AN ABSTRACT OF THE THESIS OF

Daniel R. Vincent for the degree of Doctor of Philosophy

in Entomology presented on November 17, 1983

Title: Characteristics of NADPH-Cytochrome P-450 Reductase in the House Fly (Musca domestica, L.) and the Blow Fly (Phormia regina, Meigen).

Abstract approved: Leon C. Terriere

NADPH-cytochrome P-450 reductase, an important enzyme in the cytochrome P-450-dependent microsomal monooxygenase system, was purified from insecticide-resistant (R) and -susceptible (S) house flies (Musca domestica, L.) and from the black blow fly (Phormia regina, Meigen). Proteolytic degradation to a P-450 - inactive form of the reductase was reduced during the procedure by the use of specific protease inhibitors (leupeptin and chymostatin) as well as by careful timing of other key steps. Following the several steps in the procedure, the homogeneous enzymes (M_r = 74,000 for either strain of M. domestica, and 75,000 for P. regina) was purified ca. 360-fold, achieving specific activities ranging from 32 to 40 µmol cytochrome c reduced/min/mg protein. Each of these purified enzymes was competent in supporting either epoxidation or O-demethylation

reactions involving soluble house fly cytochrome P-450 and synthetic phospholipid.

Comparisons of kinetic behavior, absolute spectra, response to ionic strength, amino acid composition, or immunochemical cross-reactivity indicated that the three reductases were nearly identical. However, the purified R-fly enzyme was more sensitive to the NADPH analogue, 2'-AMP, (Ki = 187 μM) than the other two reductases. These insect enzymes were distinctively different from rat or rabbit liver P-450 reductases in their response to ionic strength, sensitivity to 2'-AMP, amino acid composition, and antigenic determinants.

Microsomal reductase activity was lowest in pharate adult flies (ca. 50 μmol product/min/mg protein) and increased to a maximum (ca. 120 μmol product/min/mg protein) 24 to 48 hr after emergence. The activity then assumed a rather constant value (ca. 80 μmol product/min/mg protein) through 5 days of age. These values were similar in the three insects.

Treatment of the three flies with phenobarbital, a microsomal monooxygenase inducer, caused the reductase activity to increase about 2.5-fold in the two house fly strains and 5-fold in the blow fly strain. P-450 also increased several fold, as did monooxygenase activities.

Characteristics of NADPH-Cytochrome P-450 Reductase in the House Fly (*Musca domestica*, L.) and the Blow Fly (*Phormia regina*, Meigen)

by

Daniel R. Vincent

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

Completed November 17, 1983

Commencement June, 1984

APPROVED:

Redacted for Privacy

Dr. Leon C. Terriere, Professor of Entomology, in charge of major

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Date thesis is presented November 17, 1983

Typed by Cherri Spence for Daniel R. Vincent

Acknowledgements

This work has received consistent and needed support from my colleagues in the departments of Entomology, Biochemisty and Biophysics, and Agricultural Chemistry. I am particularly indebted to my advisor, Dr. Leon C. Terriere, who patiently and firmly spurred me to succeed in this project. His fatherly role in this achievement cannot be overstated.

Much appreciation is extended to the members of my committee, Drs. Chris Bayne, Terry Miller, Jeff Miller, and Victor Brookes, for their time and concern. Their counsel was timely and helpful.

The work presented in this thesis would indeed have been difficult, if not strategically impossible, without the friendship, and technical and theoretical assistance of Dan Farnsworth and Dr. Alison Moldenke. Their contributions to the production of soluble insect P-450 and the reconstitution of its activity, as well as the hundreds of measurements necessary for the induction studies deserve prominence.

The many suggestions dedicated cooperation and friendship of Dr. Rene Feyereisen are also gratefully acknowledged.

Finally, I thank my family, without whose support and love this adventure would never have come to be. Financial suppport came at important times from relatives; and constant encouragement helped me overcome the frustration of unexplainable results and of frequent, but mild, depressions. Melissa, my sweet wife, deserves the greatest share of honor for her patience, courage, and long

suffering. Our children Mika, Curtis, and Grant also deserve credit for their ability to make our memories of Corvallis all the richer.

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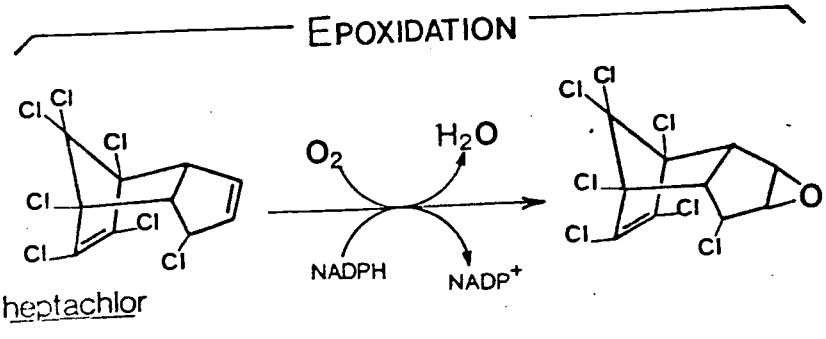
CHARACTERISTICS OF NADPH-CYTOCHROME P-450 REDUCTASE IN THE HOUSE FLY (*Musca domestica*, L.) AND THE BLOW FLY (*Phormia regina*, Meigen)

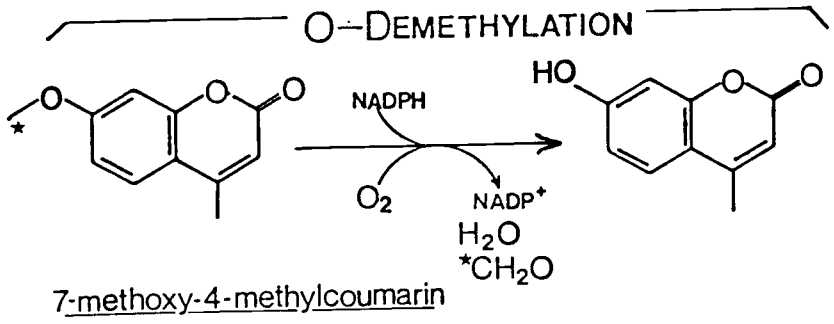
Chapter 1

Introduction and Literature Review

The cytochrome P-450-dependent microsomal monooxygenases constitute a remarkable assembly of enzymes that participates in the regulation of endocrine and pheromone titre, as well as in the detoxification of environmentally important chemicals such as insecticides and drugs. This system is also known as the mixedfunction oxidases, MFO (Mason, 1957), or more recently as the polysubstrate monooxygenases, PSMO (Nebert, 1980). It is situated primarily in the smooth endoplasmic reticulum (Terriere, 1968), and its importance in insect physiology was emphasized in a recent review (Hodgson, 1983). These enzymes perform many characteristic reactions (Kulkarni and Hodgson, 1980). Two of these (epoxidation and O-demethylation), which are germane to this study, are shown below.

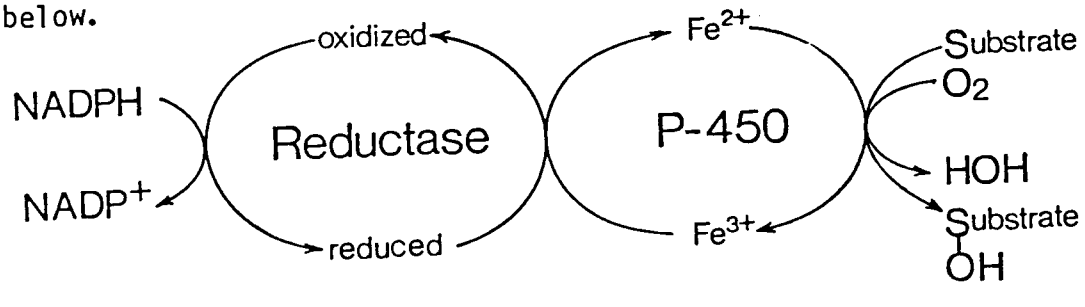


heptachlor



The activity of these monooxygenases is directed toward the oxidation of a host of physiologic substrates including juvenile hormone (Feyereisen et al., 1981; Yu and Terriere, 1978), ecdysone (Feyereisen and Durst, 1978; Terriere and Yu, 1976a; reviewed by Koolman, 1982), pheromones (Brattsten, 1979a; Blomquist et al., submitted manuscript), and environmental agents such as insecticides (Brooks, 1979) and plant allelochemics (Brattsten, 1979b). The location of this system in the membrane makes it especially suited to its task, providing a suitable environment for its lipophilic substrates and allowing for coordinated interaction of cofactors, substrates, and enzymes.

An obligatory companion enzyme in this system is NADPH-cytochrome P-450(c) reductase (EC.1.6.2.4), whose function, as its name implies, is to bridge the gap between the cytosol (NADPH) and the membrane (P-450). The scheme of this interaction is shown below.



This oxidoreductase was first described in vertebrates by B. L. Horecker (1949). The first study of this enzyme in insects was nearly 10 years later by Lang (1959), who found NADPH-dependent cytochrome c reducing activity in whole body homogenates of mosquito larvae and adults. This enzyme was first studied in mammalian liver (Horecker, 1949), and its role and characteristics are well developed in the mammalian literature (reviewed by Masters and Okita, 1980; Strobel et al., 1980). A brief review is presented below.

An understanding of the role and characteristics of NADPH-cytocrhome P-450(c) reductase has evolved over a period of more than 30 years. The first biochemical characterization of the reductase followed its purifiation from a trypsinized acetone powder of whole pig liver (Horecker, 1950). The enzyme was described as a flavoprotein (Mr = 68,000) capable of transferring electrons from NADPH to cytochrome c at a fast rate (1140 mol cytochrome c reduced/min/mol enzyme). However, it was not possible to deduce from these data the sub-cellular localization, physiologic function, or the nature of the flavin cofactors of this novel enzyme.

The cellular location of the reducatase was first reported by Williams and Kamin (1962) and confirmed immediately by Phillips and Langdon (1962). These authors proved the enzyme to be a component of liver microsomes (artifacts of the endoplasmic reticulum, Siekevitz, 1962, 1963), and concluded that their enzymes were essentially identical to that described more than a decade earlier by Horecker (1950).

At this point, it was speculated that the reductase participated in microsomal oxidase reactions (Phillips and Langdon, 1962). This theory was supported by three lines of evidence: (1) aniline hydroxylation activity suffered when the reductase was solubilized from microsomes by the protease steapsin (Sato et al., 1965), (2) cytochrome P-450 and the reductase were co-induced in rat liver by the barbiturate phenobarbital (Ernster and Orrenius, 1965), and (3) cytochrome c inhibited several microsomal monooxygenations (LaDu et al., 1957, Gillette et al., 1957; Krisch and Staudinger, 1961). Cytochrome P-450 had been previously discovered (Klingenberg, 1958; Garfinkel, 1958), described (Omura and Sato, 1964), and revealed to be the terminal oxygenase in various hydroxylations and dealkylations in microsomes prepared from adrenals (Estabrook et al., 1963) and liver (Cooper et al., 1964).

Conclusive evidence of the role of the reductase was presented several years later in two types of studies. First, Lu and Coon (1968) purified both cytochrome P-450 and NADPH-cytochrome c reductase from detergent-treated rat liver microsomes. They then demonstrated, using in vitro reconstitution techniques, that these two enzymes were the essential proteins for the omega-hydroxylation of lauric acid - a microsomal monooxygenase activity. Further experiments with this technique correlated other known microsomal activities with this system (Lu et al., 1969). The other type of study employed an antibody against reductase purified from protease-treated liver microsomes. This antibody inhibited microsomal NADPH-cytochrome c reduction (Kuriyama et al., 1969), as well as

various P-450-dependent monooxygenations (Omura, 1969). Therefore, it was firmly established that NADPH-cytochrome c reductase supplied electrons from cytoplasmic NADPH to cytochrome P-450.

It has become apparent that there are two major regions (domains) in P-450 reductase. The catalytic function of this enzyme resides in the larger domain (ca. 68,000 daltons) which can be removed intact from the microsome by proteolytic activity. The smaller domain (6-10,000 daltons), which remains in the membrane following proteolysis, is responsible for membrane interactions (Black et al., 1979; Gum and Strobel, 1981; Black and Coon, 1982). Once these two domains are separated, the enzyme can still reduce cytochrome c and other artificial substrates, but cannot interact effectively with cytochrome P-450. Solubilization of the reductase by proteases causes the separation of these two domains while detergent does not. Hence, detergent is the agent of choice for membrane solubilization when the reductase is destined for PSMO reconstitution experiments (Vermilion and Coon, 1978).

The flavin cofactor in mammalian liver reductase was considered to be FAD until Iyanagi and Mason (1973) showed that both FAD and FMN were present in the enzyme at 1 mole each per mole of enzyme. These cofactors are part of the catalytic domain. Electrons from NADPH are directed from FAD to FMN and then to the cytochrome substrate (Vermilion et al., 1981).

As previously mentioned, insect reductase was first examined, though indirectly, by Lang (1959). The first to re-examine the insect reductase were Wilson and Hodgson (1971a,b). These authors

reported the purification of house fly reductase following isobutanol treatment of abdominal microsomes. Their preparation was apparently homogeneous (M_r = 57,000), and the apoenzyme was reactivated by either FAD or FMN. However, they were not able to establish the identity of the cofactor(s).

From the data of Wilson and Hodgson (1971a,b) it was apparent that the reductase was a microsomal enzyme, and that it shared several characteristics with the mammalian enzyme, e.g., similar pH and temperature optima, kinetic mechanism and constants, and sensitivity to inhibitors. A marked difference was noted in the effect of ionic strength; the insect enzyme was apparently indifferent to this parameter, whereas the liver enzyme doubled in activity when the ionic strength was increased from near 0.15 to 0.83.

Evidence of the obligatory participation of this reductase in insect microsomal monooxygenations was provided in experiments that paralleled those mentioned for mammalian reductase. Several investigators, utilizing midgut tissues from caterpillars and crickets found that an endogenous proteolytic activity caused the reductase activity to remain in the microsomal supernatant with a concommitant loss of oxidase activity in the microsomes (Krieger and Wilkinson, 1970; Orrenius et al., 1971; Brattsten and Wilkinson, 1973a). Schonbrod and Terriere (1971) also found that xanthomattin, an eye pigment in house flies, inhibited aldrin epoxidation by house fly microsomes. They concluded that electrons from the reductase

were drawn off by this "electron sink" in a manner analogous to the monooxygenase inhibition by cytochrome c.

Additional indirect evidence contributed toward recognizing NADPH-cytochrome c reductase as a component of the insect PSMO system. Several experiments indicated that reductase and various monooxygenase activities were co-resolved in sucrose gradients following ultracentrifugation of insect homogenates (Feyereisen and Durst, 1978), and were co-induced in microsomes from house flies (Capdevila et al., 1973; Yu and Terriere, 1973; Brattsten and Wilkinson, 1973b; Feyereisen and Durst, 1980b).

The functional role of insect microsomal NADPH-cytochrome c reductase was substantiated by experiments in which the enzyme was inhibited by antibodies. Mayer and Prough (1977) isolated reductase from steapsin-treated house fly microsomes and found that the activity was inhibited by antibodies made against rat liver reductase. Crankshaw et al. (1981b) found that antibodies made against their preparation isolated from bromelain-treated armyworm midgut microsomes would not inhibit rat or rabbit liver reductase even though the antibodies were shown to inhibit liver microsomal reductase from other species (Crankshaw et al., 1981a).

