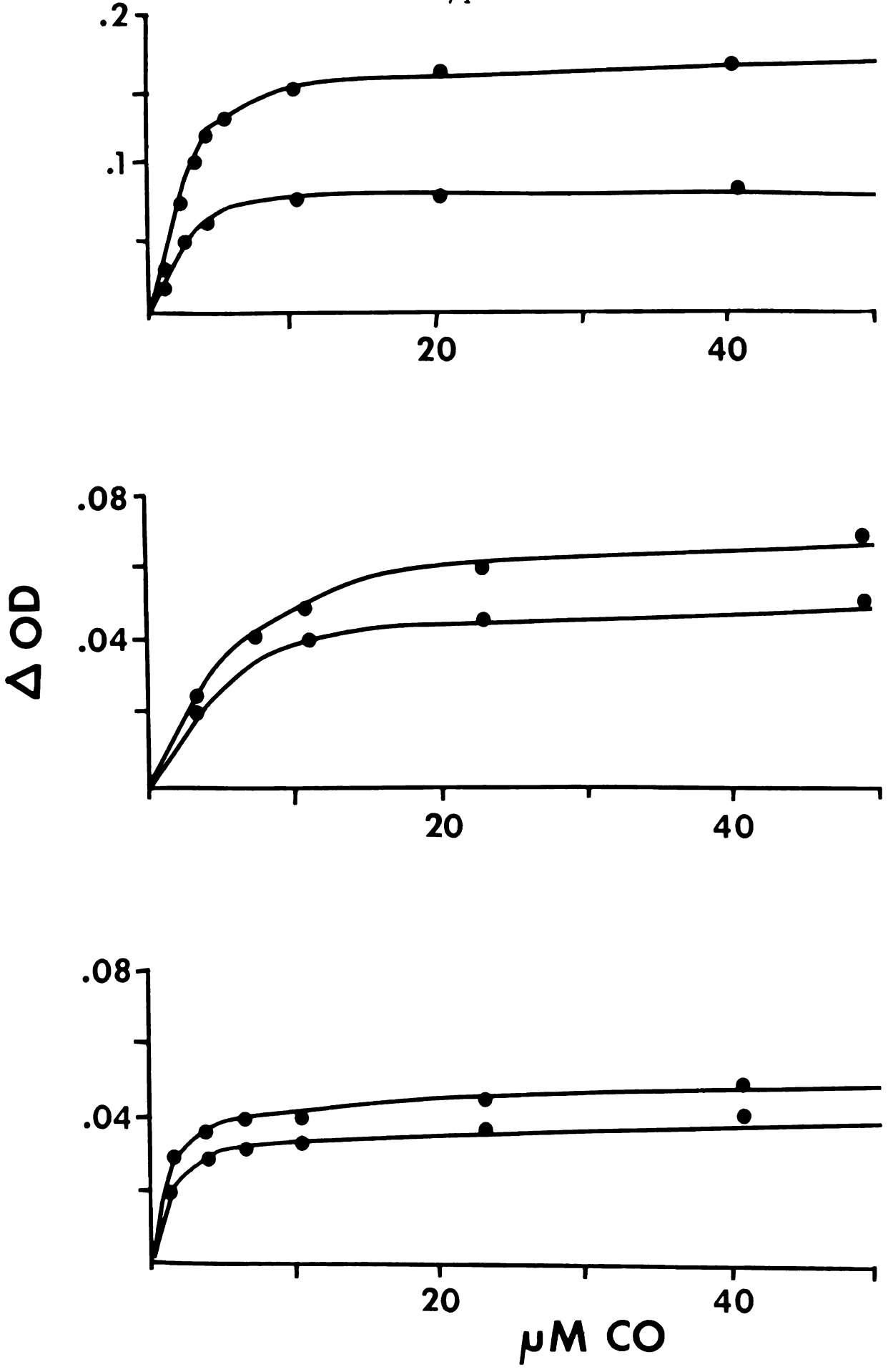
