BIOCHEMICAL EFFECTS OF POLYBROMINATED BIPHENYLS ON MICROSOMAL ENZYMES

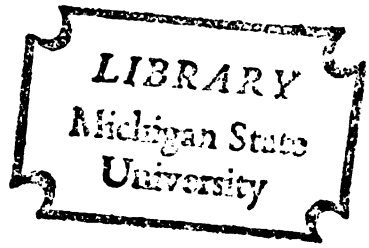
Thesis for the Degree of M. S.

MICHIGAN STATE UNIVERSITY

CATHERINE LYNNE TROISI

1975

THESIS

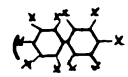


ABSTRACT

BIOCHEMICAL EFFECTS OF POLYBROMINATED BIPHENYLS
ON MICROSOMAL ENZYMES

By

Catherine Lynne Troisi

Polybrominated biphenyls ( , where any or all of the X's represent bromine atoms) were injected into rats to determine the effect, if any, on the liver microsomal enzymes responsible for the metabolism of xenobiotics. It was found that a single injection of PBB (90 mg./kg. body weight) caused a substantial increase in the liver weight to body weight ratio and in total microsomal protein, in the levels of cytochrome P_{450}, and aminopyrine demethylase, 3,4-benzpyrene hydroxlyase, and NADPH-cytochrome c reductase activities. The levels reached after a single injection of PBB were higher than the levels of induction caused by five daily injections of phenobarbital (50 mg./kg. body weight), or by one injection of 3-methylcholanthrene (20 mg./kg. body weight) or both. These levels of induction lasted for at least ten days after the injection of PBB, much longer than the effects of a single injection of Pb or 3-MC lasted.

The effect on microsomal enzymes of a chronic exposure to a low level of PBB was studied by feeding rats a diet containing 10 ppm PBB for sixteen days. After only three

days on the diet, levels of all microsomal enzymes were substantially elevated over control values, as were the liver weight to body weight ratio and total microsomal protein. These effects continued for at least two weeks after the PBB feed was withdrawn.

These studies show PBB to be a very potent inducer of liver weight, microsmal protein, and microsomal enzymes, and these effects are long-lasting. It can therefore be concluded that PBB can represent a significant environmental hazard under appropriate conditions.

BIOCHEMICAL EFFECTS OF POLYBROMINATED BIPHENYLS ON MICROSOMAL ENZYMES

By

Catherine Lynne Troisi

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Biochemistry

1975

CT 1975

ACKNOWLEDGEMENTS

I wish to thank Dr. Steven Aust for his help and guidance in conducting this research, and also the other members of my committee, Drs. John Wilson and Loran Bieber.

Robert Moore is gratefully acknowledged for his help and friendship (and the benzpyrene hydroxylase data), Ghazi Dannan for his help in the early stages of this research, as well as all the other members of Dr. Aust's laboratory.

And lastly I wish to thank Richard Stoll for helping me retain my sanity.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

PBB	Polybrominated biphenyls
PCB	Polychlorinated biphenyls
Pb	Phenobarbital
3-MC	3-methylcholanthrene
BHT	Butylated hydroxytoluene
SDS	Sodium dodecyl sulfate
NADP(H)	Nicotinamide adenine dinucleotide phosphate (reduced)
Tris	Tris(hydroxymethyl)aminomethane
i.p.	intraperitoneally
p.o.	by mouth

INTRODUCTION

The endoplasmic reticulum (microsomes) of the mammalian liver system contains the enzymes responsible for the biotransformation of a variety of substances including drugs, steroid hormones, insecticides, dyes, food preservatives and carcinogens.[1-4] The types of reactions by which these substances are metabolized are side-chain oxidations, aromatic hydroxylations, N- and O- dealkylations, deaminations, sulfoxide formations, reductions, hydrolysis and conjugations.[4-5] These reactions all require NADPH and molecular oxygen[6] and in most cases lead to inactivation of the substance being metabolized but may cause the activation of a drug, as in the case of 3,4-benzpyrene and other carcinogens. This system is referred to as a mixed-function oxidase system in the terminology of Mason[7] and consists of a cytochrome, NADPH cytochrome c reductase and a lipid (phosphatidyl choline).[8] When CO is added to a sample cuvette and both the sample and reference cuvette are reduced with dithionite, the difference spectrum shows a characteristic peak at 450 nm. This was first shown in 1958 by Klingberg[9] and Garfinkel[10] and the cytochrome involved was termed P_{450}.[11] It was later shown that cytochrome P_{450} is the terminal oxidase for the drug metabolizing system.[12] Lu *et al.*[8] using reconstituted systems showed that the specificity for hydroxylation

resides primarily in the cytochrome fraction rather than in the cytochrome c reductase or lipid fractions.

One of the important features of the system is its inducibility. The repeated administration of a substance can lead to the induction of the mixed-function oxidase system and increased metabolism of that compound.[1] This was first shown in 1954 by Brown, Miller and Miller.[13] Induction occurs only when the chemical is given *in vivo*[14] although addition of drugs to a microsomal solution does result in spectral changes, and there is evidence that the drugs are binding to cytochrome P_{450}.[15, 16] Pretreatment with ethionine to inhibit protein synthesis blocks induction *in vivo*[14] and so induction is thought to occur by a rise in enzyme synthesis and a decrease in enzyme degradation.[17, 18] There are two type of inducers: one group stimulates the metabolism of many drugs and the second group stimulates the metabolism of only a few drugs. Phenobarbital (Pb) belongs to the former group and 3-methylcholanthrene (3-MC) to the latter.[1, 4] Among the enzymes induced by Pb are aminopyrine demethylase, cytochrome c reductase and 3,4-benzpyrene hydroxylase,[1, 14, 16] while 3-MC induces the metabolism of 3,4-benzpyrene.[1]

The lack of specificity of the mixed-function oxidase system is unusual in view of the usual one enzyme-one substrate system. Over two hundred drugs, carcinogens, insecticides, and other chemicals are known to stimulate the activity of drug metabolizing enzymes in liver microsomes.[1] It was not known whether there was only one

enzyme responsible for metabolizing all of these substances or whether a separate enzyme exists for each compound. It has now been shown that at least three different cytochrome P_{450}'s exist: one of molecular weight 44,000 which is induced by Pb; one of molecular weight 50,000 which prevails in control microsomes and a cytochrome induced by 3-MC which has a difference spectrum peak at 448 (and is therefore called P_{448}) and a molecular weight of 53,000.[19, 20, 21] Other evidence for multiple drug metabolizing cytochromes includes observations by Kuntzman, et al.[22] that Pb induction stimulates the 6α, 7β, 16α, hydroxylation of testosterone by rat liver microsomes but causes the biggest increase in 16α, hydroxylation while 3-MC stimulates only the 7β hydroxylation. It has also been shown that pretreatment with 3-MC results in significant lowering of the K_M for the hydroxylation of 3,4-benzpyrene while no such decrease is found for Pb induction.[23] This suggests that 3-MC induces formation of a hydroxylase with greater affinity for the substrate than the enzyme originally present. Pederson and Aust[24] found evidence for multiple microsomal activities of aminopyrine demethylase. Pretreatment with Pb stimulates this aminopyrine demethylase activity, while pretreatment with 3-MC causes no stimulation of activity but does increase the apparent K_M. The inhibitor SKF-525A (2-diethylaminoethyl-2,2-diphenylvalerate) inhibited the activity found in microsomes induced with Pb but not in those from 3-MC treated rats.

By monitoring cytochrome P_{450} levels and microsomal enzyme activities, we therefore have a method of testing

chemicals for biochemical effects that may not be overtly noticeable. It was for this reason that the parameters followed in this study were chosen to determine what effects, if any, polybrominated biphenyls (PBB) have on rats.

In the spring of 1973, Firemaster BP-6 (containing 2.0% tetrabromobiphenyl, 10.6% pentabromobiphenyl, 62.2% hexabromobiphenyl, 13.8% heptabromobiphenyl and 11.4% others) produced by the Michigan Chemical Corporation was mistakenly added to cattle feed. As of May 22, 1975, approximately 21,000 cattle, 3,500 swine, 1,200 sheep, and 1,550,000 chickens in the state of Michigan were destroyed because of known or suspected PBB contamination according to the Michigan Department of Agriculture. The data on the toxicity of PBB is very scanty. A complete study on octabromobiphenyl (OBBP) by Norris, et al.[25] found no overt indications of toxicity at dietary levels of 10,000 1,000, 100 and 0 ppm OBBP over 30 days although enlarged livers were found at all levels and decreased packed red blood cell volumes at the 10,000 ppm dietary level was also found. A no effect level was not established. After a single injection of ^{14}C OBBP, 62% of the radioactivity was found in the feces after the first 24 hours. By day 16, however, 26% was not recovered. Radioactivity was found in all tissues. They also found that after 90 days on a OBBP diet, bromine was not eliminated from the adipose tissue of the rats and only partially eliminated from the liver after a 90 day recovery period on control diet. Norris concluded that while he could recommend the use of decabromobiphenyl oxide as a fire retardant, he did not recommend OBBP.

There have been toxicological studies done on the polychlorinated biphenyls (PCB). Although it has been available commercially for over thirty years, it is only within the last five years that its ecological and toxicological properties have been studied.