The results with antibodies supported evidence obtained in attempts at reconstituting PSMO activity with partially pure (Capdevila et al., 1975) or homogeneous (Mayer and Durrant, 1979) house fly reductase. In the former work (Capdevila et al., 1975), monooxygenase activity was restored to partially purified house fly P-450 by a relatively impure reductase preparation. Although these

data implicated the two fractions used, it was not conclusive of reductase requirement because of impurities in the reductase fraction. The reconstitution experiment in the latter work showed the homogeneous house fly reductase to be capable of reducing partially purified house fly P-450 complexed with carbon monoxide. However, there was no indication by these authors that the purified enzyme could support monooxygenase activity.

The several attempts to purify and characterize insect P-450 reductase are summarized in Table I-1. There is considerable variability in molecular weight, and kinetic constants (Km, Ki) that have been reported. The difference in molecular weight is probably related to the type of agent used to solubilize the microsomes, and the variability in the constants may also be due to the methods used. The kinetic constants (Km) for the detergent-treated preparation (Mayer and Durrant, 1979) are in good agreement with accepted values for the mammalian liver reductase, where Km_{NADPH} = 5 μM, and $Km_{cyt.\ c}$ = 10 μM (Dignam and Strobel, 1977; Strobel *et al.*, 1980). However, the Ki for 2'-AMP for the insect enzymes is more than an order of magnitude larger than the value for rat liver reductase (ca. 10 μM, Yasukochi and Masters, 1976).

Mayer and Durrant (1979) obtained an apparently intact P-450 reductase following the solubilization of house fly microsomes with non-ionic detergent. Their purified enzyme contained equimolar amounts of FAD and FMN (1 mole each per mole enzyme), had a molecular weight of 83,000 (at least 4,000 daltons larger than mammalian reductase), catalyzed the reduction of cytochrome *c* via a

Bi Bi ping-pong mechanism, and was capable of reducing insect P-450. As mentioned earlier (page 8), these authors did not demonstrate the reconstitution of monooxygenase activity with their enzyme, an important step after achieving reductase purification. Strobel *et al.* (1980) found it difficult to compare the kinetics of the insect reductase purified by Mayer and Durrant with the liver enzyme because Mayer and Durrant had ommitted kinetic data in their report. Finally, attempts to repeat the purification of house fly P-450 reductase met with considerable difficulty (Vincent *et al.*, in press), requiring that a more reliable method be developed.

The literature contains few references documenting the change in reductase activity during insect development. Capdevila *et al.* (1973) monitored reductase activity (μmol reduced/min/10 g flies) in adult female house flies of the insecticide-resistant Fc strain. They found that the reductase held a rather constant level from 4 to 10 days following eclosion. Feyereisen and Durst (1980a) examined reductase activity in the fat body of the last larval instar of *Locusta migratoria*. They reported that fluctuations in specific activity (nmol reduced/min/mg protein) for reductase and P-450-dependent ecdysone 20-monooxygenation were positively correlated with changes in ecdysone titre in the 7 days of that instar. Ahmad and Forgash (1975) also examined NADPH-cytochrome *c* reductase activity during the larval instars of *Lymantria dispar*. However, their methods and results are subject to doubt. Only five points were monitored during a 20 day period of development, and the method of assay was not specific for cytochrome *c* reduction.

Reductase activity has also been examined in several insect PSMO induction studies. Specific activity of the reductase was shown to increase in microsomes from midgut and fatbody of southern armyworm larvae when these insects were exposed to diets containing various methyl-substituted toluenes (13.5 µmol inducer/g diet) or phenobarbital (0.25%). 3-methylcholanthrene, a potent inducer of PSMO enzymes in rat liver (Conney, 1982) was an ineffective agent in those experiments (Brattsten and Wilkinson, 1973b; Brattsten et al., 1980).

Capdevila et al. (1973) showed that microsomal reductase activity increased when adult house flies were exposed to either 0.5% PB or 5% naphthalene in the diet. These authors also demonstrated that the enzyme levels returned to normal in 48 to 72 hr when the agents were withdrawn. This last observation was supported by the studies of Yu and Terriere (1973), who found that microsomal reductase activity could be increased by phenobarbital (1% in the diet) or by combinations of PB and dieldrin or juvenile hormone. The treatments also increased the tolerance of the flies to propoxur, but this disappeared in 48 to 72 hr after treatment ended.

Ecdysone was also shown to induce microsomal reductase activity in the fat body of the last instar of L. migratoria (Feyereisen and Durst, 1980b). Microgram quantities of the hormone increased the reductase activity 1.5-fold, four hours after being injected into the hemocoel. These authors postulated from their two studies (Feyereisen and Durst, 1980a,b) that the hormone was responsible for

the genetic control of this and associated PSMO activity in this tissue.

Moldenke et al. (1983) studied the effects of various monoterpenes found in mint upon the microsomal PSMO system in the variegated cutworm, a pest of mint. Reductase activity in midgut microsomes from sixth instar larvae was unaffected by doses ranging from 0.01 to 0.10% of the compounds in the diet. However, reductase activity in midgut microsomes made from the carcass (midsection of the worm, less the midgut) of these insects was induced by higher doses in the range tested.

Although our understanding of insect P-450 reductase has been advanced by the foregoing reports, many problems still remain to be solved, some of which are the subject of this dissertation. There is a need to develop a reliable method for preparing the enzyme from insect tissues so that it can be used in PSMO reconstitution experiments. These experiments will gather knowledge of the function of the various isozymes of insect P-450 that is inaccessible by other methods. These data will also provide insight into the interaction of P-450 with other membrane enzyme systems (Terriere, 1979).

More work is needed to understand the biochemical properties of insect P-450 reductase. As is evident in Table I-1, the biochemical data within one species are widely variable, and there is little information comparing the properties of the enzyme among other insect species. The confusion on the biochemistry of insect

reductase will subside as more data are made available for comparison.

Data concerning the development of the reductase in insects are scarce, and there has been no effort to compare its levels in R and S strains. Also, more information on induction of reductase activity would help to understand its characteristics in R and S strains, as well as its expression relative to P-450 and associated activities. Considering the current interest in purifying and characterizing insect P-450 isozymes (Terriere and Yu, 1979; Yu and Terriere, 1979; Naquira et al., 1980; Moldenke et al., in press) such data would be useful to determine optimum conditions for purifying the reductase.

Table I-1. Purification of insect NADPH-cytochrome P-450 (c) reductase prior to 1980.

	Wilson and Hodgson 1979a,b	Mayer and Prough 1977	Mayer and Durrant 1979	Crankshaw et al. 1979a,b
Insect Tissue	house fly abdomen	whole house flies	whole house flies	southern armyworm midgut
Method Solubilization	iso-butanol	lipase/ protease	detergent	protease
Physical Properties				
MW	57,000	65-70,000	83,000	70,000
cofactors	FAD, FMN	1 mol ea FAD, FMN	1 mol ea. FAD, FMN	1 mol ea. FAD, FMN
Biochemical Properties				
optimum pH	ca.8	7.8	---	ca. 8
optimum temperature	ca. 40°C	---	---	ca. 36°C
ionic strength	no effect	no effect	---	no effect
K_{NADPH}	0.2 µM	23.5 µM	5.0 µM	46.6 µM
$K_{cyt.\ c}$	15.0 µM	16.6 µM	12.7µM	32.1 µM
mechanism	Bi Bi ping-pong	Bi Bi ping-pong	Bi Bi ping-pong	Bi Bi ping-pong
Ki_{NADP^+}**	13.0 µM	14.0 µM	6.9 µM	15.0 µM
$Ki_{2'\text{-}AMP}$*	---	640 µM	187 µM	201 µM
PSMO Reconstitution	---	---	yes**	---

* $NADP^+$ and 2'AMP are competitive inhibitors.
** Reduced P-450 - carbon monoxide complex.

NADPH-Cytochrome P-450 Reductase from the House Fly, *Musca domestica*. Improved Methods for Purification, and Reconstitution of Aldrin Epoxidase Activity.

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Short title: House Fly P-450 Reductase.
Oregon Agricultural Experiment Station technical paper number 6734.
Accepted for publication in *Insect Biochemistry*, April 15, 1983.

Abstract

NADPH-cytochrome P-450 reductase was purified from microsomes prepared from adult phenobarbital-induced house fly abdomens. The reductase was purified 360-fold from a detergent solubilizate of these microsomes by affinity chromatography techniques. The homogeneous reductase had an apparent molecular weight of 74,000, as judged by sodium dodecyl sulphate-polyacrylamide gel electrophoresis. Its specific activity ranged from 32 to 40 µmol cytochrome c reduced/min/mg protein, and the enzyme was active in reducing house fly cytochrome P-450 in a reconstituted aldrin epoxidase assay. An important factor in the purification procedure was the inhibition of membrane-bound proteases by chymostatin and leupeptin. Attempts to purify the reductase in the absence of these inhibitors yielded a proteolytically processed form of the reductase that was active with artificial substrates, but did not function with cytochrome P-450 in a reconstituted MFO assay.

Key words: NADPH-cytochrome P-450 reductase, Musca domestica, phenobarbital-induction, reconstitution, aldrin epoxidase, MFO, protease inhibitors, affinity chromatography, cytochrome P-450.

Introduction

NADPH-cytochrome P-450 reductase (EC.1.6.2.4) is an important component of the microsomal monooxygenase system (mixed function oxidase: MFO). In a variety of higher organisms, this membrane-bound flavoprotein transfers reducing equivalents from NADPH to cytochrome P-450 for the oxidative metabolism of hormones as well as drugs and other xenobiotics.

The reductase has been purified and characterized in mammals (Masters and Okita, 1980), and has been used in reconstitution with cytochrome P-450 and phospholipid to determine the substrate specificity of mammalian MFO systems (Lu and West, 1978). Characterization and use of purified reductase from insect tissues has been difficult to achieve. Early efforts to purify the flavoprotein from insects employed solvents (Wilson and Hodgson, 1971) or proteases (Mayer and Prough, 1977; Crankshaw, et al., 1979) to solubilize the reductase from microsomes. Similar procedures used on mammalian tissues caused a processed form of the reductase that reduced artificial electron receptors (eg. cytochrome c, dichlorophenol-indophenol), but would not associate with the membrane and reduce cytochrome P-450 (Lu, et. al., 1969; Coon et al., 1973). Solubilization of insect microsomes with detergents yielded an intact reductase capable of reducing cytochrome P-450 (Capdevila et al., 1975; Mayer and Durrant, 1979). However, only Mayer and Durrant (1979) obtained a homogeneous reductase. Their enzyme had a molecular weight 83,000 (larger than any purified from

mammalian tissues) and was active in reducing the cytochrome P-450 : carbon monoxide complex. Nevertheless, they did not demonstrate a mono-oxygenase activity with their enzyme.

We have reexamined the procedures of Mayer and Durrant (1979) for obtaining NADPH-cytochrome P-450 reductase from the house fly (Musca domestica, L.). This report presents improvements which result in the production of a purified enzyme of molecular weight 74,000 which is active in reducing house fly aldrin epoxidase.

Materials and Methods

Chemicals. Biobeads SM2 were from Bio-Rad Laboratories, Richmond, CA. Emulgen 913 was a gift from KAO-Atlas Co., Ltd., Tokyo, Japan. Ultrogel AcA 44 was purchased from LKB Instruments, Inc., Rockville, MD. 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate (CHAPS) was from Pierce Chemical Co., Rockford, IL. AGNADP-Type IV, AGNADP-Type II and AG2',5'-ADP-Type II were obtained from P-L Biochemicals, Milwaukee, WI. Bovine serum albumin, chymostatin, DEAE-Sepharose CL-6B, leupeptin, Lubrol PX, molecular weight standard mixture, and sodium cholate were all purchased from Sigma Chemical Co., St Louis, MO. Phenylmethanesulphonyl fluoride (PMSF), agarose-cytochrome c were from U. S. Biochemicals, Cleveland, OH. Rabbit liver and rat liver NADPH-Cytochrome P-450 reductase were the kind gifts of Dr. Minor J. Coon (University of Michigan, Ann Arbor, MI, USA), and Dr. Henry W. Strobel (University of Texas, Houston, TX, USA), respectively. All other reagents were of the highest

purity commercially available. Buffers were filtered through a type HA, 0.45 micron Millipore^{R} filter prior to use.

Maintenance of Insects. Adult, diazinon-resistant house flies (Rutgers strain) possessing high levels of mono-oxygenase system enzymes (Yu and Terriere, 1977) were used for these experiments. The photoperiod was 16 hr L:8 hr D, and the diet consisted of a dry mixture of instant powdered milk, granulated sugar, and powdered egg yolk (12:12:1, by volume) with water ad libitum.

Preparation of Reductase. 2 days following adult eclosion, sodium phenobarbital (0.1%) was added to the drinking water, and at 5 days the dose was increased to 0.25%. 7- to 8-day old induced flies were fasted for 5 to 8 hr and females were induced to oviposit. The flies were then removed from the cage and anaesthetized with carbon dioxide. 4 to 5 g of flies were put into pint jars, and after the flies had recovered from the effects of the gas, they were frozen (-16°C) overnight (16 hr).

The frozen flies were broken apart by briskly shaking the jars for 15-20 sec. Abdomens of 10-12 g of flies were put into tubes containing 20 ml of cold grinding buffer (50 mM Tris-HCl, pH 7.7, 150 mM KCl, 1 mM EDTA) and held on ice (0-4°C) until homogenization. 10 µl of a freshly prepared stock of PMSF, (200 mg/ml ethylene glycol monomethyl ether) were added to each tube and the contents homogenized for 20 to 30 sec in a Polytron homogenizer (Brinkman Instruments) operated at low power.

The crude homogenate was filtered through four layers of cheesecloth and centrifuged for 15 min at 10,000 g (5°C). The supernatant was decanted through glasswool and then recentrifuged at 105,000 g for 70 min (5°C). The pellets were resuspended in grinding buffer (with PMSF, 0.4 mM) to the original volume of homogenate. The resuspension was centrifuged at 12,000 g for 10 min (5°C). The washed microsomes were finally sedimented at 105,000 x g for 50 min (5°C). The pellets were suspended in Tris resuspension buffer (TRB; 50 mM Tris-HCl, pH 7.7, 20% (v/v) glycerol, 1 mM EDTA, 0.1 mM dithiothreitol) with fresh PMSF (0.4 mM) to 15-25 mg protein/ml and frozen (-16°C) until further use.

Frozen microsomes (500 mg microsomal protein) were thawed and diluted to 5 mg microsomal protein/ml with cold TRB. Proteinase inhibitors were then added to the microsomes to give the following concentrations: PMSF, 8 mg%; chymostatin and leupeptin, 2.5 mg%. (Chymostatin was dissolved in dimethyl sulphoxide, and leupeptin hemisulphate was dissolved in TRB. These solutions were made fresh daily.) CHAPS in cold TRB was added dropwise to give 1.25 mg CHAPS/mg microsomal protein and the mixture was stirred for 10 min. Protamine sulphate in TRB was then added slowly to the solution to an endpoint of 0.04 mg protamine sulphate/mg microsomal protein. The resultant cloudy suspension was stirred 5 min and then centrifuged at 105,000 g for 50 min (5°C).

The following procedures were performed at 10°C. Emulgen 913 (10% (v/v) in TRB) was added to the supernatant to give 0.4 mg Emulgen 913/mg microsomal protein and the mixture was then pumped

onto twin columns (2.4 cm i.d. x 3 cm) of DEAE-Sepharose CL-6B at 0.8 ml/min/column. The columns were previously equilibrated with D-1 buffer (TRB containing 0.1% Emulgen 913 and 0.4 mM PMSF). After loading the sample, the columns were washed with D-1 buffer until a baseline on the protein monitor ($A_{280-310}$) was observed (usually 40-80 ml). The columns were subsequently washed with D-1 buffer containing 0.08 M KCl, and the reductase was then eluted from the columns by addition of D-1 containing 0.4 M KCl.