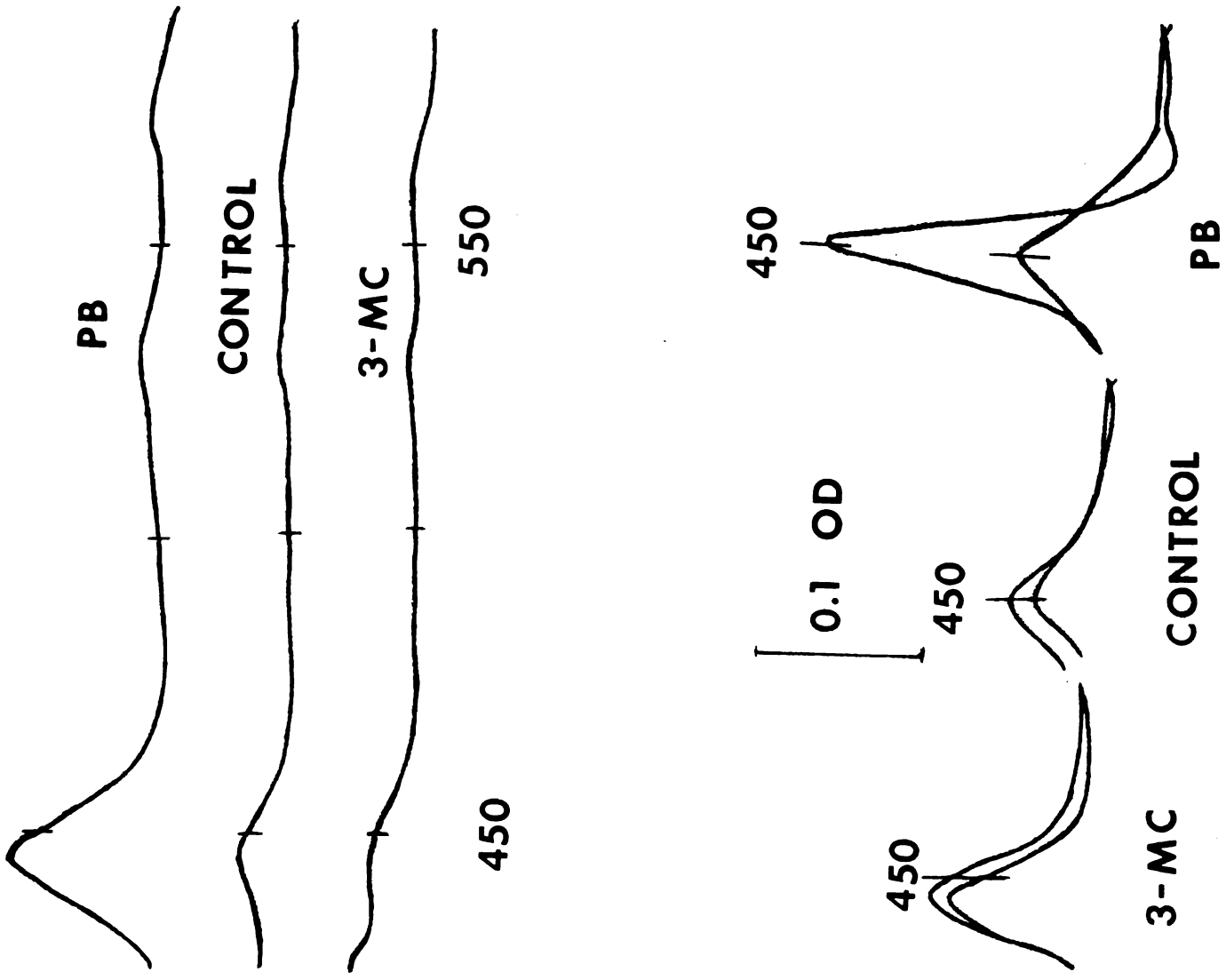