PCB's are not very toxic when given as a single dose or as a few repeated doses to birds and mammals. The minimum lethal dose in rats is approximately 2.5 gm./kg. and the 14 day LD_{50} is 4.25 gm./kg. with a 95% confidence interval of 2.8 to 6.4 gm./kg..[26] The acute oral dose toxicity decreases in rats with an increase in chlorine percent[27] and this may be due to poorer absorption of the higher chlorinated compounds.[28]

There are no manifestations of toxicity in rats at 100 or 1000 mg./kg. PCB p.o. and rats given 100 mg./kg. PCB every other day for three weeks also showed no signs of toxicity. Body weights were not significantly different from controls; liver weights were higher but kidney, heart, spleen and adrenal gland weights were not. A single i.p. dose of 100 mg./kg. of Aroclor 1242 (Monsanto Chemical Company, St. Louis; 42% chlorinated) increased liver weight, microsomal protein and N-demethylation of aminopyrine activity at 1, 5, and 10 days.[26]

Dietary studies have also been done on PCB. Aroclor 1242 was fed to rats for four weeks at levels of 0.5, 5, 50 and 500 ppm. At the higher levels increased liver weights were found and an increase in cytochrome P_{450} levels was found at 50 and 500 ppm, as well as induction of pentobarbital hydroxylation and N-demethylase activity. Nitroreductase activity was induced at 0.5 ppm PCB in the

diet.[29] Alvares, et al.[30] found that PCB increases benzpyrene hydroxylase activity and P_{448} levels. Biphenyl alone does not induce microsomal protein.[31]

Rat reproduction studies by Kimbrough[28] show a decreased survival rate of pups at dietary levels of 100 ppm of Aroclor 1248 (48% chlorinated) and a 5 ppm dietary level of exposure to dams increased the liver weight to body weight ratio in weanling rats. Secretion of PCB in milk and transplacental passage has been observed in mammals.[28]

Several reports have indicated that PCB may alter the immune response[28] and Bruckner et al.[26] speculate that the symptoms of acute, oral toxicity implicate neurological and/or muscular involvement, and dehydration may also be involved. Species differences are marked and minks, for example, are very susceptible to the toxic effects of PCB and a daily intake of 30 ppm of PCB results in death in about six months.[28]

Kimbrough[28] feels that chronic toxicity of PCB is more important in establishing effects than short-term exposure studies, and this would also seem to be true in studying the toxicity of PBB. Farber and Baker[32] found that hexabromobiphenyl is 2.5 times more potent on a weight basis than Aroclor 1254 (54% chlorinated) as a microsomal inducer. The fact that the dissociation energy for the C-Br bond is less than for the C-Cl bond may play a role in this.[33]

The purpose of this thesis is to study the effects that PBB has on the mixed-function oxidase system. Both dietary and single injection studies were done, as well as a repeated dose study. Cytochrome P_{450} levels and the activities of

microsomal enzymes for certain drug metabolisms were monitored. In this way, it was hoped to determine how PBB affects the drug metabolizing system of the rat.

MATERIALS AND METHODS

Chemicals

Firemaster BP-6 (PBB) was manufactured by the Michigan Chemical Corporation, Chicago, Illinois, and was a gift from Robert Ringer, Department of Poultry Science, Michigan State University. Phenobarbital was purchased from Merck and Co., Inc., Rahway, N.J. 3-MC, 3,4-benzpyrene, $NADP^+$, NADPH, cytochrome c, isocitrate and $NADP^+$-isocitrate dehydrogenase were all purchased from Sigma Chemical Co., St. Louis, Mo. CO was obtained from the Matheson Co., Inc., Joliet, Illinois. All other chemicals used were of reagent grade quality and obtained from the usual sources.

Animals

Male rates of the Sprague-Dawley strain were used in all experiments. They were purchased from the Spartan Research Animals, Inc., Haslett, Michigan, and the average body weights were between 250 and 300 grams. Water and Purina Rat Chow were available ad libitum. For the dietary studies, rat chow was ground in a Wiley Mill and mixed in a Hobart Mixer with 60 mls. of corn oil added for each kilogram of feed. The PBB was slowly added to one kg. of feed, mixed for approximately one-half hour and then the rest of the feed was added and the feed plus PBB was mixed for two to three hours, scraping

the sides of the bowl often to insure proper distribution of the PBB. Pb-induced microsomes were prepared by administering the stated number of daily i.p. injections (this varied with each study) of 50 mg./kg. body weight in saline solution. For one study, the microsomes were induced by adding 0.1% Pb to the drinking water for fourteen days prior to sacrifice. 3-MC-induced microsomes were prepared by injecting the animals i.p. with one injection of 20 mg./kg. body weight 3-MC in corn oil. PBB-induced microsomes were prepared by injecting 90 mg./kg. body weight PBB in corn oil.

Preparation of Microsomes

The animals were weighed and then exsanguised. The livers were perfused in situ by injecting 10 mls. of cold 1.15% KCl + 0.2% nicotinamide into the portal vein, so that blanching of the liver was observed. The liver was removed, weighed and minced with scissors while being kept in the cold. Livers from the two or three rats used for each data point were mixed together at this point. The tissue was homogenized using four strokes of a Potter-Elvehjem homogenizer equipped with a Teflon pestle, in four volumes of cold 1.15% KCl + 0.2% nicotinamide. The homogenate was centrifuged at 15,000 x g for 20 minutes to pellet the nuclear and mitochondrial fractions. The supernatant was then centrifuged at 105,000 x g for 90 minutes. In some studies, the microsomes were washed immediately by resuspending the pellet in 15 mls. of 0.3M sucrose, 0.1M pyrophosphate, and then centrifuged at 105,000 x g for

90 minutes. These are referred to as washed microsomes. In other studies, washing took place after storage of the microsomes.

To store the micosomal solution, the pellet was rehomogenized in Tris HCl buffer (0.05M, pH 7.5) containing 50% glycerol, and 2% BHT in ethanol was added to the solution for a final concentration of 0.01%. The microsomes were stored under argon at -15°C. Protein was determined by the method of Lowry.[34]

Cytochrome P_{450} Determination

The microsomal solution was diluted in 0.2M Na_2PO_4 (pH 7.6) containing 33% glycerol to obtain approximately 2 mg./ml. protein. The solution was divided between two cuvettes, one of which had CO bubbled through until saturated, and then both were reduced with dithionite. The difference spectra was obtained from 500 to 400 nm. on a Coleman 124 Recording double beam spectrophotometer. The difference extinction coefficent used was 91mM/cm.[11]

Aminopyrine Demethylase Determination

The N-demethylase activity was determined by assaying the rate of production of formaldehyde according to the method of Nash.[35] Reaction mixtures contained microsomes (equal to 10 nmoles of cytochrome P_{450}), 16 µmoles $MgCl_2$, an NADPH generating system (9.4 µmoles isocitrate, Tris Na salt, 0.52 µmoles $NADP^+$, and 0.05 units/ml. of isocitrate dehydrogenase, type IV) and 20 µmoles of aminopyrine recrystallized from hexane. Reaction mixtures were made up to 5 mls. with

0.05M Tris HCl, pH 7.5, and incubated aerobically at 37° on a Dubnoff Metabolic Shaker. One ml. aliquots were withdrawn at specific times over fifteen minutes, and added to one ml. of 10% trichloroacetic acid. After allowing ten minutes for the protein to precipitate, two mls. of Nash Reagent (2M $NH_4C_2H_3O_2$; 0.05M CH_3COOH; 0.02M 2,4-pentadione) was added and the mixtures heated at 60° for 15 minutes. The assay mixtures were centrifuged at 2000 x g for 15 minutes to remove precipitated protein, and read at 412 nm on a Coleman Jr. Spectrophotometer equipped with a flow cell.

NADPH-Cytochrome c Reductase Determination

The rate of cytochrome c reduced was followed at 550 nm on a Coleman 124 Recording double beam spectrophotometer. Reaction mixtures contained 710 nmoles of cytochrome c and 0.1 μmoles of NADPH, and was made up to a total volume of one ml. with 0.3M PO_4 buffer pH 7.3 containing 10mM EDTA. The extinction coefficent used was 21.0 x $10^3 M^{-1} cm^{-1}$.[36]

RESULTS AND DISCUSSION

The first question to be considered is whether PBB causes any discernible changes in the liver, and does it induce cytochrome P_{450} levels. To answer these questions, one group of rats was given one injection of 90 mg./kg. body weight PBB in corn oil and another group was given one injection of Pb (50 mg./kg. body weight). It has previously been shown in our lab that corn oil does not effect the microsomal enzymes. Liver weight to body weight ratio, total microsomal protein, and cytochrome P_{450} levels were followed. This is shown in Figures 1 and 2. There were no gross abnormalities of the liver observed, although occasionally fatty deposits would be found on the livers of rats injected with PBB. It was immediately obvious that PBB was effecting changes and that these changes were of a larger magnitude than those caused by Pb injection. The changes caused by PBB also lasted considerably longer than those caused by Pb. Pb-induced microsomes had parameters back to control levels after five days (except for total microsomal protein), while PBB-induced microsomes had elevated levels even after 10 days. Total microsomal protein increased four times in the PBB-injected rats but only 2.5 times in the Pb-injected rats; P_{450} levels were raised three times in the PBB rats versus two times in the Pb rats, and the liver weight to body weight

Figure 1.--Liver parameters of rats given one injection of PBB (09 mg./kg. body weight). Data points are for two rats, unwashed microsomes.