Fractions high in reductase activity were pooled, 0.5 mg each of chymostatin and leupeptin were added, and the pool was concentrated to 6 ml in an Amicon concentrator (PM-10 membrane), then dialyzed for 1 hr against not less than 100 vol of 10 mM KPO_4 pH 7.7, 20% (v/v) glycerol, 1 mM EDTA, 0.1 mM dithiothreitol, 0.1% (v/v) Emulgen 913, 0.01% NaN_3, and 0.4 mM PMSF (A-1 buffer). The concentrate-dialyzate was partitioned into 2, 3 ml fractions and frozen (-16°C).

Each 3 ml fraction was thawed individually and applied to a 1 cm i.d. x 19 cm column of AGNADP-Type IV equilibrated with A-1 buffer. The column was subsequently washed with buffer A-1 until a baseline was recorded on the monitor (usually 20-30 ml). Four ml of 1.5 mM $NADP^+$ in buffer A-1 were applied to desorb the bound reductase. Flow rate was maintained at 0.4 ml/min during the loading and washing of the column and increased to 0.8 ml/min when the $NADP^+$ was added. $NADP^+$ desorbed fractions high in reductase activity were retained and pooled with similar fractions from subsequent runs. After each run the column was equilibrated with

buffer A-1. (Prior to prolonged storage of the column, the buffer was exchanged to 0.1 M KPO_4, pH 7.7, 20% (v/v) glycerol, 1 mM EDTA, 0.1 mM dithiothreitol, 0.1% Emulgen 913, 0.01% NaN_3). Pooled reductase containing fractions were concentrated to 3 ml and applied to a 2.4 cm i.d. x 50 cm column of Ultrogel AcA 44 equilibrated with 150 mM Tris-HCl, pH 7.7, 10% (v/v) glycerol, 1 mM EDTA, 0.1 mM dithiothreitol, 0.1% (v/v) Emulgen 913, 0.01% NaN_3, and 0.4 mM PMSF. Flow rate was near 0.5 ml/min. Two peaks of reductase activity were generally observed eluting from this column. The first contained reductase of molecular weight near 74,000, and the second reductase had a molecular weight of approximately 64,000. The first peak was then diafiltered in the Amicon concentrator (PM-10 membrane) against TRB buffer, and applied to a 1 cm i.d. x 3 cm column of DEAE-Sepharose CL-6B equilibrated with D-1 buffer. The column was subsequently washed with 10-20 ml of TRB to remove the Emulgen 913 and the reductase was eluted from the column by TRB containing 0.5 M KCl. The desorbed reductase was concentrated to approximately 2 ml in the Amicon cell, excess FAD and FMN were added, and the mixture was diafiltered against at least 100 vol of 0.1 M $NaPO_4$, pH 7.5, 20% (v/v) glycerol, 1 mM EDTA, and 0.1 mM dithiothreitol. This final preparation contained homogeneous reductase, as judged by sodium dodecyl sulphate polyacrylamide gel electophoresis (SDS-PAGE), and was suitable for reconstitution experiments.

Preparation of Cytochrome P-450. Partially purified house fly cytochrome P-450 for reconstitution tests of the reductase was isolated from adult flies which had not been treated with sodium phenobarbital. At 7- to 8-days following emergence, adult flies were collected, anaesthetized with CO_2, and put into screen-covered jars. After recovering from the effects of the gas, the flies were then chilled. Abdomens were excised from the chilled flies, washed, and held in ice cold (0-4°C) buffer (0.1 M $NaPO_4$ pH 7.5, 10% (v/v) glycerol, 0.4 mM PMSF) until homogenization. Microsomes were prepared from these abdomens according to Yu and Terriere (1979) with modifications specified by Moldenke and Terriere (1981). Briefly, the abdomens were homogenized in a motor-driven teflon-glass tissue grinder. The crude homogenate was filtered through four layers of cheesecloth and centrifuged for 10 min at 1000 g (5°C). The supernatant was collected through a glasswool plug and centrifuged for 15 min at 10,000 g (5°C). Again the supernatant was collected through a glasswool plug and microsomes were sedimented by ultracentrifugation at 105,000 g (5°C) for 65 min. Pelleted microsomes were suspended to 4 mg protein/ml in phosphate resuspension buffer (PRB; 0.1 M KPO_4, pH 7.5, 20% (v/v) glycerol, 1 mM EDTA, 0.1 mM dithiothreitol) with fresh PMSF (0.4 mM).

Solubilization of the microsomes immediately followed their preparation. CHAPS was added dropwise to an endpoint of 1.25 mg CHAPS per mg microsomal protein. The solubilizate was stirred on ice for 30 min and then centrifuged 65 min at 105,000 g (5°C).

The supernatant was decanted through glasswool and held at 4-5°C overnight.

The following procedures were performed at 10°C. The supernatant was diluted 5-fold with buffer T (0.1 M KPO_4, pH 7.25, 20% (v/v) glycerol, 1 mM EDTA, 0.1 mM dithiothreitol) and loaded onto a 2.4 cm i.d. x 6 cm column of tryptamine-Sepharose equilibrated with buffer T, 0.1% CHAPS. (The tryptamine-Sepharose was a gift from Dr. D. E. Williams, Oregon State University, Corvallis, OR, USA; its synthesis is described in Williams and Buhler, 1982). The column was subsequently washed with equilibration buffer until a baseline was observed on the protein monitor. Two cytochrome P-450 peaks were eluted from the column. The first peak ($P\text{-}450_a$) was desorbed by buffer T containing 0.1% (w/v) CHAPS, 0.1% (w/v) Lubrol PX, and the second peak ($P\text{-}450_b$) eluted in Buffer T with 0.2% (w/v) sodium cholate and 0.5% (w/v) Lubrol PX. Detergents were removed from the two cytochrome fractions in a two-step process. Each fraction was dialyzed against three changes of 20 vol of PRB (1 hr for each change) and then treated with BioBeads SM2. Following detergent removal the cytochrome fractions typically contained 1.5 to 3.0 nmol cytochrome P-450/mg protein and were suitable for reconstitution experiments.

Reconstitution of Aldrin Epoxidase Activity. Partially-pure cytochrome P-450 and pure reductase were incubated with 5 µg of dioleoyl phosphatidylcholine at constant volume in 0.1 M $NaPO_4$ (pH 7.5) for 5 min at room temperature (20-25°C) (Coon, 1978). To this

mixture were added 30 µl of NADPH regenerating system (10 units/ml glucose-6- phosphate dyhydrogenase, 106 mM glucose-6-phosphate, 3.8 mM $NADP^+$) and buffer to 0.49 ml. The reaction was initiated with 10 µl aldrin (stock solution of 50 µm aldrin in ethylene glycol monomethyl ether), and incubated in a shaker bath at 34°C for 15 min. The reaction was terminated by adding 3 ml glass-distilled hexane, and product was measured according to Yu et al. (1971).

Analytical Procedures. Protein concentration was estimated by the method of Bradford (1976) with bovine serum albumin as standard. Cytochrome P-450 content was estimated at 20°C according to Omura and Sato (1962). NADPH-cytochrome c reductase activity was routinely measured at 30°C according to Williams and Kamin (1962) with reducing equivalents supplied by NADPH regenerating system (see preceding paragraph). One unit of activity is defined as 1 µmol cytochrome c reduced/min/mg protein. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis was performed according to Laemmli (1970).

Results and Discussion

The procedures described here result in the preparation of an homogeneous enzyme which reduces house fly cytochrome P-450. This is an essential requirement for the further study of the insect MFO system. Our methods represent improvements of the procedures of Mayer and Durrant (1979) in microsome preparation and

solubilization, prevention of enzyme degradation by the use of protease inhibitors, and reconstitution of MFO activity.

Microsomes containing 50-57 units (μmol cytochrome c reduced/min) of reductase activity and approximately 500 mg protein were obtained from the abdomens of 140 to 150 g of flies (Table II-1). Washing the microsomes reduced the amount of extraneous protein and thus enhanced the specific activity of the reductase. Freezer storage for up to 12 weeks caused only a small (5-10%) loss of protein and reductase activity. Furthermore, the use of abdomens, instead of whole flies as specified by Mayer and Durrant (1979), permitted greater recoveries of reductase per gram of tissue. This advantage was also noted by Wilson and Hodgson (1971) who determined that the use of abdomens constituted an initial purification step.

Control of the action of endogenous proteases was important for the recovery of the native form (intact; active in reducing cytochrome P-450) of the reductase. Mayer and Durrant (1979) and Guengerich et al. (1981) indicated that PMSF provided adequate control of proteolytic activity through all steps of their respective purification schemes for house fly and human liver reductases. Preliminary experiments in this laboratory proved PMSF to be effective against soluble protease during microsome preparation, but ineffective against membrane-bound proteolytic activity released during microsome solubilization. Further experiments indicated that the membrane-bound proteases were inhibited by a mixture of chymostatin and leupeptin (lyosomal protease inhibitors) which permitted greater recovery of native

reductase. These results corroborate the observations of Masters and Okita (1980) who found this class of protease inhibitor to be superior to PMSF during the purification of reductase from liver microsomes.

Although Mayer and Durrant (1979) used the nonionic detergent, Emulgen 913, for microsome solubilization, we found that the use of the zwitterionic detergent CHAPS (Hjelmeland 1980) resulted in less conversion of cytochrome P-450 to its enzymatically inactive counterpart, cytochrome P-420. Preliminary experiments indicated that the P-450 fractions generated during reductase purification may be a valuable source for further purification of insect P-450. Hence, protection of the P-450 enzymes, as well as the reductase, was considered important. Continued use of CHAPS beyond solubilization was unnecessary. Emulgen 913 had less tendency to inactivate soluble P-450, provided better separation of proteins during ion-exchange chromatography, and did not interfere with affinity chromatography. Recovery of reductase activity in the CHAPS solubilizate ranged from 110 to 115% (Table II-1). Elevated recoveries have previously been observed in detergent-solubilized preparations of house fly (Capdevila et al. 1975; Mayer and Durrant, 1979) and rat liver (Guengerich and Martin, 1981) microsomes. It is not yet clear whether this increase in activity is due to allosteric effects or to factors associated with membrane organization.

The use of protamine sulphate in the crude solubilizate proved advantageous in two respects. An appreciable decrease in total protein without loss of reductase activity was observed when

protamine sulphate was administered at the ratio specified in the procedure. Also, protamine sulphate precipitate sedimented faster and formed a firm pellet in the presence of 20% glycerol buffer. Similar advantages were noted in a procedure for purifying rat liver reductase (Dignam and Strobel, 1977).

The recovery of activity and fold-purification of the reductase through affinity chromatography approximated that obtained by Mayer and Durrant (1979) (Table II-1). However, a substantial loss (65%) occurred during gel filtration on Ultrogel AcA 44. In part, this is due to elimination of the inactive form (i.e. proteolytically processed, unable to reduce cytochrome P-450) of the reductase. Additional causes of this loss are unknown. Following this step the reductase was purified 360-fold, achieved its final level of activity (32-40 units/mg protein) and was distinctly yellow in colour.

Stepwise elution of adsorbed proteins on the first DEAE-sepharose column (Figure II-1) yielded results comparable to those of Mayer and Durrant (1979) who used gradient elution techniques. The reductase was readily adsorbed on this column, but eluted efficiently at the prescribed salt concentration.

The largest increase in purity (11-fold over the previous step) resulted from affinity chromatography on AGNADP-type IV gel (Figure II-2). Recovery of activity ranged from 80 to 100%. Other affinity adsorbants (eg. AG 2', 5'-ADP-Type II, AGNADP-type III, and agarose-cytochrome c) were tested, but did not bind the reductase. A key factor to the success of this chromatographic step was the pH

of the buffer. When the pH dropped to 7.5 or lower, the reductase was not retained on the column. Sample volume was of secondary importance on this column. 3 ml or less of sample seemed to optimize resolution; however, good results were obtained with sample volumes as large as 4.5 ml. Native reductase, as well as inactive reductase, bound to the affinity column. These were separated by gel permeation chromatography on Ultrogel AcA 44 (Figure II-3).

The quality of the reductase at various key points during the purification procedure was determined by SDS-PAGE (Figure II-4). Preliminary experiments indicated that proteolysis of the reductase was great in the eluate from the initial DEAE column unless chymostatin and leupeptin were present. Although these protease inhibitors were not lost during concentration procedures, their protective effect diminished during prolonged dialysis. Therefore, the time of dialysis did not exceed 1 to 2 hr. This is in contrast to the procedure of Mayer and Durrant (1979) who dialyzed their sample overnight.

An estimation of the molecular weight of house fly reductase included rat liver and rabbit liver reductases as internal standards (Figure II-5). House fly reductase (74,000) was smaller than both mammalian reductases (rat, 82,000; rabbit, 83,000). Previous estimates for reductase from the house fly (83,000, Mayer and Durrant (1979) and the southern armyworm (80,500, Crankshaw et al., 1981) exceed our estimate by six- to eight-thousand daltons. Molecular weight estimation by SDS-PAGE, although considered a valid

procedure (Dignam and Strobel, 1977; Guengerich et al., 1981), gives varying values when used by different laboratories. Size estimates for rat liver reductase vary from 74,000 daltons (Guengerich et al., 1981) to 79,500 (Dignam and Strobel, 1977). Since such great variability is apparent in the literature, it was considered important to include the mammalian reductases in this experiment at least to determine the relative size of the house fly reductase.

The function of the reconstituted aldrin epoxidase system was dependent upon the native reductase (Table II-2). Assay mixtures devoid of the native reductase or cytochrome P-450 showed no formation of product (dieldrin). Likewise, inclusion of the inactive reductase in place of the native reductase in the reconstitution showed no product formation. Increases in the reductase:P-450 ratio led to increases in epoxidation activity to a point where the system was saturated with reductase. Even so, optimum reconstituted activity was only 25% of the activity present in microsomes. Preliminary experiments indicated that removal of detergents, especially Emulgen 913, was essential to achieve reconstitution. This contrasts with the procedures of both Capdevila et al., (1975) and Mayer and Durrant (1979) who supplanted the phospholipid moeity with non-ionic detergents. It has been shown that detergents cause artifactual variability in reconstituted mammalian MFO systems (Lu and West, 1978). Apparently, the MFO activity is stimulated at low concentrations of detergent and inhibited at high concentrations. Since no precautions to determine detergent concentration were mentioned by Mayer and Durrant (1979),

their results were probably, in a strict sense, serendipitous. It is our opinion that the use of phospholipid provides more consistent results.

Our procedures can be employed to further examine the mono-oxygenase system in insects. In particular, substrate specifities of cytochrome P-450 enzymes can be studied and relationships between MFO system components can be evaluated. Also, using this procedure will make it possible to further evaluate the contributions of the MFO system in applied problems such as insecticide resistance.

Acknowledgements

The authors wish to thank Mrs. Seham Ali for her excellent technical assistance. Helpful advice of Dr. Rene Feyereisen and David E. Williams was greatly appreciated. This work was supported by USPHS grant no. ES 00362-23.

Fig. II-1. Ion-exchange chromatography of house fly NADPH-cytochrome P-450 reductase on DEAE-Sepharose. Procedures are described in text. Enzyme activity is μmol cytochrome c reduced/min/ml.