.2
.1
20
40
.08
.04
ΔOD
20
40
.08
.04
20
40
μM CO

Fig. 23a.--Metopirone difference spectra obtained in the reduced state of microsomes from control animals and animals pretreated with phenobarbital and 3-methylcholanthrene.

Fig. 23b.--CO difference spectra in the presence of metopirone obtained in the reduced state of microsomes from control animals and animals pretreated with phenobarbital and 3-methylcholanthrene.


PB
CONTROL
3-MC
550
450
450
0.1 OD
450
450
3-MC
CONTROL
PB

spectral change with each type of microsome. An extinction coefficient for the interaction of metopirone with the heme of P-450 was obtained by calculating the amount decrease in absorbance at 450 nm in a CO difference spectrum with PB microsomes and assuming that this amount of P-450 was responsible for the difference in absorbance from 446 nm to 480 nm in the metopirone difference spectrum. An extinction coefficient at pH 7.3 in KPO_4 buffer of 146 $cm^{-1}m\underline{M}^{-1}$ was calculated.

A procedure was then developed for the solubilization and partial purification of P-420, the soluble cytochrome product of P-450 in hopes of using this product for further studies on the multiplicity of microsomal oxidases. The first question concerned the multiplicity of P-450 itself while the second involves the possibility of using P-420 as a substrate for NADPH-cytochrome P-450 reductase and for obtaining difference spectra. Steapsin (0.072%) solubilized all of the NADPH-cytochrome C and 80-90% of the b_5 present while only 25-30% of the P-420 was solubilized. It was found that a 37°C incubation for 60 minutes at a pH of 7.5 under anaerobic conditions was optimum for this solubilization step.

The next step involved solubilization of the P-420 with snake venom (Naja Naja; 0.5 mg/ml). The optimum conditions for this step were 20 minutes at 37°C, pH 9.5 in an aerobic atmosphere. This procedure solubilized all of the cytochrome P-420.

The final step involved a 0-75% ammonium sulfate fractionation of the venom-solubilized P-420. Essentially all of the solubilized P-420 was precipitated out of solution, and could be separated and redissolved. The final concentrations in the prep were generally 6-8 nmoles P-420/mg protein without any cytochrome b_5 present for PB microsomes, and 2-4 nmoles P-420/mg protein when either 3-MC or control microsomes were used. Electrophoretic gels have shown that a substantial purification had taken place, but some impurities still remained.

The solubilized P-420 was then tested in the same manner as P-450 for its CO binding ability with or without metopirone. Both metopirone and dieldrine had an identical effect on the binding of CO to the soluble P-420 as compared to whole microsomal P-450. The absorbance peak had shifted from 420 nm to 416 nm when metopirone was present.

Studies were then done on NADPH reduction of the solubilized P-420 in hopes of showing specific reductases for each P-450. P-420 was isolated from PB pretreated animals and reduced by NADPH with whole microsomes as the source of reductase from all three types of animals (control, PB, and 3-MC). It was found that all three types were capable of reducing P-420 without any preference as to the extent and rate of reduction.

Optimum conditions for P-450 reductase were found for further investigation of multiple reductases. These

conditions were 0.05 M PO_4 buffer with 15% glycerol, pH 7.0. It was found that the initial rate of P-450 reduction was directly proportional to the glycerol concentration found in solution. Fifteen per cent glycerol was chosen due simply to the fact that it produced a rate which could easily be studied.

DISCUSSION

The discovery that many drugs were metabolized by a microsomal mixed-function oxidase, coupled with the discovery of cytochrome P-450, led to the belief that this single oxidase system was responsible for all of the mixed-function oxidase reactions carried out by the liver microsomes. Many studies were carried out to show that all compounds which were substrates for the system were competitive inhibitors of the metabolism of each other. For example, ethylmorphine demethylation was competitively inhibited by other drugs and steroids.[49] Lipid peroxidation was inhibited by drugs which were metabolized.[50] However, evidence then started to accumulate which said all of the reactions were not catalyzed by the same oxidase. Induction by phenobarbital or 3-methylcholanthrene gave microsomal enzymes with altered kinetic parameters. Then it was discovered that PB and 3-MC induction resulted in two different cytochrome P-450's. These results suggested that multiplicity of microsomal mixed-function oxidases must exist.