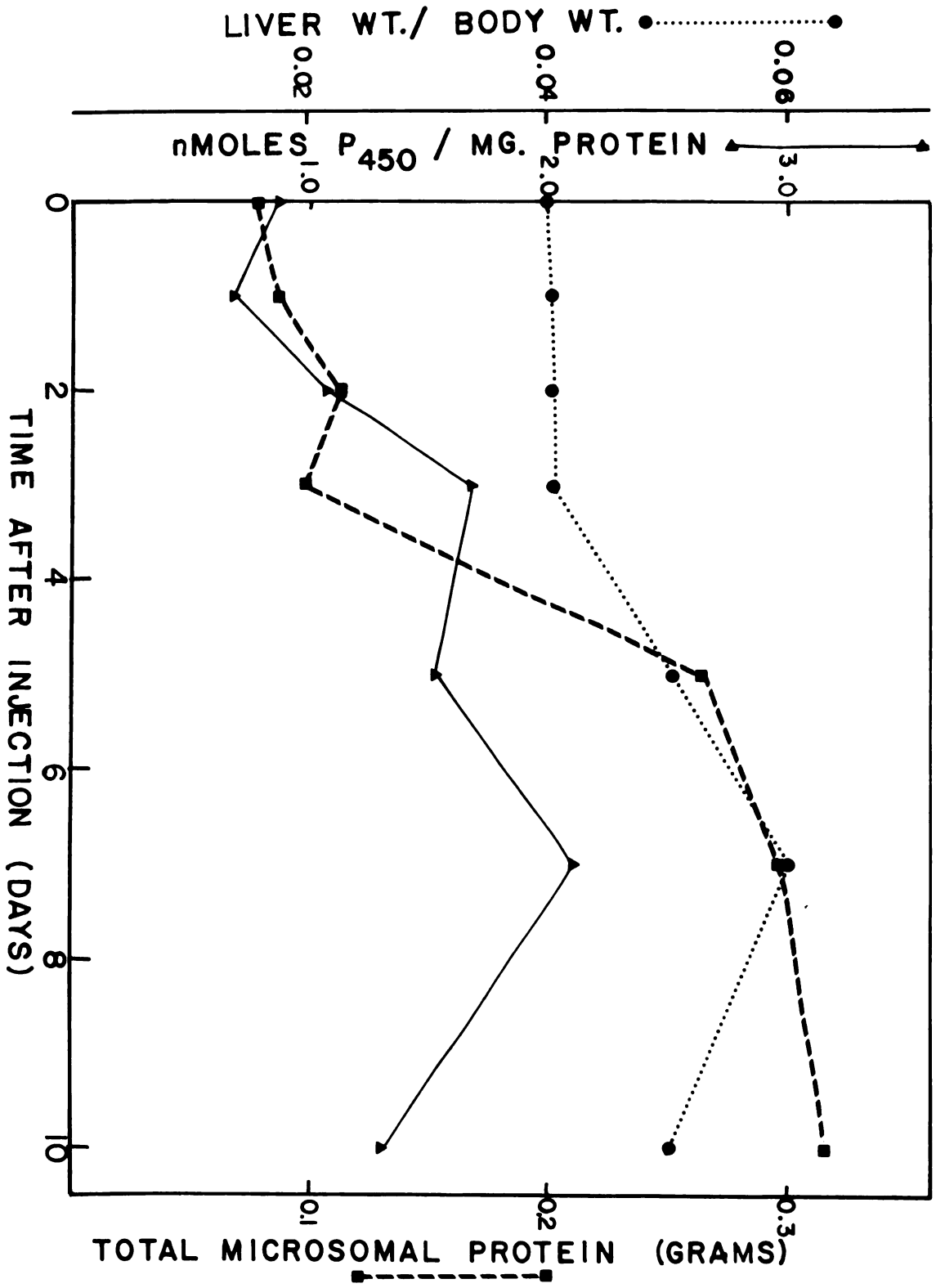
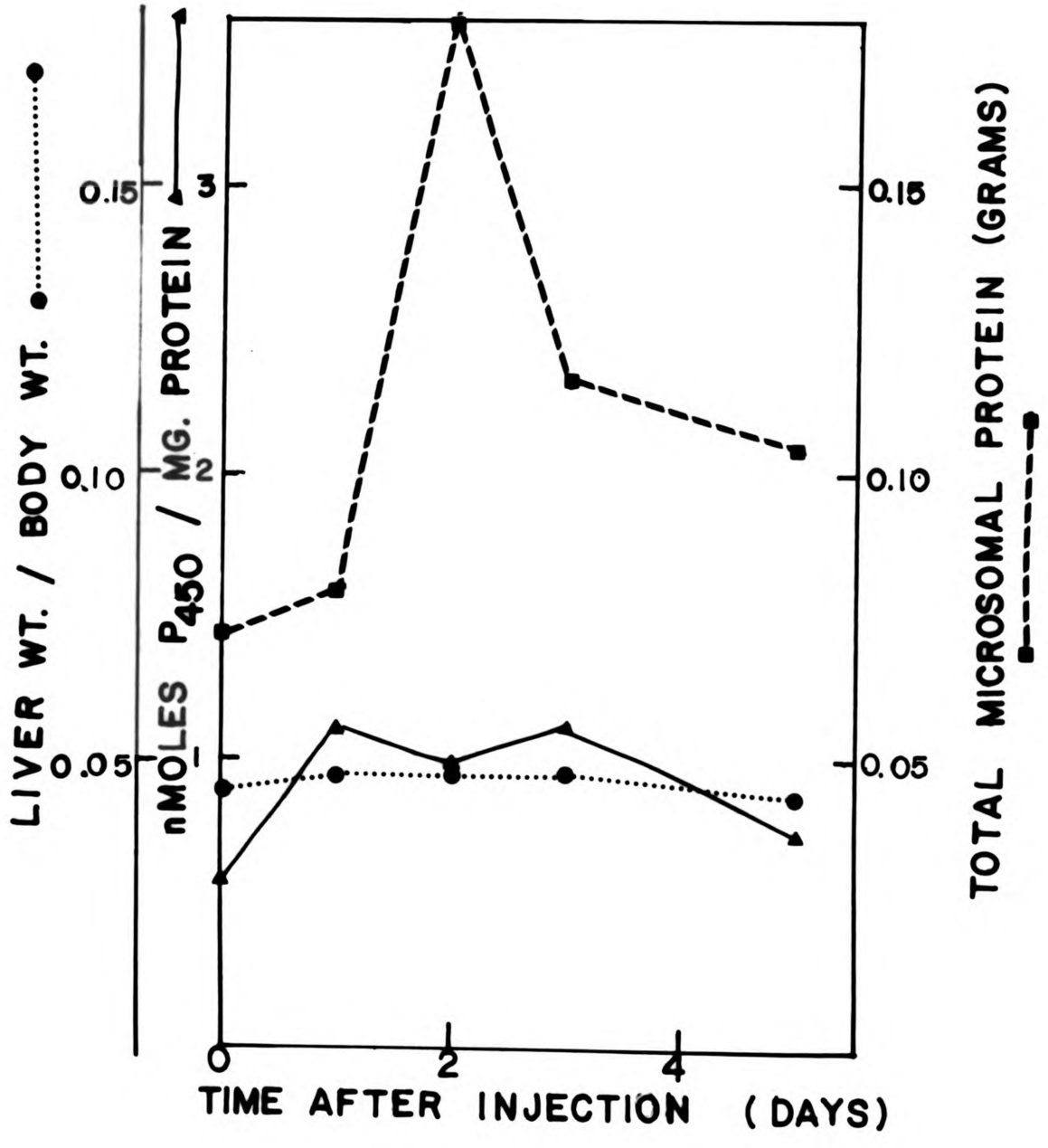

LIVER WT./ BODY WT.
0.02
0.04
0.06
nMOLES P450 / MG. PROTEIN
1.0
2.0
3.0
0
2
4
6
8
10
TIME AFTER INJECTION (DAYS)
0.1
0.2
0.3
TOTAL MICROSOMAL PROTEIN (GRAMS)

Figure 2.--Liver parameters of rats given one injection of Pb (50 mg./kg. body weight). Data points are for two rats, unwashed microsomes.


LIVER WT. / BODY WT.
nMOLES P450 / MG. PROTEIN
TOTAL MICROSOMAL PROTEIN (GRAMS)
0.15
0.10
0.05
3
2
1
0
2
4
TIME AFTER INJECTION (DAYS)

ratio increased one and a half times in the rats injected with PBB but not at all in those injected with Pb. Since we could not be sure that the dose of Pb was maximally inducing the microsomal enzymes, these experiments were repeated and this time the Pb rats received daily injections of 50 mg./kg. body weight Pb. Although the differences in the amount of induction were less between the Pb and PBB induced microsomes, the results were similar to the first experiment shown in Figure 1.

In order to determine if PBB and Pb act similarly on the liver and microsomal enzymes, rats were given 0.1% Pb in the drinking water for fouteen days in order to maximally induce the microsomal enzymes. They were then given one injection of PBB (90 mg./kg. body weight) and the parameters given above were followed for three days (Table 1).

Table 1

Liver parameters of rats given Pb-H_2O for fourteen days and then one injection of PBB (90 mg./kg. body weight) Data points are for two rats, unwashed microsomes.

Time (days)	$\frac{\text{liver wt.}}{\text{body wt.}}$	Total Microsomal Protein (grams)	$\frac{\text{nmoles } P_{450}}{\text{mg. protein}}$
0	0.066	0.294	2.22
1	0.061	0.418	2.21
3	0.058	0.651	2.15

Liver weight to body weight in ratio remained constant, but total microsomal protein more than doubled, as did total nmoles of cytochrome P_{450} even though nmoles P_{450}/mg. protein

remained constant. If PBB were acting identically on the liver microsomes to Pb, then we would expect no changes in any of these parameters. Since changes were found, it was concluded that PBB is a much more potent inducer of microsomal enzymes than Pb is. It also induces microsomal protein to a much greater extent than Pb does, so it must be acting differently on the microsomes. Another important conclusion of this experiment is that PBB does not destroy cytochrome P_{450}, and is characteristic of some halogenated compounds which form free radicals.[37]

The next thing to look at was which microsomal cytochrome P_{450}'s are induced by PBB. This is relevant to study because, as explained in the Introduction, there are two types of inducers known: Pb falls into the first category of general inducers of microsomal enzymes, while 3-MC belongs to the category of specific microsomal enzyme inducers.[1, 4] There were three groups of rats: one group was given one injection of 90 mg./kg. body weight PBB; a second, one injection of 20 mg./kg. body weight 3-MC; and a third group was given five daily injections of 50 mg./kg. body weight of Pb. The microsomes were washed, and cytochrome P_{450}, NADPH-cytochrome c reductase, and aminopyrine demethylase activity levels were followed versus time (Figures 3-8). Benzpyrene hydrosylase data is generously provided by Robert Moore, using the method of Gieler et al.[38] This data is shown in Table 2.