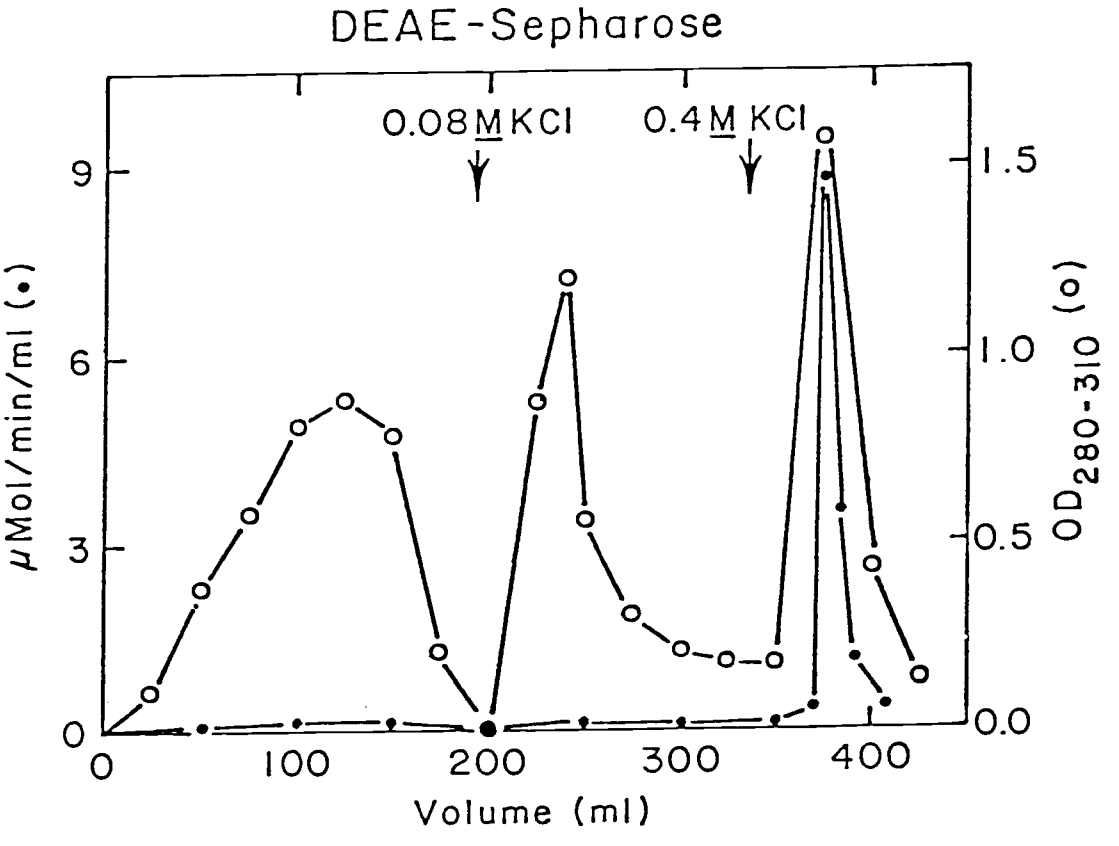
DEAE-Sepharose
0.08M KCl
0.4M KCl
μMol/min/ml (•)
OD280-310 (o)
Volume (ml)
0
3
6
9
0.0
0.5
1.0
1.5
0
100
200
300
400

Fig. II-1.

Fig. II-2. Affinity chromatography of DEAE-Sepharose eluate containing NADPH-cytochrome P-450 reductase activity on AGNADP-type IV. Procedures are described in text. Enzyme activity is μmol cytochrome c reduced/min/ml.

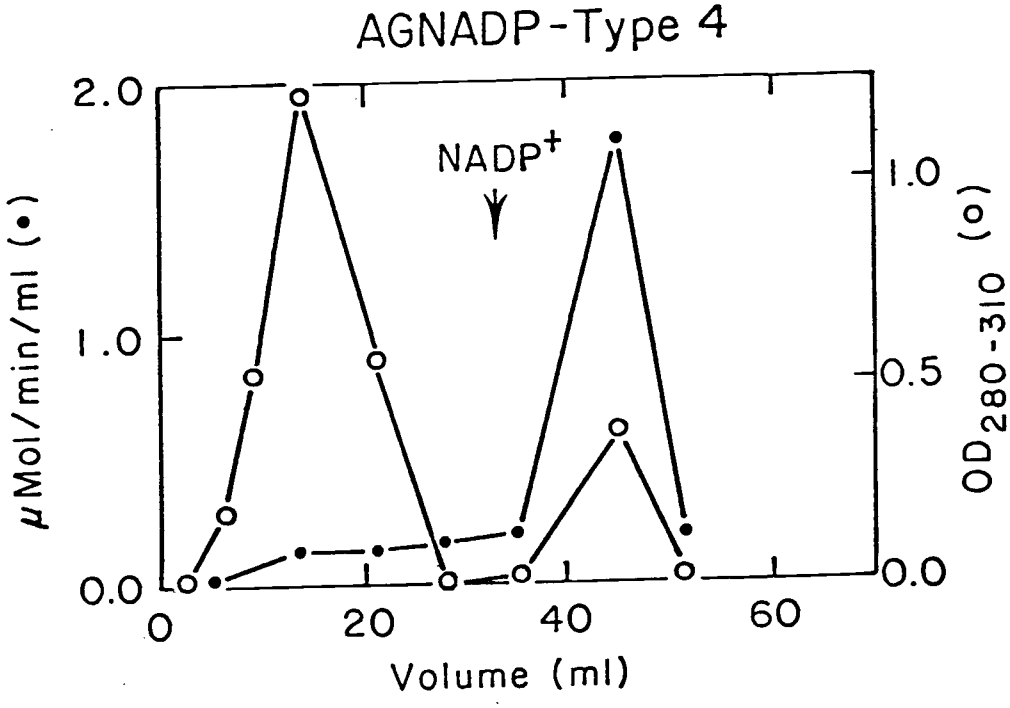
AGNADP-Type 4
NADP+
2.0
1.0
0.0
μMol/min/ml (•)
1.0
0.5
0.0
OD280-310 (o)
0
20
40
60
Volume (ml)

Fig. II-2.

Fig. II-3. Gel permeation chromatography of affinity eluate containing NADPH-cytochrome P-450 reductase activity on Ultrogel AcA 44. Procedures are outlined in text. Enzyme activity is μmol cytochrome c reduced/min/ml.

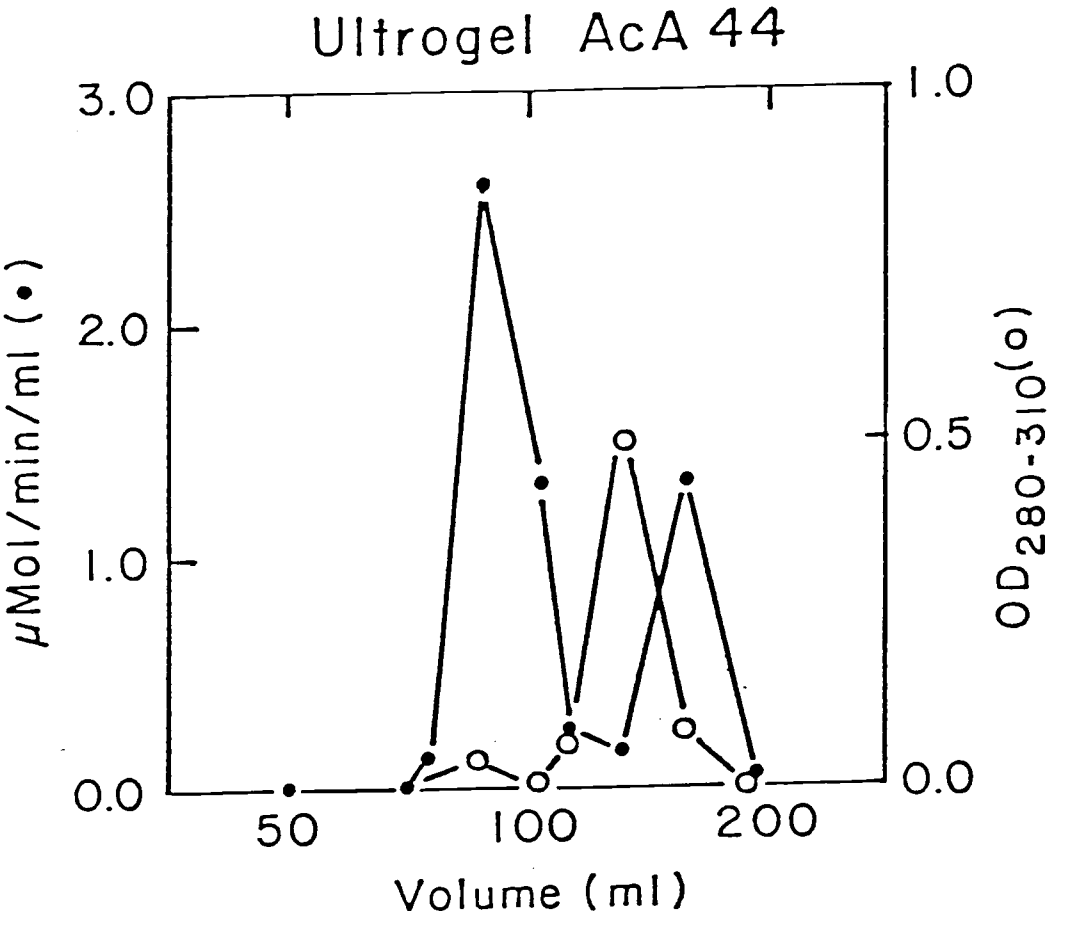
Ultrogel AcA 44
3.0
2.0
1.0
0.0
μMol/min/ml (•)
1.0
0.5
0.0
OD280-310(o)
50
100
200
Volume (ml)

Fig. II-3.

Fig. II-4. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (Laemmli, 1970) of fractions during purification of house fly NADPH-cytochrome P-450 reductase. Channels 1, 5 and 10 are protein standards. Channel 2, CHAPS-solubilized, protamine sulphate-treated microsomal supernatant; channel 3, DEAE-Sepharose column pool (0.4 M KCl fractions); channel 4, concentrate of DEAE eluate; channel 6, dialyzate of concentrated DEAE pool; channel 7, non-binding fractions from affinity column; channel 8, $NADP^+$ desorbed reductase from affinity column; channel 9, homogenous house fly cytochrome P-450 reductase. The gel was 12 cm in length and 7.5% acrylamide.

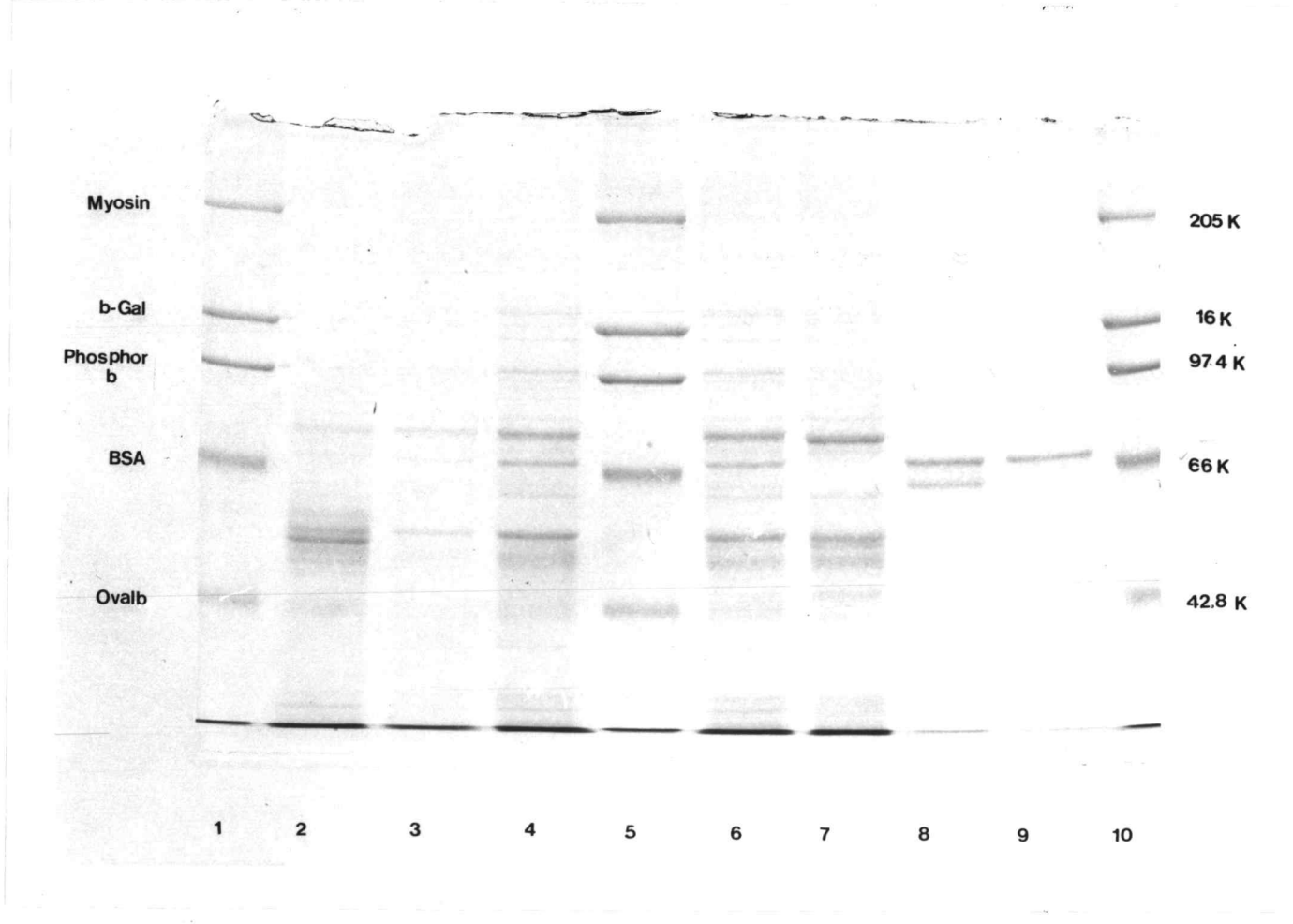
Myosin
b-Gal
Phosphor b
BSA
Ovalb
205 K
16 K
97.4 K
66 K
42.8 K
1
2
3
4
5
6
7
8
9
10

Fig. II-4.

Fig. II-5. Estimation of molecular weight of house fly NADPH-cytochrome P-450 reductase by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (Laemmli, 1970). Rat liver and rabbit liver reductases were included as internal standards. Proteins used to form standard curve were ovalbumin (42,500), bovine serum albumin (66,000), phosphorylase-b (97,500) and β-galactosidase (116,000). Gels were 7.5% acrylamide and 16 cm in length.