The best evidence for multiplicity of aminopyrine demethylase activity is the fact that Lineweaver-Burk plots of demethylase activity in control microsomes is not linear.

These results are suggestive of at least two activities with differences in K_m for aminopyrine. Therefore, it is impossible to determine whether other compounds undergoing oxidation can competitively inhibit aminopyrine demethylation. Lineweaver-Burk plots of control activity is not linear and the addition of inhibitors introduce another degree of complexity depending on which activity is inhibited.

The data published by Wada and coworkers,[51] is very good evidence for more than one enzyme for the hydroxylation of aniline. Essentially linear Lineweaver-Burk plots were found for the hydroxylation of aniline; however, nonlinearity was evident when the inhibitor prednisolone was included in the incubation mixture. The K_m's of the two enzymes appear to be similar, however, the differences in inhibition by prednisolone show up by kinetic evaluation.

Alvares et al.[52] have shown a difference in K_m's for the hydroxylation of benzpyrene by microsomes obtained from animals induced by phenobarbital and 3-methylcholanthrene.

The results of this study, and of Pederson and Aust[29] suggest that the aminopyrine demethylase activity at low substrate concentrations can be induced by PB while the activity at high substrate concentrations can be induced by 3-MC.

A similar investigation of ethylmorphine demethylase activity did not reveal large changes in kinetic parameters

upon induction with PB or 3-MC. However, substrate competition experiments with ethylmorphine and aminopyrine suggested that phenobarbital induction resulted in less competition between the two substrates. PB must induce some enzyme(s) which are specific for only ethylmorphine or aminopyrine.

Microsomes from female rats consistently show less aminopyrine demethylase activity. It has been suggested that this involves differences in hormonal balance. Activity in male rats is thought to be induced by such hormones as testosterone, because this androgen is much more prevalent in the male than in the female. It was also thought that the differences in aminopyrine demethylation in male and female rat microsomes could involve kinetic parameters. That is the low K_m component may contribute less to the overall aminopyrine demethylase activity, resulting in lower activities, especially at low substrate concentrations. However, Lineweaver-Burk plots of aminopyrine demethylase in female microsomes were similar to that in microsomes from male rats. Pretreating female rats with testosterone increased activity at low concentrations of substrate, however, activity was still less than activity in microsomes from male rats. Testosterone had no effect on activity in microsomes from male rats. Gillette, et al.,[53] have shown that the state of pregnancy affects the kinetic parameters of the metabolism of a

number of substrates. Other investigators have reported similar results for the NADPH-linked electron transport system and the terminal oxidase.[54] Others have reported variations of the detoxification enzymes during development.[55]

The differences in inhibition of aminopyrine demethylation in control, PB- and 3-MC-microsomes is also suggestive of multiple activities with different susceptibility to inhibition. Lineweaver-Burk plots of aminopyrine demethylase activity in all three types of microsomes in the presence of various inhibitors indicated some similarities among inhibitors.

The extent of inhibition by DDT, metopirone and dieldrin varied depending upon the type of microsome used. These compounds inhibited aminopyrine demethylase activity far less in 3-MC microsomes than in either control or PB microsomes. They were among the most potent inhibitors for aminopyrine demethylase activity in PB microsomes, suggesting that these compounds were inhibiting a component which was responsible for less of the total activity in 3-MC microsomes and a far greater percentage in PB microsomes.

SKF-525-A and SKF-8742-A, on the other hand, were much more potent inhibitors of the N-demethylase activity in 3-MC microsomes. Takayangi, et al.,[56] have shown that one particular inhibitor, SKF-525-A, and a few other drugs

were not only competitive, but also noncompetitive and mixed-type according to the substrate used and to the species of animals used.