In each parameter studied, PBB was a better inducer than either Pb or 3-MC, except for benzpyrene hydroxylase where 3-MC was slightly higher in its inducing power. It did take PBB longer to induce this enzyme to its highest level

Figure 3.--Liver parameters of rats given one injection of PBB (90 mg./kg. body weight). Male rats were injected on day 0 with PBB. Data points represent three rats on days 0-3 and two rats on days 5 and 7. Data is for washed microsomes. Open symbols refer to ten day control values.

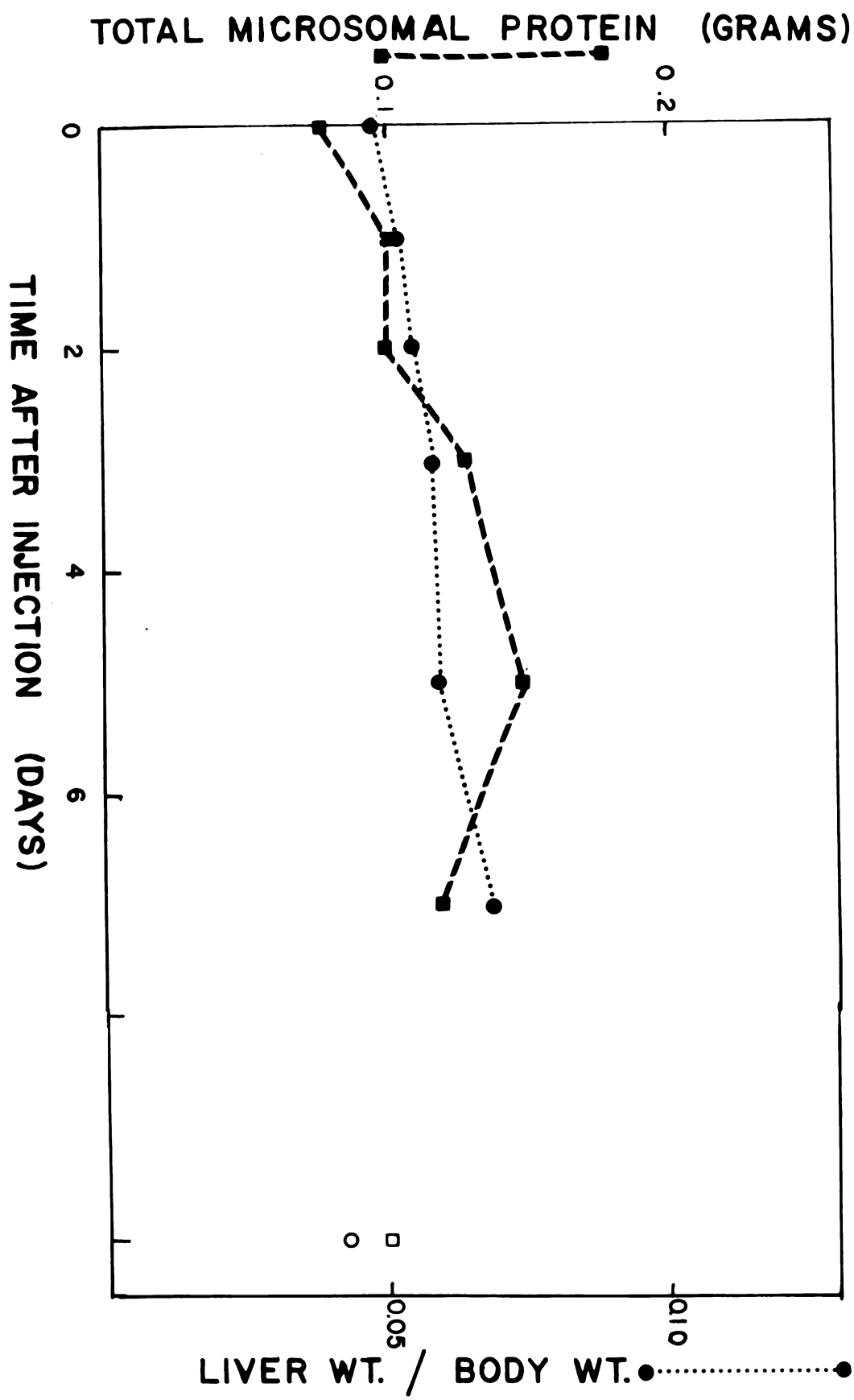

TOTAL MICROSOMAL PROTEIN (GRAMS)
0.1
0.2
TIME AFTER INJECTION (DAYS)
0
2
4
6
0.05
0.10
LIVER WT. / BODY WT.

Figure 4.--Drug metabolism of rats given one injection of PBB (90 mg./kg. body weight). Male rats were injected on day 0 with PBB. Data points represent three rats on days 0-3 and two rats on days 5 and 7. Data is for washed microsomes. Open symbols refer to ten day control values.

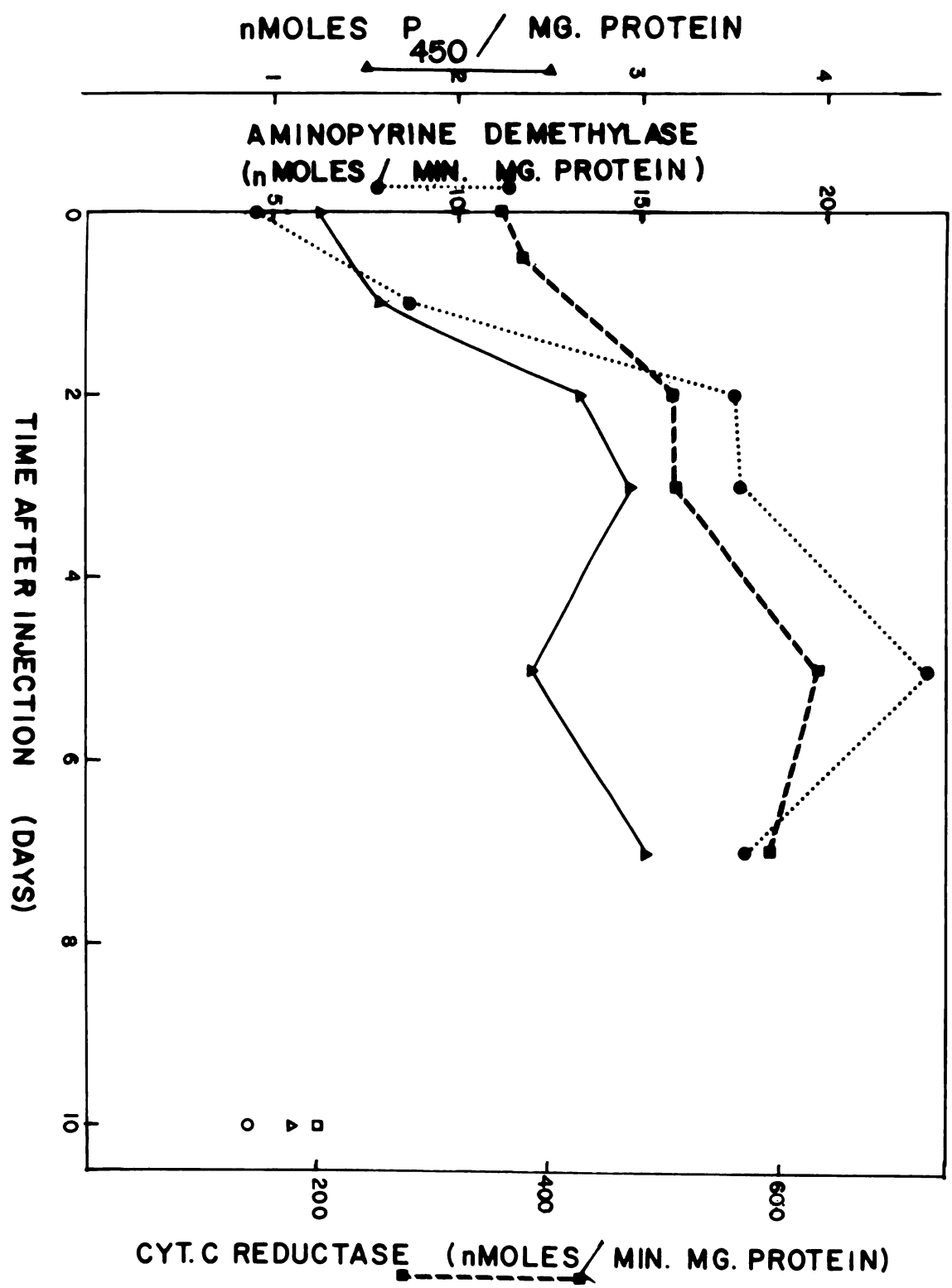

nMOLES P450 / MG. PROTEIN
AMINOPYRINE DEMETHYLASE
(nMOLES / MIN. MG. PROTEIN)
TIME AFTER INJECTION (DAYS)
CYT. C REDUCTASE (nMOLES / MIN. MG. PROTEIN)

Figure 5.--Liver parameters for rats given five daily injections of Pb (50 mg./kg. body weight). Male rats were injected with Pb on days 0-4. Data points represent three rats on days 0-3 and two rats on days 5-10. Data is for washed microsomes. Open symbols refer to ten day control values.