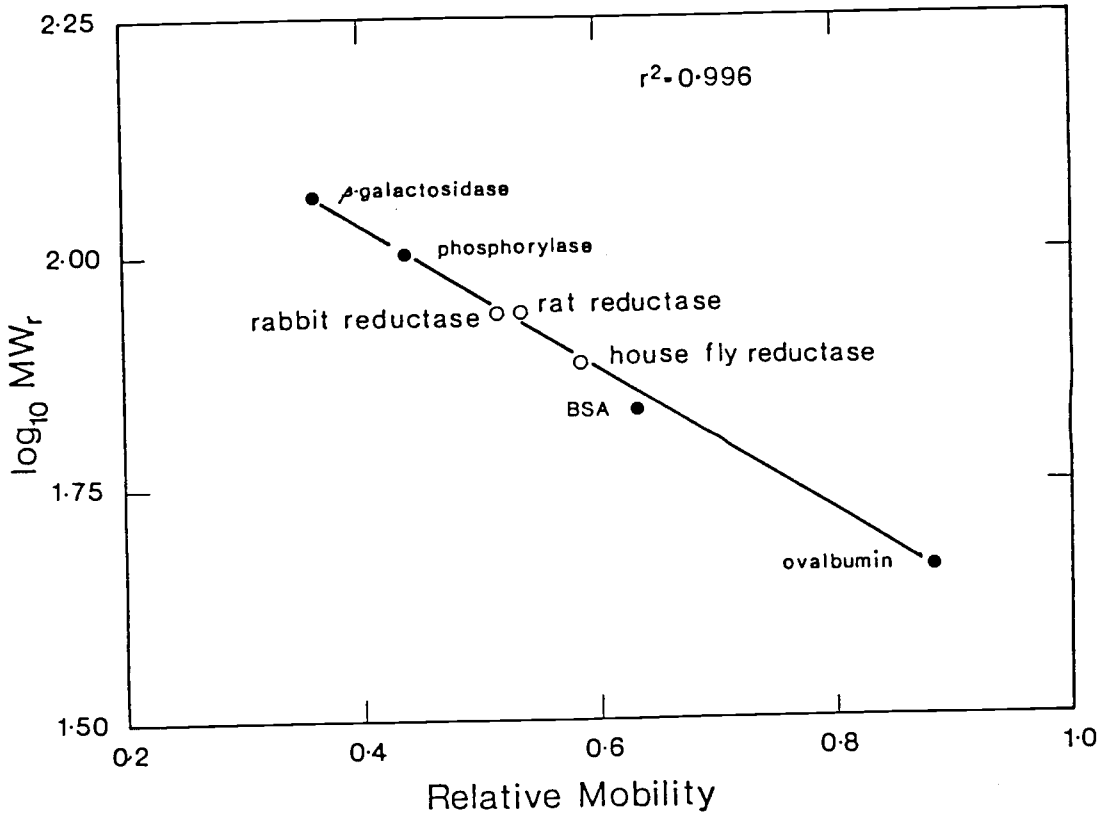
2·25
2·00
1·75
1·50
$\log_{10} MW_r$
r^2 - 0·996
β-galactosidase
phosphorylase
rabbit reductase
rat reductase
house fly reductase
BSA
ovalbumin
0·2
0·4
0·6
0·8
1·0
Relative Mobility

Fig. II-5

Table II-1. Purification of NADPH-cytochrome P-450 reductase from M. domestica

Preparation	Total Protein (mg)	Total Activity (μmol/min)	Cytochrome C Reduction (μmol/min/mg)	Enzyme Yield (% microsomes)	Fold Purification (microsomes)
Thawed microsomes	500.0	55	0.1	100	---
Solubilized microsomes	220.0	65	0.3	120	3
DEAE-Sepharose column eluate	40.0	55	1.4	100	14
AGNADP-Type 4 column eluate, concentrate	3.3	50	15.0	90	150
Utrogel AcA 44 column eluate, concentrate	0.4	14	36.0	25	360
DEAE-Sepharose column diafiltrate	0.3	14	36.0	25	360

Values are representative of a typical experiment. In four separate experiments the mean and standard error for final specific activity and final enzyme yield were respectively 36 ± 1.3 μmol cytochrome c reduced/min/mg, and 25 ± 3.4%.

Table II-2. Reconstitution of aldrin epoxidase activity with purified house fly NADPH-cytochrome P-450 reductase and partially purified house fly cytochrome P-450.

Components	pmol P-450a	Units Reductase	Aldrin Epoxidation[1] (pmol dieldrin/min/pmol P450)
1. Reductase (HMW)[2], P-450a[3]	14	0.25	0.33
2. Reductase (HMW), P-450a	14	0.5	0.37
3. Reductase (HMW)	0	0.5	0
4. P-450a	14	0	0
5. Reductase (LMW)[2] P-450a	14	1	0

[1]Reconstitution is described in "Materials and Methods" section.

[2]HMW, high molecular weight; LMW, low molecular weight.

[3]P-450a preparation is described in "Materials and Methods" section.

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Characterization of NADPH-Cytochrome P-450 Reductase from Susceptible and Resistant House Flies (*Musca domestica*, L.) and the Blow Fly (*Phormia regina*, Meigen)

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Abstract

NADPH-cytochrome c reductase was purified by affinity chromatographic techniques from microsomes prepared from the abdomens of insecticide-resistant (R) and -susceptible (S) house flies and of the black blow fly. Data are presented which describe (1) the ability of these purified enzymes to support an *in vitro* reconstitution of monooxygenase activity, (2) the changes in their activity observed in buffers of varying ionic strength, (3) comparative kinetic behavior between their microsomal and purified forms, (4) their immunochemical characteristics, and (5) their amino acid composition. The insect reductases were found to be very similar in all of these tests. Substrate binding constants were 5 μM for NADPH, 12 μM for cytochrome c, and the catalytic mechanism was interpreted as ordered Bi Bi. The inhibitory constant of the resistant fly for 2'-AMP, an analogue of $NADP^+$, was 187 μM, whereas no association of the inhibitor was observed below concentrations of 400 μM for either the susceptible house fly or the blow fly. However, the data are insufficient to suggest that the reductase is a significant factor in insecticide resistance. Compared to the same enzymes from rat and rabbit liver, the insect reductases show a different ionic strength optimum (0.14), have distinct antigenic determinants, and have different levels of acidic and basic amino acids in the membrane-binding peptide.

Key Word Index: *Musca domestica*, *Phormia regina*, NADPH-cytochrome c reductase, reconstituted monooxygenase activity, kinetics, immunology, amino acid composition, membrane-binding peptide.

Introduction

NADPH-cytochrome c reductase (EC.1.6.2.4) has a pivotal role in the microsomal polysubstrate monooxygenase system in which both natural and synthetic lipophilic substrates are oxidatively metabolized by insects and other organisms. We recently reported the purification of this enzyme (hereafter P-450 reductase) from house fly microsomes and its use in reconstituting aldrin epoxidation activity with partially purified house fly cytochrome P-450 and synthetic phospholipid (Vincent et al., in press).

Most of the information concerning the properties of this reductase has come from studies involving partially degraded forms (cleaved reductase) which retain catalytic action for artificial substrates such as cytochrome c or dichlorophenol indophenol, but do not support P-450-dependent monooxygenations (Wilson and Hodgson, 1971; Mayer and Prough, 1977; Crankshaw et al., 1979). Such forms lack a peptide (6,000 to 10,000 daltons) that is responsible for membrane interactions (Gum and Strobel, 1981). Mayer and Durrant (1979) succeeded in preparing the P-450-active enzyme, but their report of its characteristics did not provide kinetic data which Strobel et al. (1980) have stressed are necessary for an understanding of its catalytic mechanism.

The present communication describes the biochemical, kinetic and immunochemical characterization of P-450 reductase isolated from M. domestica and P. regina. The insect enzymes are compared, as appropriate, to the analogous enzymes from rat and rabbit liver.

Materials and Methods

Reagents. Reagents and their sources were as follows: 2'-adenosine monophosphate (2'-AMP), cytochrome c (type III), dioleoylphosphatidyl choline, flavin adenine dinucleotide (FAD), and flavin mononucleotide (FMN, grade I), Sigma Chemical Co., St. Louis, MO; agarose immunodiffusion tablets, Bio-Rad Laboratories, Richmond, CA; 3-[(3-cholamidopropyl)dimethylammonio]-1- propanesulphonate (CHAPS), Pierce Chemical Co., Rockford, IL; Crowles double stain, Polysciences Inc., Warrington, PA; 7-methoxy-4-methylcoumarin and phenylmethylsulphonyl fluoride (PMSF), Calbiochem, La Jolla, CA; 7-hydroxy-4-methylcoumarin, Aldrich Chemical Co., Milwaukee, WI; and phenobarbital (USP grade), J. T. Baker Chemical Co., Phillipsburg, NJ. Sources of reagents for the purification of NADPH-cytochrome P-450 reductase from house fly microsomes are given elsewhere (Vincent et al., in press). P-450 reductase from rat and rabbit liver were the kind gifts of Drs. Henry W. Strobel (University of Texas, Houston, TX, USA), and Minor J. Coon (University of Michigan, Ann Arbor, MI, USA). All other reagents were of the highest quality commercially available. Buffers were filtered (Millipore^{r}, type HA, 0.45 μm) prior to use.

Insects. The insects used in these experiments were from colonies continuously maintained at our laboratory. Rutgers is a multi-resistant (R) strain of M. domestica and has been shown to have a high level of PSMO activity, whereas NAIDM is a susceptible (S) strain and possesses low PSMO activity (Moldenke and Terriere,

1981). P. regina is susceptible to insecticides and has a low complement of P-450 and associated monooxygenase activities (Terriere and Yu, 1979). However, these three flies have comparable constitutive levels of microsomal P-450 reductase (Vincent et al., ms. in prep.). The colonies were maintained as described elsewhere (house flies, Moldenke and Terriere, 1981; blow fly, Terriere and Yu, 1979). Flies used in the preparation of purified P-450 reductase were treated with phenobarbital in the drinking water in order to induce the enzyme (Vincent et al., in press). Reductase in microsomes of phenobarbital-treated house flies was identical kinetically to that in control microsomes (Table III-2).

Enzyme preparation. Microsomes used in these experiments were prepared from abdomens of live female flies by the method prescribed for reductase preparation in Vincent et al. (in press). The washed microsomes were resuspended in 0.05 M TRIS-HCl (pH = 7.7), 20% (v:v) glycerol, 1.0 mM EDTA, 0.2 mM PMSF (buffer A) and frozen in aliquots (-16°C). Prior to analyses, the thawed microsomes were diluted with ice cold 0.05 M TRIS-HCl buffer (pH = 7.7) and centrifuged (5°C) for 65 min at 105,000 g to ensure that the reductase used in the subsequent assays was membrane-bound. The microsomal pellet was then resuspended in buffer A and held on ice for the duration of the assays (6 to 10 hr).

P-450 reductase was prepared from fly microsomes by methods described by Vincent et al. (in press), and stored frozen (-70°C). Thawed enzyme was mixed with excess FMN and FAD, diafiltered (Amicon^R cell, PM-10 membrane), oxidized with potassium

ferricyanide and diafiltered against 0.05 M potassium phosphate buffer (pH = 7.7) (final dialysis gradient after ferricyanide addition was greater than 30,000 to 1). The reductase from all three diptera contained essentially equimolar amounts of FAD and FMN at a ratio of 2 mol flavin per mol enzyme. The ratios were based upon relative molecular weights of 74,000 for M. domestica (both strains) and 75,000 for P. regina which were determined by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The NAIDM reductase was particularly labile; greater than 50% of the P-450 reductase (74,000 daltons) had converted adventitiously to the partially degraded form (68,000 daltons) following freezer storage for 1-2 months. Therefore, NAIDM P-450 reductase was used immediately following its purification.

Reconstitution of PSMO activity. Purified P-450 reductase was tested for its ability to support the O-demethylation of 7-methoxy-4-methylcoumarin (Feyereisen and Vincent, in press) by partially purified house fly cytochrome P-450-2 (Moldenke et al., in press). The cytochrome and reductase were preincubated (25°C) with 5 µg dioleoyl phosphatidylcholine (total vol = 0.20 ml) for at least 1 min, and then diluted to 0.93 ml with 0.1 M sodium phosphate buffer (pH = 7.5). 60 µl of NADPH- regenerating system (10 units/ml glucose-6-phosphate dehydrogenase, 106 mM glucose-6-phosphate, 3.5 mM $NADP^+$) were added and the mixture was brought to 30°C. 10 µl of substrate (stock of 10.0 mM 7-methoxy-4-methylcoumarin in 100% ethanol) were added and the reaction was followed in a Perkin-Elmer 650-105 spectrofluorometer thermostated to 30°C. Wavelengths (and

slit) settings were: excitation at 360 nm (3 nm slit), emission at 460 nm (5 nm slit). The nanomolar extinction coefficient for the product, 7-hydroxy-4-methylcoumarin, was 0.78/cm at 30°C. This activity was inhibited by either carbon monoxide or antibodies raised against the house fly P-450 reductase (Feyereisen and Vincent, in press), indicating the participation of P-450 and P-450 reductase in this reaction.

Kinetic analyses. Kinetic behavior of the reductase was assayed at 30° ± 0.1°C employing cytochrome c as substrate (Williams and Kamin, 1962). The quantity of enzyme used was within the limits of linearity for the reaction and was held constant for each experiment.

In assays to determine the effect of pH upon reductase activity, the reaction buffer was supplanted by either 0.05 M potassium phosphate (pH 5.00 to pH 7.75) or TRIS-HCl (pH = 7.00 to pH = 10.0), both of which had been adjusted to equivalent ionic strength by the addition of KCl. Reductase was tested for its stability to pH by diluting stock solutions into the various buffers and incubating the dilutions for 15 min at 30°C. 20 µl of each of these incubates were then assayed at pH = 7.70 which preliminary experiments had shown to be the pH of optimum activity. The effect of ionic strength upon reductase activity was determined by replacing the reaction buffer with 0.05 M TRIS-HCl, pH = 7.7, and adjusting the ionic strength with KCl. Reductase activity was then assayed as previously described. NADPH in the kinetic experiments

was also supplied by the regenerating system and the concentration was verified spectrophotometrically ($E_{340\ nm}$ = 6.2/mM/cm).

Immunology. Double immunodiffusion analysis (Ouchterlony, 1962) employed 0.2% CHAPS in the agarose gel to facilitate protein movement. The antibody had been generated in rabbits against Rutgers P-450 reductase and partially purified from the serum by ammonium sulphate precipitation (Feyereisen and Vincent, in press). After 48 hr of incubation, the gels were rinsed several times with distilled water and stained (Crowles double stain) to visualize precipitin lines.

Amino acid analyses. The amino acid composition of fly reductase was determined from acid hydrolysates (20 hr and 70 hr) by the method of Spackman et al. (1958). The residues were color developed with a ninhydrin/DMSO/lithium acetate reagent and resolved on a single 6 mm column (Dionex DC-6A resin) in a Beckman 120B Amino Acid Analyzer. Quantitation of cysteine as cysteic acid was by the procedure of Spencer and Wold (1969). Tryptophan was determined by the UV-absorbance method of Edelhoch (1967). Both P-450 reductase and cleaved reductase from each fly were analyzed thereby providing the composition of the two major domains of the enzyme, the hydrophobic domain (or membrane-binding peptide), and the hydrophilic (catalytic) domain (Black and Coon, 1982).

Analytical methods. Protein was measured by the protein-dye technique of Bradford (1976) with bovine serum albumin used as standard. Cytochrome P-450 was quantified at 20°C in an Aminco

DW-2a spectrophotometer by the method of Omura and Sato (1964). Relative molecular weight (M_r) determination was by SDS-PAGE using a discontinuous buffer system (Laemmli, 1970). Analyses of individual flavins were by the fluorometric method of Faeder and Siegel (1973). A molar extinction coefficient of 1.07×10^4/ cm at 454 nm was used to quantitate flavin in the reductase (Iyanagi and Mason, 1973).

Results and Discussion

Proof that the product resulting from our isolation and purification procedures is indeed P-450 reductase is seen in the reconstitution experiments (Table III-1). Both house fly enzymes were similar in their behavior in the reconstituted system; however, the blow fly reductase saturated the system at a much lower reductase:P-450 ratio. Although these data do not give a clear reason for the apparent increased efficacy of the blow fly reductase in this system, the difference in character (e.g. size, amino acid composition) of the membrane-binding peptide of the P. regina enzyme is the most likely explanation.