Hexobarbitol, which was not a good inhibitor of activity in control microsomes, was a strong inhibitor of aminopyrine N-demethylase activity in both types of induced microsomes. Other studies showed that structural analogs of some compounds did not inhibit N-demethylase activity in the same manner.[57] These results have been reported for other substrates also.

Due to the variability of inhibition in the three types of microsomes, DDT, metopirone and dieldrin were then chosen for further studies in this system.

A plot of activity vs. inhibitor concentration (DDT) was not hyperbolic as classical kinetics would predict,[58] but instead yielded three linear segments dividing the total aminopyrine demethylase activity into three components based on their affinity for DDT. In practice this plot of activity vs. the concentration of inhibitor was useful, in that for a system of multiple activities, at any substrate level it was possible to estimate the contribution of any one particular activity to the total activity.

The fact that complete inhibition could not be obtained at very high concentrations of inhibitor could not be explained by partial competitive inhibition, as described by Palmer.[59] In partial competitive inhibition

the fraction of activity remaining at high inhibitor concentrations would be independent of enzyme concentration. In this system, however, the fraction of total activity remaining at high inhibitor concentrations was altered by induction; increased by 3-MC induction and decreased by PB induction. This fact applies to any argument for only one enzyme with multiple sites for the demethylation of aminopyrine. The only alternative would be that the induced enzyme is not a different enzyme but rather one in a different environment, which affects the kinetic parameters. Such a system seemed unlikely and might still be considered separate enzymes.

The suggestion that these activities involve separate proteins with different specificity came from the fact that the individual activities can be preferentially induced by PB or 3-MC and the activities induced by these compounds have completely different kinetic properties. Upon induction by PB pretreatment of animals only one component seemed evident in a Lineweaver-Burk plot because of its low K_m and high activity. However, upon the addition of increasing levels of DDT to the incubation mixture, three components were evident. One, preferentially induced by PB, was extremely active and easily inhibited by DDT, dieldrin and metopirone. A second, present in all types of microsomes, was responsible for the majority of the activity in control microsomes and was moderately sensitive

to DDT and insensitive to either metopirone or dieldrin. The third component was preferentially induced by 3-MC pretreatment and was insensitive to all three of these inhibitors. The later component had a high K_m for aminopyrine so it could only be observed at high substrate concentrations. This activity seemed to be present in all types of microsomes.

The K_m of the phenobarbital induced component (5×10^{-4} M) can be fairly accurately estimated from a Lineweaver-Burk plot of activity in "highly" induced PB microsomes because of its low K_m and high activity. The K_m of the DDT-insensitive component can be obtained from a Lineweaver-Burk plot of aminopyrine demethylase activity in the presence of sufficient DDT to completely inhibit the other components. By this method a K_m of 1.3×10^{-2} M was obtained. The K_m of the third component can only be estimated by plotting a double reciprocal plot from activities obtained at a DDT level between 50 and 150 μM. By using this method a K_m of 4.2×10^{-4} M was obtained. Therefore, the total aminopyrine demethylase activity involved three enzymatic activities, two with low K_m's and a high K_m activity.

By the use of increasing levels of the inhibitor DDT it was possible to show that the degree of induction of aminopyrine demethylase cannot be accurately assessed by only assaying for total activity. Since total activity was made up of multiple activities, it was necessary to

find which activity had been induced. For example (Figure 12), phenobarbital induction caused about a four-fold increase in total activity (2m$\underline{M}$ aminopyrine), however, in actuality it had induced a ten-fold increase in one particular component.

Dieldrin and metopirone separated the total aminopyrine demethylase activity into two components; one sensitive to inhibition and one insensitive. The insensitive fraction could be shown to consist of two activities by a Lineweaver-Burk plot in the presence of 100 $\mu\underline{M}$ dieldrin. Such a plot was biphasic presumably because of the presence of two activities with large differences in their K_m for aminopyrine. The fact that PB slightly induced the high K_m component was also evident.