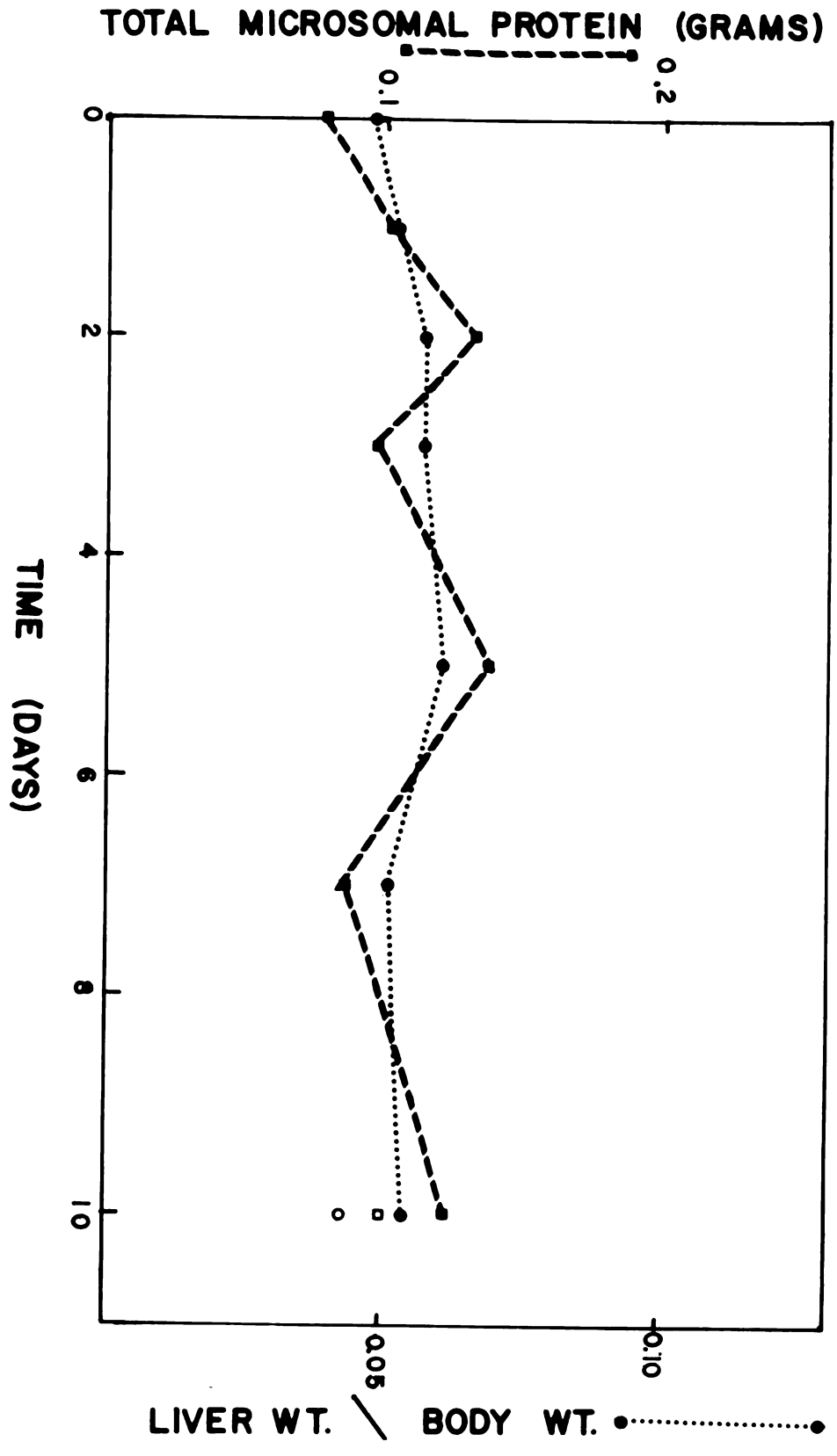

TOTAL MICROSOMAL PROTEIN (GRAMS)
0.1
0.2
0
2
4
6
8
10
TIME (DAYS)
0.05
0.10
LIVER WT. / BODY WT.

Figure 6.--Drug metabolism data of rats given five daily injections of Pb (50 mg./kg. body weight). Male rats were injected with Pb on days 0-4. Data points represent three rats on days 0-3 and two rats on days 5-10. Data is for washed microsomes. Open symbols refer to ten day control values.

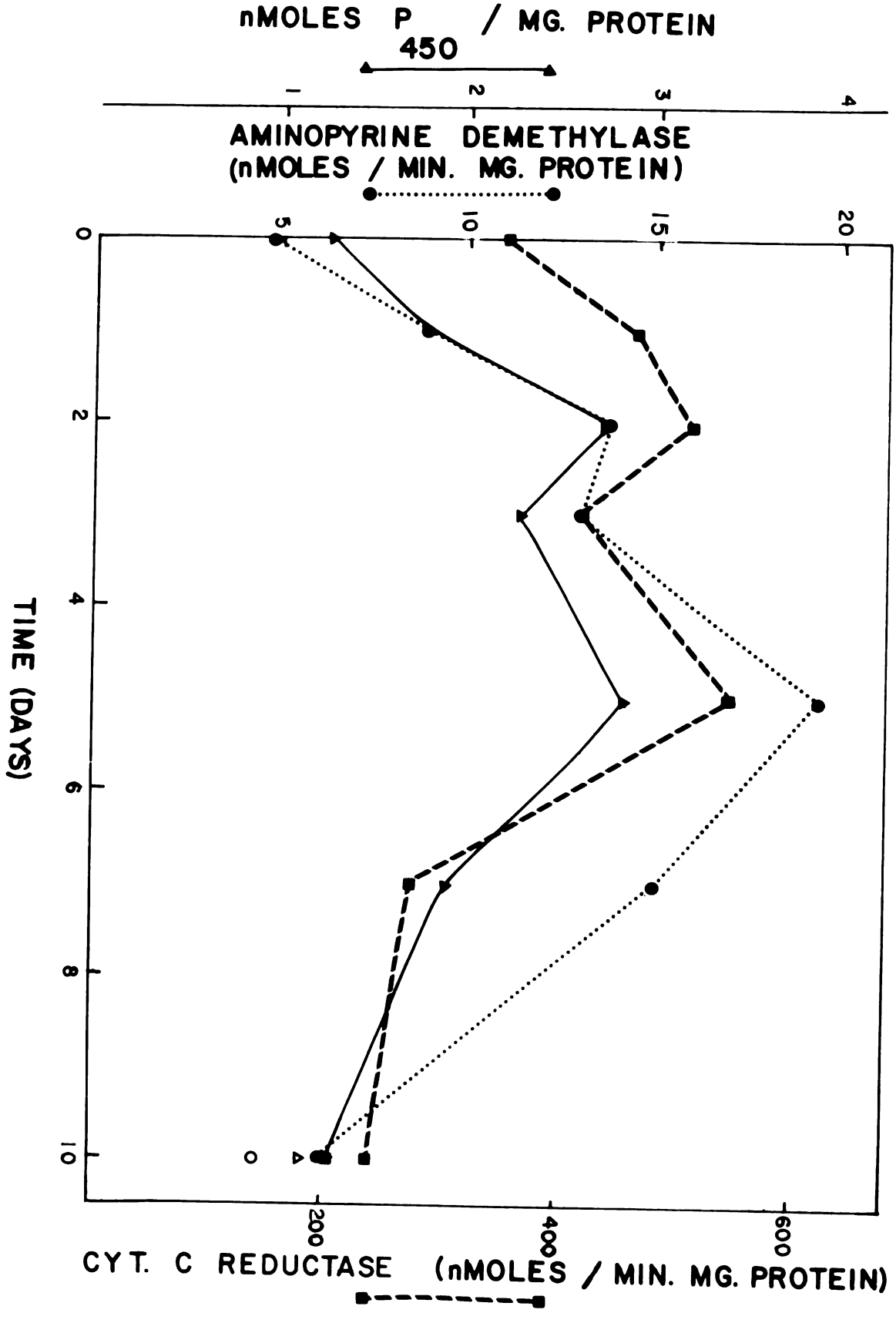

nMOLES P / MG. PROTEIN
450
1
2
3
4
AMINOPYRINE DEMETHYLASE
(nMOLES / MIN. MG. PROTEIN)
5
10
15
20
TIME (DAYS)
0
2
4
6
8
10
0
200
400
600
CYT. C REDUCTASE (nMOLES / MIN. MG. PROTEIN)

Figure 7.--Liver parameters of rats given one injection of 3-MC (20 mg./kg. body weight). Male rats were given one injection of 3-MC on day 0. Data points represent three rats on days 0-3 and two rats on day 5. Data is for washed microsomes.