The absolute spectra (visible region) of fully oxidized house fly and blow fly P-450 reductases were found to be nearly identical (Fig. III-1). Absorbance maxima were at 380 nm and 450 nm and the upper value displayed a shoulder at 484 nm. These values are typical of this oxidized flavoprotein regardless of its source, or of its molecular weight (Kamin et al., 1965; Crankshaw et al., 1979; Mayer and Durrant, 1979; Oprian and Coon, 1982).

Data for the temperature dependence of M. domestica and P. regina reductase activity were similar to those already published for cleaved reductase from the house fly (Wilson and Hodgson, 1971) and the southern armyworm (Crankshaw et al., 1979). Arrhenius plots [10^3/T (°K) vs log (activity)] for the dipteran reductases were linear (10°C to 35°C) and permitted estimates of activation energies of 43 kJ/mol for P. regina, 41 kJ/mol for NAIDM, and 48 kJ/mol for Rutgers (plots not shown). These values were in agreement with values obtained for microsomal reductase, and were within the range of values observed for armyworm (31.65 kJ/mol) and rat liver (60.16 kJ/mol) reductases obtained from bromelain-treated microsomes by Crankshaw et al. (1979).

Optimum activity was observed for microsomal and purified fly reductases when the reaction was between pH 7.7 and 8.5 (data not shown). The purified enzymes were stable to incubation in buffers ranging from pH 6.0 to 10.0, while in microsomes the reductase was stable from pH 4.5 to 11. These data indicate that the enzyme itself is resistant to pH changes, and that the membrane acts to prevent reductase damage in adverse acid-base conditions.

Ionic strength was found to have a profound, but equivalent, influence upon the activity of M. domestica and P. regina P-450 reductases (Fig. III-2). Activity was maximum in dilute buffer (/2 = 0.12) and decreased by 40% as the ionic strength reached 0.8. This result disagrees with earlier studies of this characteristic based on degraded forms of the reductase from insect tissues, in which the enzyme was found to be indifferent to changes in ionic

strength (Wilson and Hodgson, 1971; Mayer and Prough, 1977; Crankshaw et al., 1979). However, our results support the suggestion of Crankshaw that this characteristic distinguishes between insect reductase and its mammalian liver counterpart for which maximum activity is observed at $\Gamma/2 = 0.8$ (Phillips and Langdon, 1962; Yosukochi and Masters, 1976).

Representative plots of the kinetic data are shown for the microsomal (Fig. III-3) and purified (Fig. III-4) forms of P-450 reductase. Lineweaver-Burke plots of the data for microsomal reductase activity yielded a series of parallel lines each with a break in the slope near 10 μM cytochrome c. The parallel lines are characteristic of an uncompetitive association of some compound with the enzyme-NADPH complex, and could be interpreted in a two substrate enzyme as representing a Bi Bi ping-pong catalytic mechanism (Cleland, 1970). This mechanism is one in which the reductase is first reduced by NADPH and then, following the release of NADP, reduces the cytochrome substrate. Under these conditions, the change in slope evident in the lines would mean that two enzymes were involved in the reaction.

When kinetic experiments were completed for the purified reductases (Fig. III-4), the lines of the kinetic plot converged on the x-axis and did not change in slope. This plot indicated that the catalytic mechanism was ordered Bi Bi (Cleland, 1970) and that there was only one enzyme involved. Furthermore, it prompted a reconsideration of the assumption made in the microsomal experiments, that the interaction of reductase with P-450 was not

appreciable at cytochrome c concentrations exceeding 1 μM. An alternative explanation of this situation is that the change in slope at 10 μM is where the reductase-P-450 interaction (Km = 0.9 μM, Strobel et al., 1980) is overcome by cytochrome c. Why the lines in the range of 20 to 50 μM cytochrome c remain parallel in the microsomal plot is not understood.

As mentioned, the convergent lines evident in Fig. III-4 are consistent with an ordered Bi Bi mechanism of electron transfer (eg. where both NADPH and cytochrome are bound to reductase before electron transfer is effectuated). However, Mayer and Durrant (1979) reported that their preparation of house fly reductase exhibited Bi Bi ping-pong kinetics. Their interpretation supported the earlier studies of cleaved insect reductase solubilized by either proteases (Mayer and Prough 1977; Crankshaw et al., 1979) or iso-butanol (Wilson and Hodgson, 1971). Both kinetic mechanisms have been proposed from studies of mammalian liver P-450 reductase. Strobel (Dignam and Strobel, 1977; Strobel et al., 1980) has suggested that the ordered Bi Bi mechanism is more likely on the grounds that $NADP^+$ was a competitive inhibitor and that other workers (Yasukochi and Masters, 1976) had not controlled their assays for optimum activity factors (primarily ionic strength and temperature). The same argument can be extended to the disagreement concerning the kinetic mechanism of insect P-450 reductase. Mayer and Durrant (1979) assayed the kinetic behavior of their reductase preparation at the same ionic strength as ours, but at a lower temperature (25°C). An increase in activity of 50% would have been

realized in their assays by raising the temperature to 30°C. This difference might account for the disparity between our observations and theirs by making differences in the slopes of kinetic plots more apparent. However, such a comparison of results is difficult because kinetic data are not shown by Mayer and Durrant (1979). This matter should be clarified as more kinetic data are collected for P-450 reductase from other organisms.

The binding constants for NADPH and cytochrome c were comparable for the microsomal and purified forms of the reductase from M. domestica and P. regina (Table III-2). Turnover numbers for cytochrome c were also similar, but there was an upward trend correlated with pesticide susceptibility. These values are in agreement with those of Mayer and Durrant (1979) and compare favorably with values for liver reductase (Strobel et al., 1980).

The effect of 2'-AMP (an analogue of $NADP^+$) upon the reductase was determined in duplicate tests. Only the R-fly enzyme was inhibited at the 2-AMP concentrations tested (competitive, Ki = 187 μM). This value is identical to that obtained by Mayer and Durrant (1979) for R-fly reductase, and is the only evidence collected so far that the P-450 reductase differs between R and S strains of M. domestica. It is very much larger than the value for liver reductase (Yosukochi and Masters, 1976).

Double immunodiffusion analysis (Ouchterlony, 1962) using rabbit anti-house fly antibodies indicated a high degree of homology between antigenic sites in insect proteins, and a low degree of homology between insect and mammalian liver P-450 reductases (Fig.

III-5). No precipitin lines were observed when antigens were challenged with preimmune rabbit serum. The antibody preparation we used was able to discriminate between the high and low molecular weight forms of the Rutgers reductase indicating that the membrane-binding peptide of the protein was itself antigenic (Feyereisen and Vincent, in press).

The amino acid compositions of the dipteran reductases are presented in Table III-3. Substantial differences between R and S strains of M. domestica are in arginine (Δ14), histidine (Δ8), isoleucine (Δ5), and methionine (Δ4) residues. Between M. domestica and P. regina reductases substantial differences are evident in alanine (average of Δ4), cysteine (average of Δ8), methionine (average of Δ8), and lysine (average of Δ7). Caution must be exercised in emphasizing the differences in cysteine, methionine, or histidine because of their relatively low frequencies in the three proteins. Analysis of the data by the method of Cornish-Bowden (1983) yielded an SΔn value of 50 for the test between any of the three insect proteins. A value of 500 was calculated from the comparison of the insect reductases with either the rat (Knapp et al., 1977; Williams et al., 1983) or the rabbit (Black et al., 1979) enzymes. For proteins of this size, a strong case of relatedness is indicated by SΔn values below 284, a weak case is between 284 and 670, and no case for values exceeding 670. These data lend support to the conclusions drawn from the immunochemical comparison(s), that the insect proteins are closely related in their structure,

whereas the insect and mammalian liver enzymes are distantly related.

Relative amino acid composition for the membrane-binding peptide was calculated from the difference between compositons of intact and degraded forms of the insect reductases. These were compared to calculated values for rat and rabbit liver peptides (Table III-4). Percentages of acidic and hydrophobic residues differ between Rutgers and NAIDM, whereas blow fly reductase contains more acidic and less basic character than the house fly proteins. The mammalian peptides differ substantially from the fly proteins, the former having considerably higher percentages of acidic residues and considerably lower percentages of basic residues. The data suggest that this part of the molecule is widely variable between insects and mammals. Due to catalytic, cofactor, and protein solubility requirements, one would expect many sequences in the catalytic domain to be highly conserved; and indeed, this appears to be the case, for the domain has a very comparable molecular weight from species to species and amino acid composition is very similar, at least between insects and mammals (Crankshaw et al., 1981). On the other hand, the membrane-binding peptide would need to satisfy variable requirements regarding lipid and protein interactions from species to species. It follows that this region would be subject to more variability in its composition.

It is apparent from these studies that the P-450 reductase of the house fly and the blow fly are closely related in activity and structure, whereas the insect and mammalian reductases are distantly

related in structure but closely related in function. The reductase from R house flies is similar to that of S house flies in terms of molecular weight, amino acid composition, operating parameters (pH, ionic strength, kinetic mechanism, and constants), and in its concentration in the microsomal membranes (Vincent et al., ms. in prep.), but is more susceptible to the inhibitor 2'-AMP. More information is needed to determine if this difference is connected in any way with the high microsomal oxidase activity of the Rutgers strain and its high level of resistance to insecticides. Major differences between insect and rat or rabbit reductases include the effect of ionic strength upon activity, 2'-AMP inhibition, antigenic determinants, and relative amino acid composition in the membrane-binding peptide.

Acknowledgements

The authors thank Melissa Vincent for the maintenance of insects, Drs. Robert R. Becker and Tom J. Bailey for amino acid analyses, Dr. Alison F. Moldenke for providing the soluble P-450-2, and Dan E. Farnsworth and Seham Ali for their technical assistance. This work was supported by USPHS Grant No. ES 00362-25.

Fig. III-1. Absolute spectra of oxidized NADPH-cytochrome P-450 reductase from P. regina and M. domestica (NAIDM strain). Enzymes were prepared as described in Materials and Methods. The spectra for the Rutgers and NAIDM strains were identical.

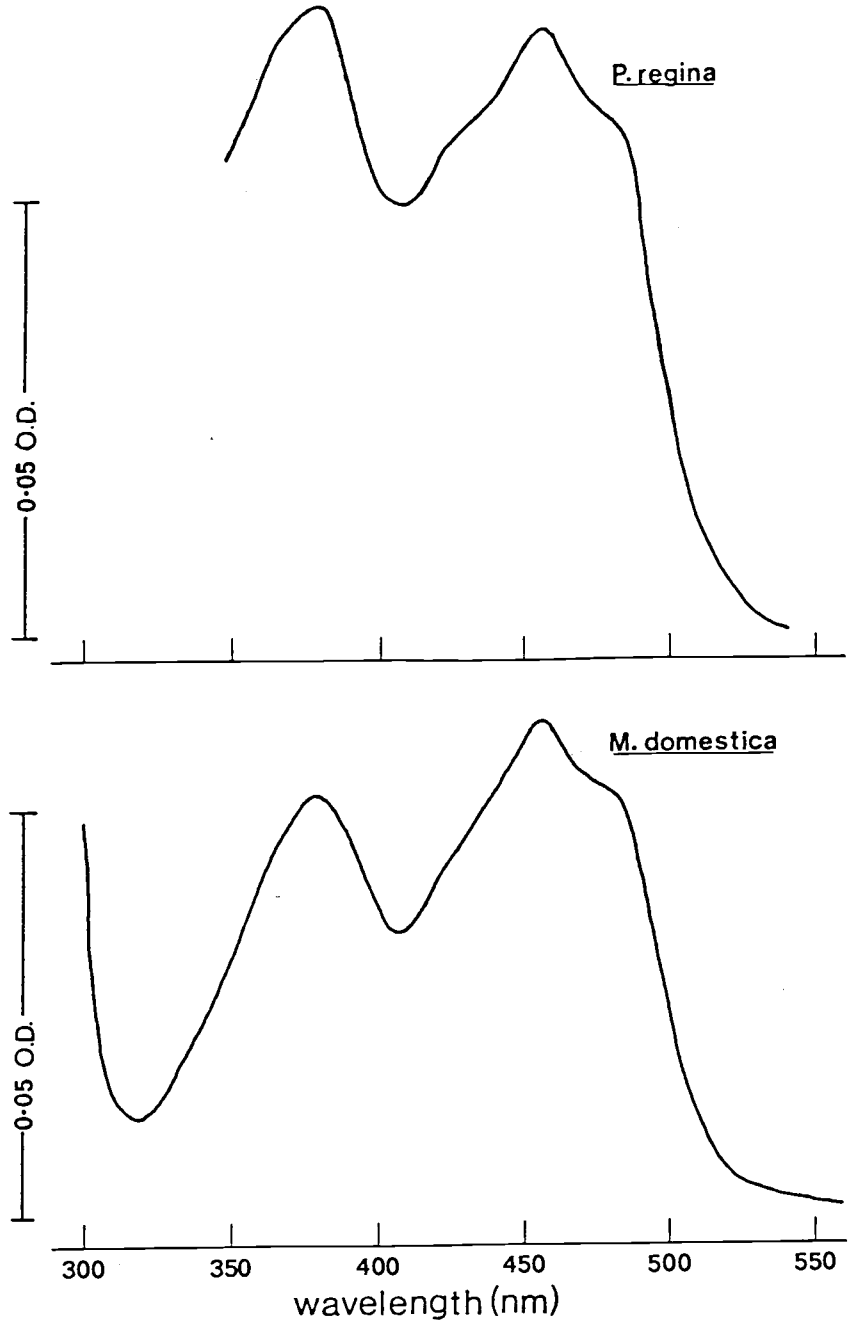
P. regina
0·05 O.D.
M. domestica
0·05 O.D.
300
350
400
450
500
550
wavelength (nm)

Fig. III-1.

Fig. III-2. The effect of ionic strength upon the activity of NADPH-cytochrome P-450 reductase purified from *P. regina*. Activity in arbitrary units based on change in A_{550}/min at 30°C. Identical results obtained for both strains of *M. domestica*.