The dieldrin and/or metopirone sensitive component could be shown to be induced by PB by comparing the v vs. [I] plots for both control and PB microsomes. This latter finding also confirmed that the PB-induced component was present, although difficult to observe in control and 3-MC microsomes.

Inhibition studies on the DDT insensitive component by other drugs indicated that only one activity existed. All three types of classical kinetic inhibition were also shown to exist for this component.

Since both metopirone and dieldrin gave similar inhibition of this system, the assumption was made that the

mechanism of inhibition for these compounds was the same. Substrate difference spectra suggested that both metopirone and dieldrin bind to the heme group of P-450 in both the oxidized and reduced states of the P-450 particle. However, Imai and Sato[60] have suggested that substrates combine with a site in P-450 close enough to interact with the activated oxygen molecule on the heme group, and that the spectral changes induced by substrates are due to a secondary effect of the substrate binding to the non-heme moiety of P-450 on the ligand state through changes in conformation. Other investigators[61] have also shown ligand binding was dependent upon type of induced microsome used.

Two possible mechanisms could explain these results. The inhibitors could prevent the binding of substrate to the P-450 or they could prevent oxygen binding. A pure oxygen atmosphere did not overcome dieldrin inhibition; however, metopirone and dieldrin did decrease the binding of CO. If the binding of CO and oxygen are related, perhaps these compounds inhibit by preventing the formation of the "activated oxygen complex." Correlating the decrease in binding of CO, the loss of demethylase activity and ligand binding (metopirone, measured at 446 nm) suggested that this compound binds the heme which had been preferentially induced by PB. The fact that not all of the aminopyrine demethylase activity was lost and/or total inhibition of CO binding were not obtained, provides

evidence that at least one other heme protein is present in this microsomal mixed-function oxidase system.

Murphy, et al.,[62] have shown the appearance of more than one P-450 fraction upon subfractionation of rat liver microsomes by use of rate-zonal centrifugation. PB and 3-MC induction were found to have a very distinctive effect on the distribution of cytochrome P-450 within the microsomal vesicles. The altered distribution observed with 3-MC induction was inhibited by pretreating the animals with thioactamide and ethionine. These results suggest that the P-450 produced after treatment with 3-MC is preferentially formed in, or bound to a vesicle distinct from that which contains the P-450 in the normal or phenobarbital-induced animal.

By making the assumption that the extent of P-450 lost in the CO binding experiment paralleled the increase in the metopirone binding spectrum at 446 nm, an extinction coefficient for metopirone-P-450 was calculated to be 146 cm^{-1} $m\underline{M}^{-1}$.

Previous studies on the induction of P-450 by phenobarbital and 3-methylcholanthrene have shown that the absorbance maximum for P-450 in a CO-difference spectrum depended upon which inducer was used.[63] Control microsomes had an absorbance maximum at 449 nm, which could be shifted to 448 nm upon induction with 3-MC or to 450 nm with PB induction. Investigators therefore argued for the

existence of two microsomal cytochromes; one induced by 3-MC which absorbed at 448 nm and another absorbing at 450 nm which was induced by PB.

If metopirone binds to the P-450 induced by PB such that it cannot bind CO, the P-450 remaining should be cytochrome P-448. Therefore, this inclusion of metopirone in both the sample and reference cell should cancel out all P-450 and leave P-448 to obtain a CO-difference spectrum at 448 nm. However, the maximum occurred at 451 nm. Estabrook, et al.,[64] reported that this maximum occurred at 454 nm. The CO-difference spectrum of 3-MC microsomes in the presence of metopirone showed a slight decrease in absorbance without any shift in the maximum. Estabrook proposed the existence of a P-448 - P-454 interconvertible cytochrome form based upon their reaction with CO. They had concluded that metopirone changed the equilibrium established between the two functionally different forms of cytochrome P-450. They further had shown that a decrease in one form appears as an "inhibition" of aminopyrine or hexobarbitol metabolism, while the concomitant increase of the other form paralleled the "stimulation" in the ring hydroxylation of acetanalide.