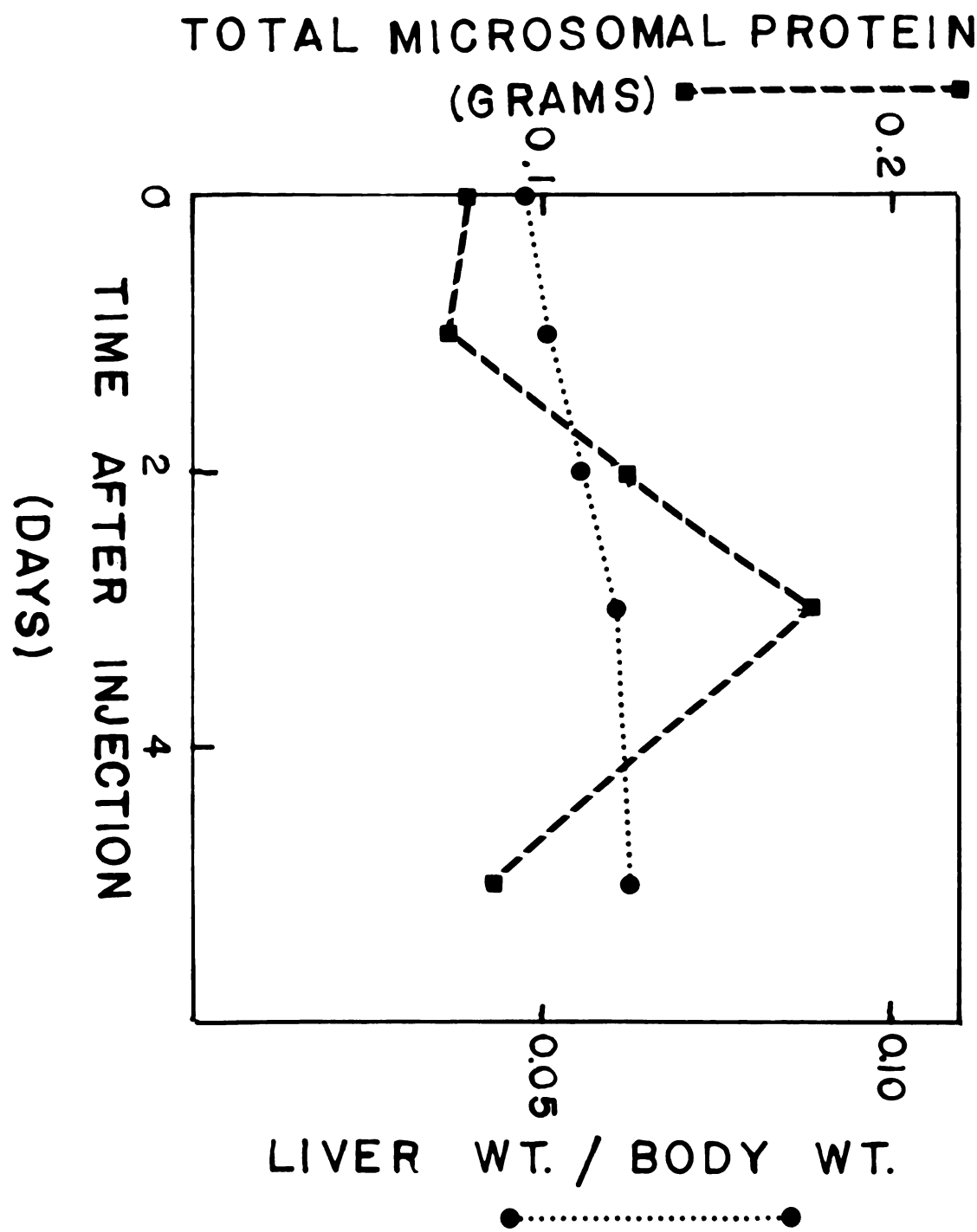

TOTAL MICROSOMAL PROTEIN
(GRAMS)
0.1
0.2
0
2
4
TIME AFTER INJECTION
(DAYS)
0.05
0.10
LIVER WT. / BODY WT.

Figure 8.--Drug metabolism data of rats given one injection of 3-MC (20 mg./kg. body weight). Male rats were given one injection of 3-MC on day 0. Data points represent three rats on days 0-3 and two rats on day 5. Data is for washed microsomes.

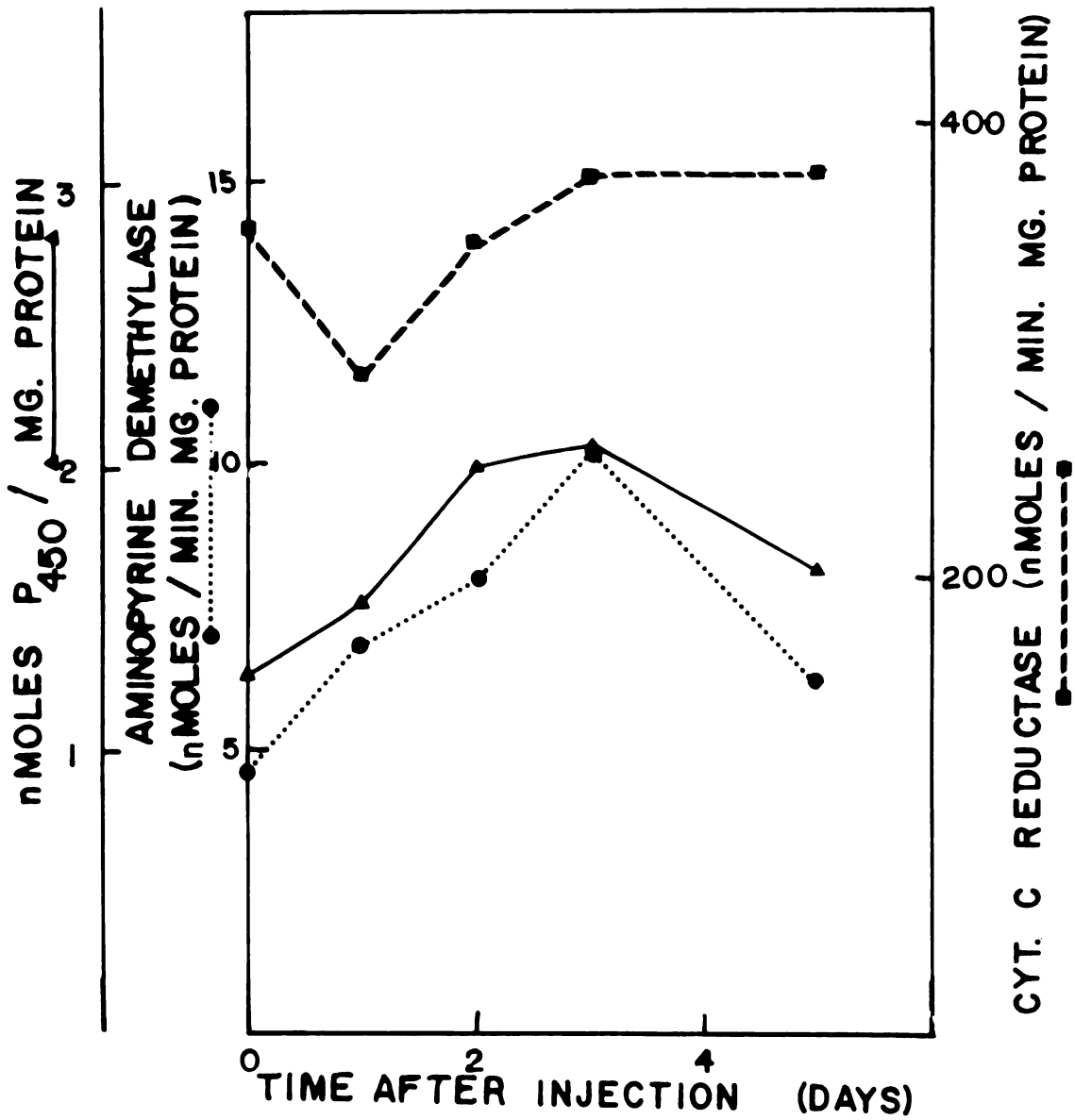

nMOLES P450 / MG. PROTEIN
AMINOPYRINE DEMETHYLASE (nMOLES / MIN. MG. PROTEIN)
CYT. C REDUCTASE (nMOLES / MIN. MG. PROTEIN)
TIME AFTER INJECTION (DAYS)
0
2
4
5
10
15
200
400

Table 2

Benzpyrene hydroxylase data for rats given either one injection of PBB (90 mg./kg. body weight), one injection of 3-MC (20 mg./kg. body weight), or five daily injections of Pb (50 mg./kg. body weight), on days 0-4. Data represents three rats on days 0-3 and two rats on days 5-10. Data is for washed microsomes and is courtesy of Robert Moore.

Treatment	Day	Benzpyrene Hydroxylase nmoles hydroxybenzpyrene (min. mg. protein)
control	0	0.74
PBB	1	1.33
PBB	2	2.65
PBB	3	3.23
PBB	5	3.58
PBB	7	5.23
Pb	1	1.30
Pb	2	1.57
Pb	3	2.41
Pb	5	1.02
Pb	7	1.10
Pb	10	0.72
control	10	0.72
3-MC	1	5.28
3-MC	2	6.66
3-MC	3	4.20
3-MC	5	2.93

as compared to the time required for 3-MC to induce it to its highest levels. Aminopyrine demethylase activity levels are especially impresssive -- PBB induces the activity almost six times over control values, while Pb microsomes had 4.3 times control activity of aminopyrine demethylase and 3-MC induced microsomes had 2.3 times control activity. The liver weight to body weight ratio, total microsomal protein, NADPH-cytochrome c reductase and cytochrome P_{450} levels were also induced to a greater extent by PBB-injection than by five Pb injections or one 3-MC injection. Since PBB induces all enzymes studied, we decided to see if a combination of both Pb and 3-MC would duplicate the effects of PBB. Rats were given five daily injections of Pb (50 mg./kg. body weight) and 32 hours after the last injection, they were given one injection of 3-MC (20 mg./kg. body weight). The rats were sacrificed 38 hours later. This data is presented in Table 3 (again, benzpyrene hydroxylase data is courtesy of Robert Moore). The 3-MC injection did not change the liver weight to body weight ratio, cytochrome P_{450} levels, aminopyrine demethylase levels or NADPH-cytochrome c reductase levels, but benzpyrene hydroxlase activity increased seven times over the five day Pb value (i.e. enzyme level at time of injection of 3-MC). It appears as if PBB is a more potent inducer than either Pb or 3-MC and does not just follow the combined pattern of the two, since drug metabolism total activities are much higher in PBB-induced microsomes, than in Pb + 3-MC induced microsomes.

If a single dose of PBB affects the liver and microsomal enzymes this much, we decided to test what a repeated dose

TABLE 3

Liver parameters and drug metabolism data for rats given five daily injections of Pb (50 mg./kg. body weight), and one injection of 3-MC (20 mg./kg. body weight). Data is for washed microsomes; control values represent three rats, 5-day Pb and Pb + 3-MC represent two rats. Benzpyrene hydroxylase data is courtesy of Robert Moore.

Treatment	$\frac{\text{liver wt.}}{\text{body wt.}}$	P_{450} ($\frac{\text{nmoles}}{\text{mg. protein}}$)	Aminopyrine Demethylase ($\frac{\text{nmoles HCOOH}}{\text{min. mg. protein}}$)
control	0.048	0.98	5.45
5 day Pb	0.061	2.85	19.65
Pb + 3-MC	0.063	2.14	15.7

Treatment	Cytochrome c Reductase ($\frac{\text{nmoles}}{\text{min. mg. protein}}$)	Benzpyrene Hydroxylase ($\frac{\text{nmoles hydroxy-benzpyrene}}{\text{min. mg. protein}}$)
control	359	0.74
5 day Pb	543	1.02
Pb + 3-MC	447	7.26

would do. To study this, rats were given five daily injections of PBB (90 mg./kg. body weight), and then sacrificed 24 hours after the last injeciton. Again, no morphological abnormalities were found in the livers. The results are shown in Table 4. Curiously, these results are almost identical with those results when only a single injection of PBB (90 mg./kg. body weight) is given except for the benzpyrene hydroxylase activity. Therefore, a single dose of 90 mg./kg. body weight (corresponding to about 20 mg. PBB/rat) must be completely inducing the microsomal enzymes to the limit to which they can be induced by i.p. injections of PBB. This is consistent with the idea of the strong potency of PBB in its inducing power.