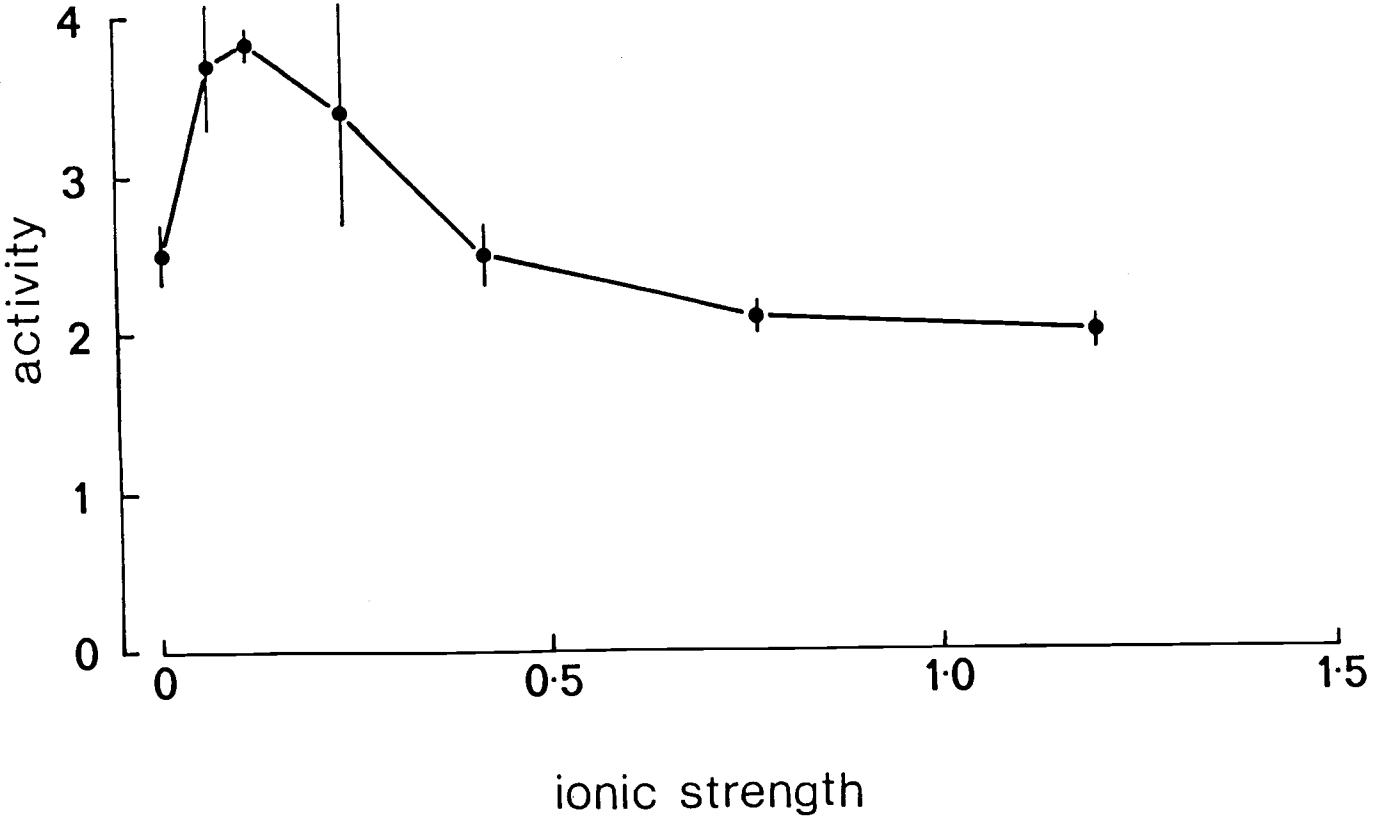

Fig. III-2.

Fig. III-3. Double-reciprocal plots of initial velocity (ΔA_{550}/min, 30°C) *vs* cytochrome *c* (μM) for microsomal NADPH-cytochrome P-450 reductase from Rutgers strain of *M. domestica*. Similar results were observed for NAIDM strain and *P. regina*.

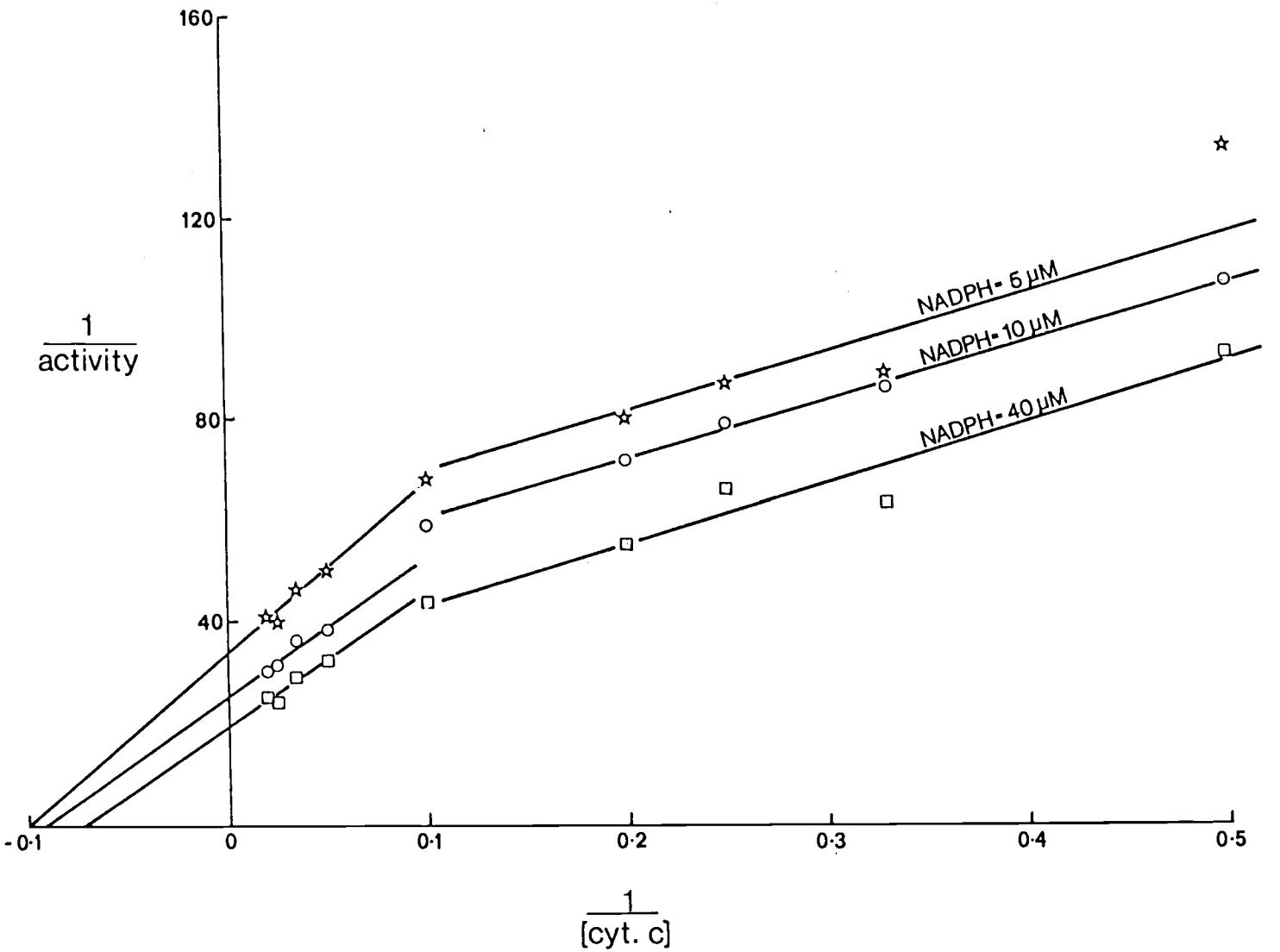
160
120
80
40
1
activity
NADPH- 5 µM
NADPH- 10 µM
NADPH- 40 µM
-0·1
0
0·1
0·2
0·3
0·4
0·5
1
[cyt. c]

Fig. III-3.

Fig. III-4. Double-reciprocal plots of initial velocity (ΔA_{550}/min, 30°C) *vs* cytochrome *c* (μM) for purified NADPH-cytochrome P-450 reductase from Rutgers strain of *M. domestica*. Similar results were observed for NAIDM strain and *P. regina*.

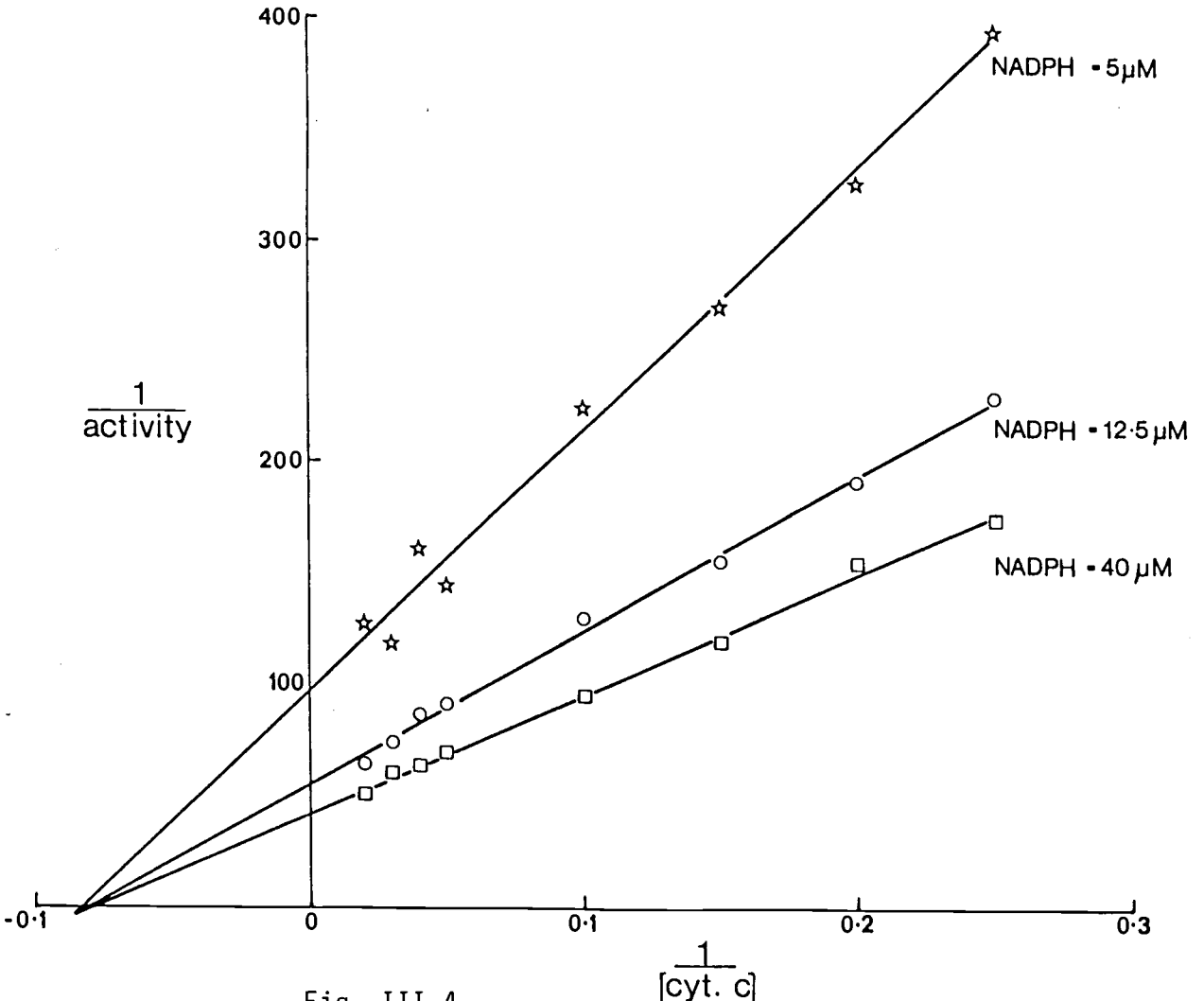
400
300
200
100
1
activity
NADPH - 5μM
NADPH - 12·5μM
NADPH - 40μM
-0·1
0
0·1
0·2
0·3
1
[cyt. c]

Fig. III-4.

Fig. III-5. Double immunodiffusion analysis (Ouchterlony, 1962) of purified NADPH-cytochrome P-450 reductase from M. domestica, P. regina, rat and rabbit using antibodies raised to Rutgers NADPH-cytochrome P-450 reductase. Insects and procedures are described in Materials and Methods.

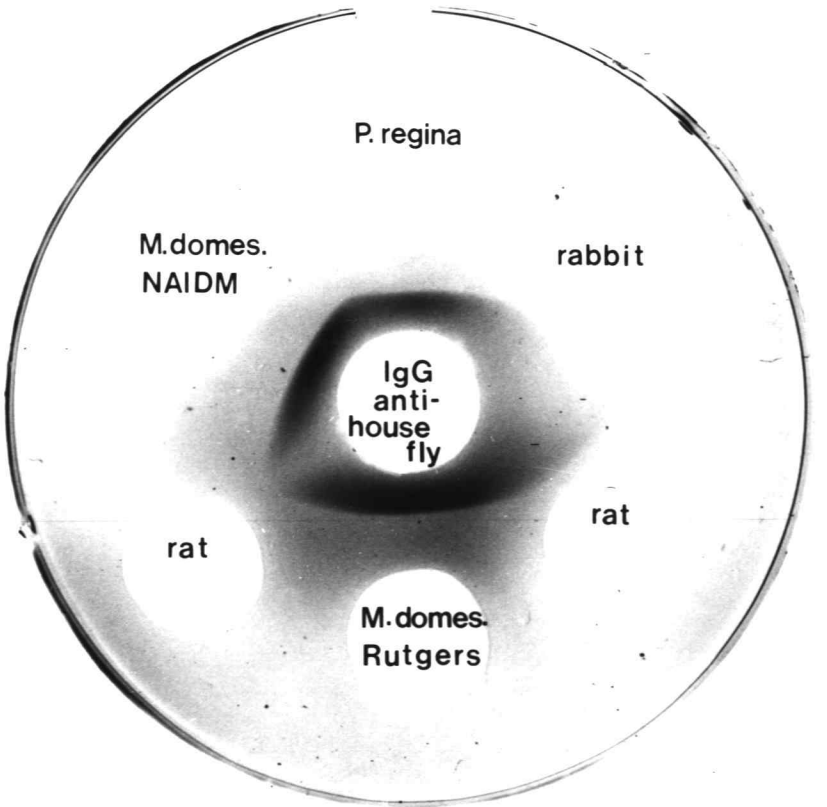
P. regina
M.domes.
NAIDM
rabbit
IgG
anti-
house
fly
rat
rat
M.domes.
Rutgers

Fig. III-5.

Table III-1. Reconstitution of O-dealkylation activity using NADPH-cytochrome P-450 reductases purified from *M. domestica* and *P. regina*.

Species	Units Reductase	Activity*
M. domestica		
Rutgers	0.00	0.00
	0.14	0.56
	0.28	1.26
NAIDM	0.00	0.00
	0.12	0.40
	0.24	1.01
	0.48	1.23
P. regina	0.00	0.00
	0.13	1.23
	0.25	1.20

* pmol 7-hydroxy-4-methylcoumarin/min/pmol P-450. Values represent single assays using 2 pmol Rutgers P-450-2 as described in text.

Table III-2. Kinetic parameters for microsomal and purified NADPH-cytochrome P-450 (c) reductases of M. domestica and P. regina.

Enzyme source	K_{NADPH} (μM)	$K_{cyt.\ c}$ (μM)	Turnover number μmol cyt. c reduced / min/μmol enzyme	K_i 2'-AMP[a] (μM)
M. domestica				
Rutgers strain				
microsomal	4.4 ± 0.9[b]	12.1 ± 1.3	--	--
purified	8.0 ± 1.3	12.2 ± 0.7	2575 ± 75	187
NAIDM strain				
microsomal	3.7 ± 0.3	11.6 ± 0.2	--	--
purified	5.3 ± 2.0	18.1 ± 1.9	3032 ± 382	No effect
P. regina				
microsomal	5.7 ± 0.6	14.2 ± 1.1	--	--
purified	6.7	16.2	3300	No effect

[a]2'-AMP was a competitive inhibitor for Rutgers. Levels of inhibitor as high as 400 micromolar had no observable effect upon either NAIDM or P. regina reductases.

[b]Values where mean and standard error of the mean are shown are from 2 or more experiments. Values without S.E.M. displayed are from single experiments.

Table III-3. Amino acid composition of NADPH-cytochrome P-450 reductase from *M. domestica* and *P. regina*.