Solubilized P-420 also gave a difference spectrum with metopirone, however the maximum occurred at 416 nm. Increasing the pH of a suspension of whole microsomes from 7.0 to 9.0 resulted in the loss of P-450 with a concomitant increase in P-420. The same type of result was obtained

with metopirone difference spectra. The peak at 446 nm was lost with the appearance of a peak at 418 nm. Therefore, just as P-420 is the breakdown product of P-450 with CO-difference spectra, P-418 is the breakdown product of P-446 with metopirone difference spectra.

Unlike P-450, however, the presence of metopirone caused a shift in the CO-difference spectrum of solubilized P-420 to a maximum at 416 nm.

The mechanism of inhibition of aminopyrine demethylase by DDT appears to be different than the mechanism by which metopirone and dieldrin inhibit. No spectral evidence for binding was obtained with DDT nor did DDT affect the binding of CO by P-450. When KCN was used as an inhibitor for aminopyrine demethylase activity, a pattern very similar to DDT inhibition was obtained. Total activity was divided into three components. Although KCN inhibition generally involves cytochromes, because of the excessive amounts of KCN needed to obtain this particular inhibition (4m$\underline{M}$) and the fact that no difference spectrum was obtained,[18] the assumption was made that KCN inhibition was not due to the binding to the heme group. Therefore, the inhibition might involve the electron transport system. However, no reversal of DDT inhibition by excessive levels of NADPH was obtained; either DDT bound irreversibly or this was not the mechanism by which DDT inhibited. DDT had no effect on cytochrome C or nitroblue tetrazolium reductase indicating that neither of these reductases were

involved in the microsomal mixed-function oxidase system or that the point of inhibition by DDT occurred at a later step in the electron transport system.

It has been known that both NADPH and NADH are needed for maximal aminopyrine demethylase activity[29,65] however, the mechanism is not known. NADH does not support demethylase activity alone or in the presence of $NADP^+$, indicating no transhydrogenase activity. In addition to the reduction of P-450 by NADPH, NADH will also reduce this cytochrome but at a slower rate. Both reactions are biphasic. NADH had no effect on the rate of reduction of P-450 by NADPH. This biphasic reduction of P-450 has been reported by other workers.[51] Both rates were found to be dependent upon pH and glycerol concentration. Usually the faster reduction rate is too fast to accurately measure, however, this rate could be observed by the inclusion of 25% glycerol in the reaction mixture. DDT had only a slight effect on the reduction of P-450 by NADPH, however, DDT stimulated the fast reduction of P-450 by NADH at least three-fold. Since DDT acted as an inhibitor of aminopyrine demethylase, the effect seen in these reductase experiments did not indicate the mechanism of DDT inhibition.

However, it was found that NADH could reverse DDT inhibition of the moderately DDT-sensitive aminopyrine demethylase activity. The component which was highly sensitive to DDT, metopirone and dieldrin inhibition was not

substantially affected. This therefore, led to the belief that DDT inhibited the two activities by two separate mechanisms. The two activities must also have different electron transport systems.

It would appear that the two components seen in Lineweaver-Burk plots of aminopyrine demethylase activity in control microsomes are made up of the two enzymes as postulated earlier.[29] However, the low K_m component does not seem to be a single activity, induced by PK. Two low K_m activities seem to exist, and one is induced by phenobarbital. The high K_m activity (induced by 3-MC) does not seem to be primarily responsible for the demethylation of aminopyrine but is capable of doing so at high substrate concentrations.