A dietary study was done to determine the effects of a long term exposure to a small dose of PBB. A level of 10 ppm PBB was put into the rat feed for 16 days. A level of 10 ppm corresponds to approximately 0.8 mg. PBB/ kg. body weight/ day. On the seventeenth day the rats were put back on control feed. Two rats were sacrificed every three days and the microsomes assayed for the usual parameters. This data is shown on Figures 9 and 10. Benzpyrene hydroxylase data (courtesy of Robert Moore) is shown on Table 5. After only three days on the diet (an intake of approximately 0.5 mg. of PBB), liver weight to body weight ratio had gone up 1.2 times, total microsomal protein had almost doubled, aminopyrine demethylase levels had increased five times, cytochrome P_{450} levels had increased 1.4 times over control values, and NADPH-cytochrome c reductase levels had gone up 1.3 times. Benzpyrene hydroxylase activity was 1.6 times control values. After six to nine

TABLE 4

Liver parameters and drug metabolism data for rats given five daily injections of PBB (90 mg./kg. body weight). The two rats were sacrificed 24 hours after the last injection. Controls received five injections of corn oil. Data is for washed microsomes. Benzpyrene hydroxylase data is courtesy of Robert Moore.

Treatment	$\frac{\text{liver wt.}}{\text{body wt.}}$	P_{450} ($\frac{\text{nmoles}}{\text{mg. protein}}$)	Aminopyrine ($\frac{\text{nmoles}}{\text{min. mg. protein}}$ HCOOH) Demethylase
control	0.049	1.29	4.56
PBB	0.065	3.42	27.6

Treatment	Cytochrome c Reductase ($\frac{\text{nmoles}}{\text{min. mg. protein}}$)	Benzpyrene Hydroxylase	$\frac{\text{nmoles hydroxy-benzpyrene}}{\text{min. mg. protein}}$
control	416		0.52
PBB	719		5.44

Figure 9.--Liver parameters of rats on 10 ppm PBB diet. Male rats were fed diets containing 10 ppm for sixteen days. They received control feed from day seventeen (indicated by the arrow) until the end of the study. Data points represent two rats. Microsomes were washed. Open symbols refer to thirty day control values.

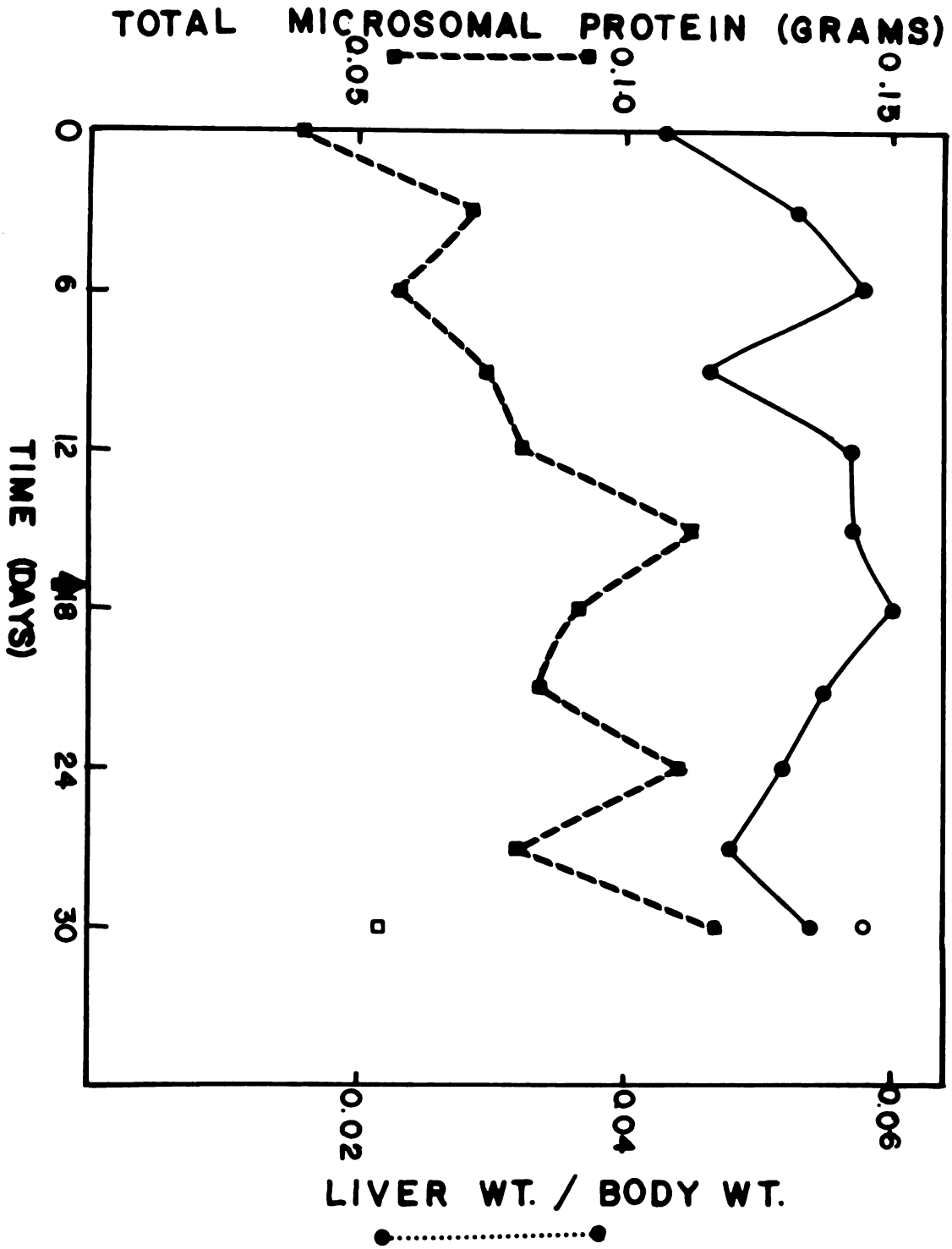

TOTAL MICROSOMAL PROTEIN (GRAMS)
0.05
0.10
0.15
0
6
12
18
24
30
TIME (DAYS)
0.02
0.04
0.06
LIVER WT. / BODY WT.

Figure 10.--Drug metabolism data of rats on 10 ppm PBB diet. Male rats were fed diets containing 10 ppm PBB for sixteen days. They received control feed from day seventeen (indicated by the arrow) until the end of the study. Data points represent two rats. Microsomes were washed. Open symbols refer to thirty day control values.

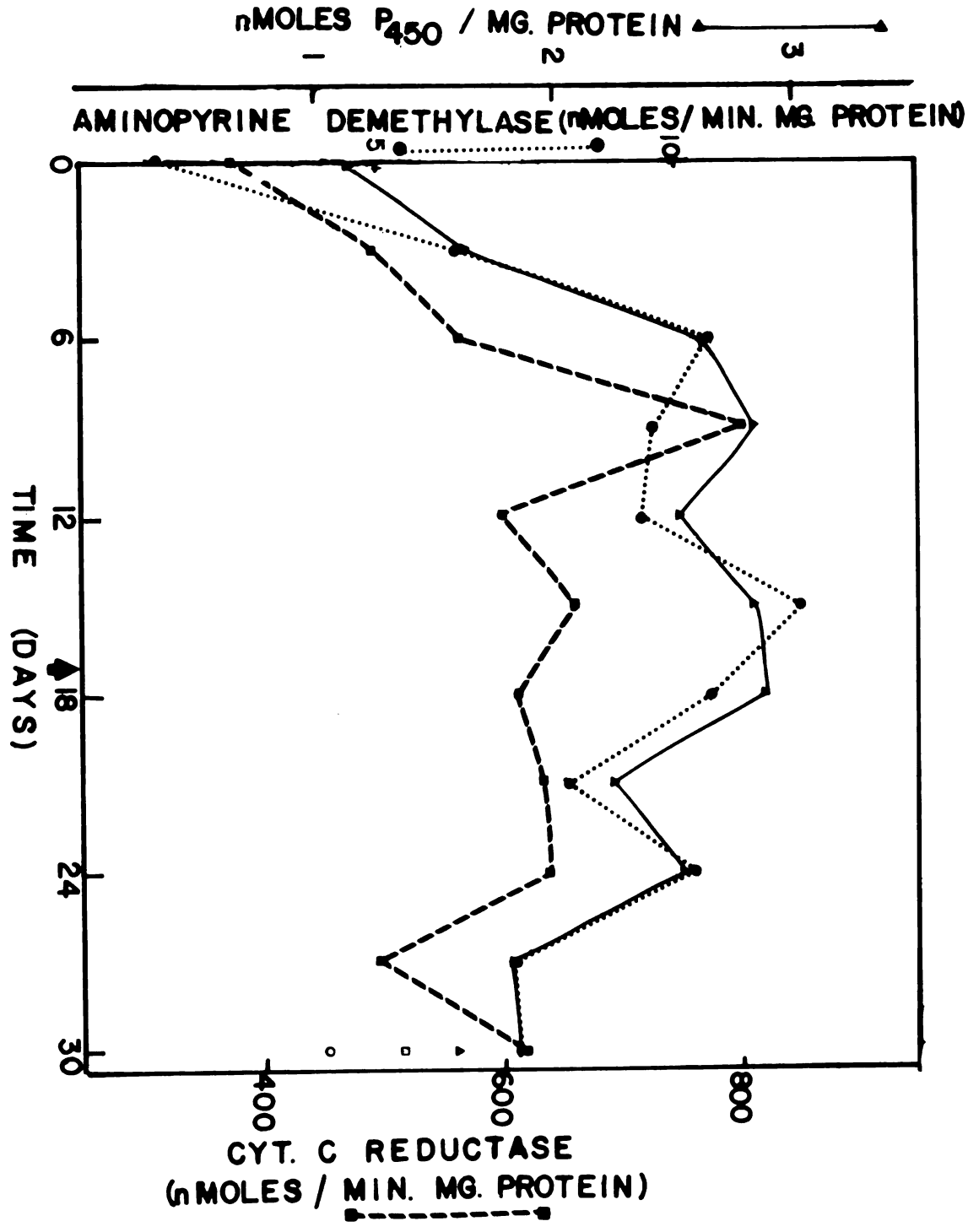

nMOLES P450 / MG. PROTEIN
1
2
3
AMINOPYRINE DEMETHYLASE (nMOLES/ MIN. MG. PROTEIN)
5
10
0
6
12
18
24
30
TIME (DAYS)
400
600
800
CYT. C REDUCTASE
(nMOLES / MIN. MG. PROTEIN)

TABLE 5

Benzpyrene hydroxylase data for rats on 10 ppm PBB diet. They received control feed from day seventeen until the end of the study. Data points represent two rats, washed microsomes. Data is courtesy of Robert Moore.

Day	Benzpyrene Hydroxylase ($\frac{\text{nmoles OH-benzpyrene}}{\text{min. mg. protein}}$)
0	0.46
3	0.74
6	1.08
9	1.11
12	0.97
15	1.20
18	0.925
21	0.93
24	0.31
27	0.57
30	0.27
control-30	0.15

days on the PBB diet (1.0 to 1.5 mg. of PBB ingested), maximal levels of the drug metabolizing enzymes were reached although total protein continued to rise. At the maximal level, aminopyrine demethylase levels were induced to ten times over control values (this is higher than with one injection of PBB [90 mg./kg. body weight]). Cytochrome P_{450} levels were up 3.2 times, cytochrome c reductase values were up 2.1 times over control values, and benzpyrene hydroxylase activity was induced 2.6 times over control values. Except for benzpyrene hydroxylase levels, a chronic exposure to PBB in the diet raises the levels of the drug metabolizing enzymes to higher levels than those obtained with a single injection (90 mg./kg. body weight). Total protein in the microsomes was also higher in the rats fed PBB, but liver weight to body weight ratio stayed about the same in the rats fed 10 ppm PBB as in those injected with PBB (90 mg./kg. body weight).

The feed was withdrawn on day seventeen and control feed given for the remainder of the experiment. During the next two weeks, the liver weight to body weight ratio decreased to below control values, but total microsomal protein was still substantially elevated over control levels. The microsomal enzyme levels also started to decline. After two weeks of control diet, cytochrome c reductase and cytochrome P_{450} levels were only slightly higher than control values, but aminopyrine demethylase levels were still almost twice as high as control values. This phenomena may be due to differential elimination of the differently brominated compounds found in Firemaster BP-6. It may be that, as found

with the PCB's[28] that the higher brominated coumpounds are absorbed less and so would be eliminated first, and it would be these compounds that are responsible for the effects that disappear first when the PBB feed is withdrawn.

It would seem that PBB is a very potent inducer of microsomal enzymes even at very low levels in the diet, and that the effects last for a considerable time after the PBB is withdrawn. This data has much significance when one considers that meat of suspected infected cattle is being sold in Michigan today.

Lastly, a study was done to determine if PBB affects the eating habits of animals in which it is injected. Over the period of one week, the average amount of feed eaten by control rats was 28.6 ± 1.3 grams/rat/day, while rats injected with 50 mg./kg. weight of PBB ate an average of 28.3 ± 1.9 grams/rat/day. There is no significant difference between these averages nor were there any differences in the eating patterns of the two groups over the week studied.

SUMMARY

PBB is not an inert chemical biologically. When animals are given as little as 0.5 mg. PBB over three days in the diet, increases are found in the levels of aminopyrine demethylase, benzpyrene hydroxylase, NADPH-cytochrome c reductase and cytochrome P_{450} as well as liver weight to body weight ratio and total microsomal protein. One dose of 90 mg./kg. body weight of PBB increases these parameters for at least 10 days and 10 ppm PBB in the diet for sixteen days keeps these levels elevated for at least two weeks. PBB appears to be a potent inducer of microsomal protein in particular -- even after rats were given a maximally inducing dose of Pb, one PBB injection (90 mg./kg. body weight) radically increased microsomal protein and also total cytochrome P_{450} levels. This would indicate, along with the data that all microsomal enzymes studied were induced, not following the pattern of Pb, 3-MC or the two combined, that PBB is a new type of inducer. It is more potent and more general in its effects than previous inducers that have been studied, and presents an enviromental hazard. SDS-gels are now being run in our lab to determine which of the cytochrome P_{450}'s are being induced by PBB. (See reference 21 for a discussion of this.)

PBB is a stronger inducer of microsomal enzymes than PCB is, and concern has already been raised over the effects of

PCB in the environment. The recent contamination of cattle feed in Michigan with PBB therefore deserves a second look, now that it has been shown that PBB does affect the liver and microsomal enzymes.

LIST OF REFERENCES

REFERENCES

1. A. H. Conney, Phar. Rev. 19, 317 (1967).

2. A. H. Conney and J. J. Burns, Science 178, 576 (1972).

3. R. Kuntzman, Ann. Rev. Phar. 9, 21 (1969).

4. A. Kappas and A. P. Alvares, Sci. Am. 232(6), 22 (1975).

5. B. Brodie, J. Gillette and B. LaDu, Ann. Rev. of Biochem. 27, 427 (1958).

6. J. Baker and S. Chaykin, J. Biol. Chem. 237, 1309 (1962).

7. H. S. Mason, Science 125, 1185 (1957).

8. A. Y. H. Lu, R. Kuntzman, S. West, M. Jacobsen and A. H. Conney, J. Biol. Chem. 247, 1727 (1973).

9. M. Klingenberg, Arch. Biochem. and Biophys. 75, 376 (1958).

10. D. Garfinkel, Arch. Biochem. and Biophy. 77, 493 (1958).

11. T. Omura and R. Sato, J. Biol. Chem. 239, 2370 (1964).

12. D. Cooper, S. Levin, S. Narasimhulu, O. Rosenthal, and R. Estabrook, Science 147, 400 (1965).

13. R. Brown, J. A. Miller, and E. C. Miller, J. Biol. Chem. 209, 211 (1954).

14. A. H. Conney and J. J. Burns, Nature 184, 363 (1959).

15. Y. Imai and R. Sato, Biochem. Biophys. Res. Commum. 22, 620 (1966).

16. L. Ernster and S. Orrening, Fed. Proc. 24, 1190 (1965).

17. H. Jick and L. Shuster, J. Biol. Chem. 241, 5366 (1966).

18. A. H. Conney, E. C. Miller and J. A. Miller, Cancer Research 16, 450 (1956).

19. A. F. Welton, and S. D. Aust, Biochem. Biophys. Res. Commum. 56, 898 (1974).

20. A. P. Alvares, G. Schilling, W. Levin, and R. Kuntzman, Biochem. Biophys. Res. Commum. 29, 521 (1967).

21. A. Y. H. Lu, W. Levin, S. West, M. Jacobsen, D. Ryan, R. Kuntzman, and A. H. Conney, J. Biol. Chem. 248, 456 (1973).

22. R. Kuntzman, W. Levine, M. Jacobsen, and A. H. Conney, Life Sci. 7, 215 (1968).

23. A. P. Alvares, G. Schilling and R. Kuntzman, Biochem. Biophys. Res. Commum. 30, 588 (1968).

24. T. Pederson and S. D. Aust, Biochem Phar. 19, 2221 (1970).

25. J. Norris, J. Ehrmantraut, C. Gibbons, R. Kociba, B. Schwetz, J. Rose, C. Humiston, G. Jewett, W. Crummett, P. Gehring, J. Tirsell, J. Brosier, Applied Polymer Symposia 22, 195 (1973).

26. J. Bruckner, K. Khanna and H. Cornish, Toxicology and Appl. Phar. 24, 434 (1973).

27. L. Fishbein, J. Chromatography 68, 345 (1972).

28. R. Kimbrough, Critical Rev. in Toxicology, Jan., 1974.

29. C. Litterst, T. Farber, A. Baker, and E. VanLoon, Toxicol. Appl. Phar. 23, 112, (1972).

30. A. H. Alvares, D. Bickers, and A. Kappas, Fed. Proc. 32, 235 (abstract) (1973).

31. D. Ecobichon and G. Johnstone, "The Influence of Pure Polychlorinated biphenyl Compounds on Hepatic Functions in Rats" presented at the 12th Annual Meeting, Soc. Toxicol. New York, N.Y. March 18-22, 1973.

32. F. Farber and A. Baker, "Microsomal Enzyme Induction by Hexabrominated Biphenyls" presented at the Soc. of Toxicol. Meeting, Washington, D.C. Mar. 10-14, 1974.

33. B. Mahan, University Chemistry, Addison-Wesley Publishing Co., Inc., Reading, Mass. 1969.

34. O. H. Lowry, N. Rosebrough, A. Farr and R. Randall, J. Biol. Chem. 193, 265 (1951).

35. T. Nash, Biochem. J. 55, 416 (1953).

36. W. Massey, Biochem. Biophys. Acta 34, 255 (1959).

37. T. F. Slater, Free Radical Mechanisms in Tissue Injury, Pion Ltd., London, 1972.

38. J. E. Gielen, F. M. Goujon, D. W. Niebert, J. Biol. Chem. 247, 1125 (1972).

MICHIGAN STATE UNIVERSITY LIBRARIES
3 1293 03178 1184