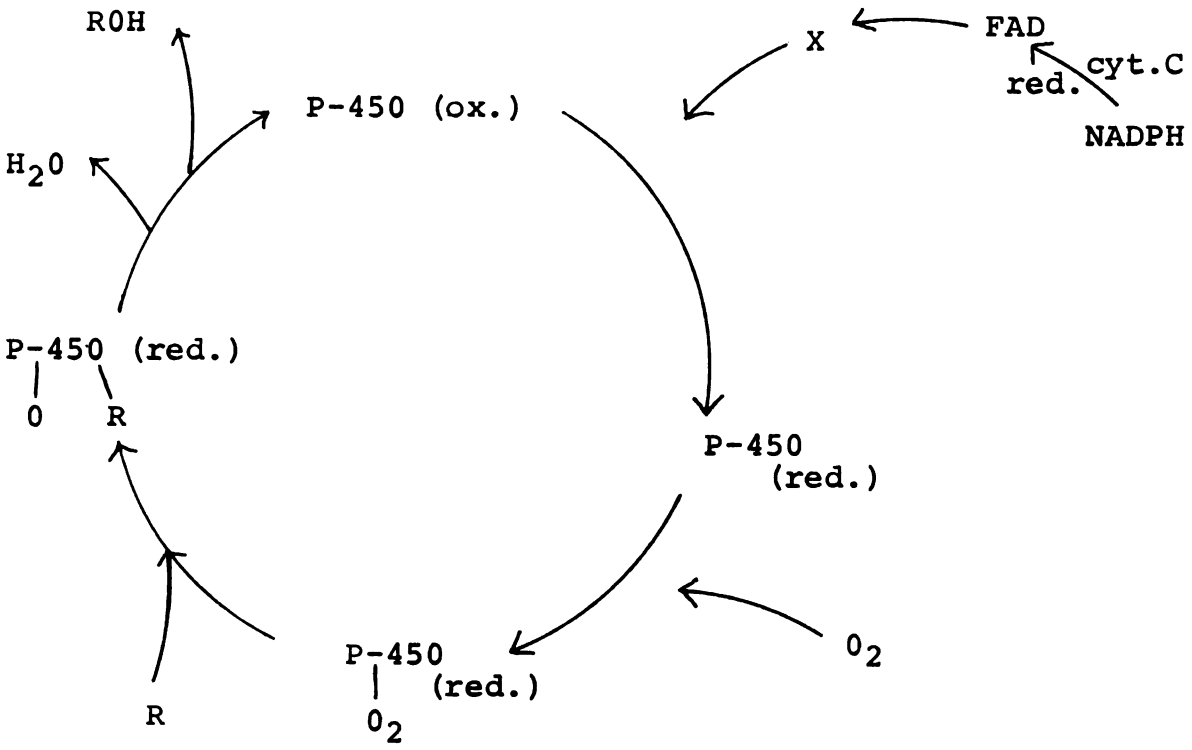
The low K_m activity which is induced by PB is also highly sensitive to DDT, metopirone, and dieldrin inhibition. The inhibition by both metopirone and dieldrin is presumably a mechanism of P-450 heme binding preventing the formation of the "active oxygen complex." The mechanism of DDT inhibition of this component is not yet understood, however, the DDT inhibition of the other low K_m component seems to involve the electron transport system. It is therefore believed that these aminopyrine demethylase activities, in addition to involving a multiplicity in P-450, have multiple reductases, perhaps one reductase system for each activity seen.

TABLE 1.--A summary of the properties of the aminopyrine demethylase activities in rat liver microsomes.

Property	Component of Aminopyrine Demethylase*		
	1	2	3
K_m	5×10^{-4} M	4.2×10^{-4} M	1.3×10^{-2} M
Induction by:	PB	?	3-MC
Stimulated by NADH	-	+	-
DDT inhibition	+	+	-
Dieldrin inhibition	+	-	-
Metopirone inhibition	+	-	-
KCN inhibition	+	+	-
P-450 Involved	450	?	448

*The three components correspond to: (1) highly DDT sensitive; (2) moderately sensitive to DDT; (3) DDT insensitive (see Figure 12)

Scheme for Microsomal Mixed-function Oxidase



(1) Possibility for 3 P-450's.

(2) NADH involved with only one P-450.

(3) Possibility for each P-450 having its own electron transport system, or sharing a common system.

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