Residue[a]	*M. domestica* Rutgers[b]	*M. domestica* NAIDM[b]	*P. regina*[c]
Asx	67	65	68
Thr	38	35	42
Ser	38	37	36
Glx	73	74	76
Pro	28	28	32
Gly	42	43	45
Ala	36	38	41
Cys	11	8	2
Val	40	43	40
Met	5	9	15
Ile	39	44	38
Leu	53	56	58
Tyr	27	23	24
Phe	33	28	27
His	14	22	14
Lys	43	46	52
Arg	50	36	42
Trp	10	10	9
	648	645	661

[a]Tryptophan determined spectrophotometrically. Other residues determined from acid hydrolyzates (see Materials and Methods).

[b]Based on M_r of 74,000.

[c]Based on M_r of 75,000.

Table III-4. Relative amino acid composition of the membrane-binding peptide of NADPH-cytochrome P-450 reductase from M. domestica and P. regina compared to values published for rat and rabbit.

Species	Amino acid composition (% of residues)[a]			
	Acidic	Neutral	Hydrophobic	Basic
M. domestica				
Rutgers[a]	16	10	48	26
NAIDM	9	9	60	22
P. regina	26	13	43	18
Rat[c]	37	18	37	8
Rabbit[d]	36	15	42	7

[a]Acidic residues were Asp, Thr, Ser, and Glu; Neutral residues were Pro and Gly; Hydrophobic residues were Ala, Val, Ile, Leu, Tyr, Phe, and Trp; Basic residues were His, Lys, and Arg.

[b]Insect values calculated from difference between intact and degraded forms of the reductase.

[c]Calculated from Gum and Strobel, 1981.

[d]Calculated from Black et al., 1979.

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Cytochrome P-450 in Insects. 6. Age Dependency and Phenobarbital Induction of Cytochrome P-450, P-450 Reductase and Monooxygenase Activities in Susceptible and Resistant Strains of *Musca domestica*, L.

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Abstract

Development and phenobarbital (PB) induction of microsomal cytochrome P-450, P-450 reductase, and two epoxidation and two O-demethylation activities were examined in chronologically timed populations of insecticide-susceptible (NAIDM) and -resistant (Rutgers) house flies. Measurements of these enzymes started with the pharate adult stage and ended 5 days following eclosion. Untreated insects of both strains had comparable reductase levels, whereas P-450 and associated monooxygenase activities were 1.5-fold or more higher in Rutgers. Maximum induction, as well as toxicity, occurred at a lower PB concentration in NAIDM than Rutgers. The drug caused consistently higher increases in enzymes and activities within 12 hr of starting treatment in both strains. When PB was withdrawn from treated flies (both strains) 48 hr after treatment began, specific activities (product/min/mg protein) in all enzymes returned to control values in 24 hr while metabolic capacity (product/min/insect) achieved control values within 48 hr. The changes in turnover numbers (pmol product/min/pmol P-450), in conjuction with the differences in the monooxygenation of the four substrates, suggest that PB treatment induced both a quantitative and qualitative change in NAIDM monooxygenation but only a quantitative change in Rutgers monooxygenation.

Introduction

The phenomenon of induction of the microsomal polysubstrate monooxygenases (PSMO) in insects by exogenous chemicals has been of considerable interest to biochemists, toxicologists, and pest managers. The metabolic response of these enzymes to environmental factors has been of value in understanding enzyme regulation and metabolic capability, and has relevance to the understanding of insect-host plant interactions (Brattsten, 1979; Terriere, 1984).

Aspects of this phenomenon which have been examined in some detail include the increase or decrease in insecticide toxicity due to induction (Agosin et al., 1969; Fuhremann and Lichtenstein, 1972; Yu and Terriere, 1973; Berry et al., 1980), the nature of the chemicals producing the response (Moldenke et al., 1983; Brattsten and Wilkinson, 1973), and the comparison of enzyme levels resulting from different inducers (Perry et al., 1971; Yu and Terriere, 1972; Terriere and Yu, 1974). Little attention has been given to the role of age in induction, the rate of enhancement of enzyme levels during induction, the rate that enzyme levels return to normal when the inducer is withdrawn, and to whether induction of the PSMO system differs between insecticide-susceptible (S) and -resistant (R) insects. Furthermore, most studies have been preoccupied with the response of cytochrome P-450, resulting in little information on the effect of inducers on cytochrome P-450 reductase, an important component of the system.

In this article, we report the results of our studies of the developmental patterns of this system before, during, and after

exposure to an inducing agent, phenobarbital, in adult house flies. In addition to the patterns for the two key enzymes, cytochrome P-450 and its reductase, we also followed the activities of this system in two epoxidation and two O-dealkylation reactions.

Materials and Methods

Reagents. The chemicals used in this study were: phenobarbital (PB), J.T. Baker Chemical Co., Phillipsburg, NJ; phenylmethylsulfonyl fluoride (PMSF) and 7-methoxy-4-methylcoumarin, Calbiochem., La Jolla, CA; 1-phenyl-2-thiourea (PTU), Eastman Chemicals, Rochester, NY; aldrin and dielderin, Shell Biosciences Laboratories, Sittingbourne, England; heptachlor and heptachlor epoxide, Chem Services, West Chester, PA; methoxyresorufin and resorufin, Molecular Probes, Inc., Junction City, OR; and 7-hydroxy-4-methylcoumarin, Aldrich Chemical Co., Milwaukee, WI. All other materials were of the highest purity commercially available. Buffers were passed through a Millipore^{R} type HA, 0.45 micron filter prior to use.

Insects. House flies and their maintenance were as described by Moldenke and Terriere (1981). NAIDM is a susceptible strain whose level of P-450 is about 1/2 that of Rutgers, a multi-resistant strain. A 3 hr emergence window established the chronological age of the populations. Female flies were induced to oviposit beginning on day 3 of the adult stage which reduced the contamination of microsomes by non-specific reproductive protein(s) (Moldenke et al.,

in press). PB was administered in distilled water beginning 24 hr following eclosion, when flies were observed to be actively feeding. The inducer solution was refreshed after 48 hr in the extended time course experiment (Figs. IV-2, IV-3). In these experiments the flies were also water-starved for 2 hr prior to dosing.

Microsome Preparation. Fifty female flies from each group were anaesthetized with carbon dioxide. After recovering, they were chilled (0-4°C), and their abdomens were excized with a razor blade and placed immediately into a Potter grinding vessel containing 20 ml of ice cold buffer (0.1 M sodium phosphate, pH = 7.5, 10% (v:v) glycerol) to which 0.1 ml PTU (10^{-1} M in ethyleneglycol monomethylether) and 0.01 ml PMSF (1.15 M in ethyleneglycol monomethylether) had been added. PTU blocked the action of tyrosinase in pharate and newly emerged adult flies (Terriere et al., 1980). PMSF inhibited the action of digestive proteases that destroy microsomal NADPH-cytochrome P-450 reductase (Vincent et al., in press). The contents were ground for 15 to 20 sec and filtered through cheesecloth. 5 ml of buffer were used to rinse the grinder and were poured through the cheesecloth into the homogenate. The homogenate was centrifuged at 5°C (10 min at 2,900 g, 10 min at 10,000 g, and 60 min at 105,000 g) and resuspended to about 4 ml in ice cold 0.1 potassium phosphate buffer (pH = 7.5) containing 20% (v:v) glycerol, 1.0 mM ethylenediamine tetraacetic acid, 0.1 mM dithiothreitol and 0.4 mM PMSF. All assays were usually completed within 2 hr of microsome resuspension, or 4 hr from the initiation of the experiment.

Analytical Procedures. Protein was determined by the method of Bradford (1976) using bovine serum albumin as standard. Cytochrome-P-450 content was estimated according to Omura and Sato (1964) at 20°C in a Aminco DW-2a recording spectrophotometer. NADPH-cytochrome c reductase activity was measured in duplicate at 30°C by the method of Williams and Kamin (1962) except that cytochrome c was 50 μM. One unit of reductase activity was defined to be one nmol cytochrome c reduced/min under the defined conditions. Aldrin or heptachlor epoxidation (AE or HE, respectively) was assayed in duplicate at 30°C by the method of Moldenke and Terriere (1981) as modified by Feyereisen (1983). Single assays of the O-dealkylation of 7-methoxy-4-methylcoumarin (MMCOD) (Feyereisen and Vincent, in press) or methoxyresorufin (MROD) (Mayer et al., 1977) were followed in a Perkin-Elmer 650-105 spectrofluorometer thermostated to 30°C. The reactions were initiated by the addition of substrate. Wavelength and slit settings were: 360 nm, 3 nm slit (excitation), 460 nm, 5nm slit (emission) for 7-hydroxy-4-methylcoumarin, and 560 nm, 3 nm slit (excitation), 580 nm, 5 nm slit (emission) for resorufin. These activities were inhibited by both carbon monoxide and antibodies raised against house fly P-450 reductase (Feyereisen and Vincent, in press) indicating the participation of cytochrome P-450 and P-450 reductase in the reactions. Reducing equivalents were supplied by NADPH-regenerating system (10 units/ml glucose-6-phosphate dehydrogenase, 106 mM glucose-6-phosphate, 3.8 mM $NADP^+$).

Results

Dose response. The six enzymes responded in parallel to induction, with median response concentrations of 0.10% PB for NAIDM and 0.15% PB for Rutgers (Fig. IV-1). NAIDM was more sensitive to the inducer, reaching maximum levels of induction, as well as exhibiting more mortality at a lower dose than Rutgers (mortality data not shown). The reason for this is not clear, but it seems likely that Rutgers, which is known to posses a more active PSMO system (Figs. IV-2, IV-3), was able to metabolize and/or excrete the PB at a faster rate. We have no explanation for the dip in response by the NAIDM strain at the 0.20% concentration.

Maximum induction was observed at 0.15% PB for NAIDM and 0.25% PB for Rutgers (Fig. IV-1, Table IV-1). P-450 content, HE, and MROD remained substantially higher in Rutgers than NAIDM, while reductase, AE, and MMCOD achieved nearly identical levels in the two strains. The data also indicate that HE increased to a greater extent than did AE: 23-fold vs 18-fold (NAIDM), 6-fold vs 3-fold (Rutgers). Although specific activities (product/mg protein) were not as high for MROD in either strain, this activity had a greater percent gain upon induction than did MMCOD (11-fold vs 7-fold in NAIDM, 5-fold vs 1.5-fold in Rutgers). Finally, increases in monooxygenase activities were more marked than were increases in structural enzymes (P-450, reductase), especially in the NAIDM strain, which is a common observation in studies of insect PSMO induction (reviewed by Terriere, 1984).

Age-dependent changes in constitutive PSMO activity. P-450 in both strains was at basal levels in pharate adults and increased to a rather stable value near 48 hr following eclosion (Figs. IV-2, IV-3). The specific content (nmol/mg) of this protein in the R-fly was nearly twice the level of the S-fly. Specific activity of reductase showed a similar pattern of development with an apparent peak in activity at 42 hr for NAIDM and 48 hr for Rutgers. This activity had comparable levels in these two strains throughout the developmental period monitored. Microsomal P-420 content [P-420/(P-420 + P-450)] averaged 20% in NAIDM and 15% in Rutgers in all of the experiments in this study.

Monooxygenation activities in NAIDM fluctuated little during the course of the experiment, except for a slight increase in HE at day 5. This was not the case in Rutgers, however, where large decreases in both AE and HE occurred between days 1 and 2. MMCOD also exhibited a slight decrease in that period, but MROD did not. These activities in the R-fly also increased greatly in the first 24 hr following eclosion and, except for the deviations already noted, fluctuated little from that value throughout the remainder of the experiment.

Activity based on the number of abdomens was calculated from these data and found to compare favorably with published developmental curves for house flies (Khan, 1970; Perry and Buckner, 1970; Hansen and Hodgson, 1971; Capdevila et al., 1973; Yu and Terriere, 1974). Also, it was evident that developmental curves were similar for both Rutgers and NAIDM during the period monitored

indicating that chronological age was reliable for making comparisons of this system in strains of the same species. This disagrees with the theory extended by Feyereisen (1983) that physiological age is the only reliable reference parameter for M. domestica.

Rates of PSMO induction and decay. Median response concentrations of PB were used in this part of the study because comparisons would be more appropriate, responses would be easily monitored, and mortality would be avoided. This regimen caused significant induction in the NAIDM PSMO system within 12 hr. Induction was apparent within 12 hr for all enzymes in Rutgers, except for reductase and MMROD which occurred between 12 and 18 hr following PB-treatment. No induction was evident at day 4 in either strain, but this trend was reversed on day 5 in all enzymes, except reductase and MROD in NAIDM, and reductase, MMCOD, and MROD in Rutgers. Maximum induction of structural enzymes and O-demethylation activities was apparent 24 hr following the start of PB-treatment, and epoxidation achieved maximum 12 hr later in both strains. A major difference between the strains was observed in enzyme turnover numbers (pmol product/min/pmol P-450) which were increased by PB treatment in NAIDM (Table IV-2), but not in Rutgers (Table IV-3).

When the inducer was removed 48 hr after treatment started, specific activities were at control levels within 24 hr (day 4). However, when the data were transformed to a per-insect basis, the enzyme activities in this group remained at maximum levels longer,

nearing control values within 48 hr (day 5). This same trend was evident in the turnover numbers for the NAIDM strain (Table IV-2).

Effect of induction on microsomal protein content. Expression of enzyme activities as a function of microsomal protein corrected for size differences between individuals, and indicated the status of the enzymes of interest relative to other microsomal protein(s). Protein content in untreated flies was near 2 mg/50 abdomens from days 0 through 2 and then increased on day 3 to near twice that value (Figs. IV-2, IV-3). This increase helped to explain decreases in constitutive enzyme levels that occurred at this age. The influx of protein was presumed to be related to physiological changes caused by vitellogenesis, a process that begins 48 to 72 hr after eclosion (Dillwith et al., 1981). Furthermore, egg-related protein has been thought to have a dilution effect upon microsomal PSMO enzyme activity (Moldenke et al., in press).

The protein levels of control and PB-treated house flies were comparable for both strains. When PB was withdrawn from a portion of the treated insects at day 3 (after 48 hr exposure to the drug) protein content rose ca. 30% on day 4. However, protein in this group returned to control on day 5. This fluctuation in protein content, which explained the rapid decline in enzyme specific activity occurring on day 4, may have been due to a release of the effects of PB treatment upon egg maturation (Yu and Terriere, 1974).

Discussion

NADPH-cytochrome P-450 reductase has a pivotal function in the PSMO system. However, little information is available on the development or induction of this key enzyme in S or R insects. Our results show that constitutive levels of reductase are comparable for Rutgers and NAIDM (Figs. IV-2, IV-3), which supports the theory that differences in PSMO activity between R and S strains are attributable to isozymes of cytochrome P-450 (reviewed by Hodgson, 1983). The control of reductase activity appears to be interfaced with the expression of P-450, as evidenced by its parallel response to PB concentration (Fig. IV-1) and its similar time frame of induction (Figs. IV-2, IV-3). Futhermore, maximum concentrations of inducer stimulated the reductase 2-fold in both strains while monooxygenase activities averaged much larger increases, especially in the NAIDM strain. These data suggest that reductase is abundant in the microsomes and does not have a primary regulatory function in the control of PSMO activity in either strain.

Induction of the PSMO system by PB occurs in a matter of hours in both Rutgers and NAIDM. The present data confirm the observations of Walker and Terriere (1970) which indicated a significant induction of heptachlor epoxidation 12 hr after applying µg quantities of dieldrin to various house fly strains. Feyereisen and Durst (1980) showed that significant induction of a specific ecdysone monooxygenase in fat body microsomes of Locusta migratoria occurred in a matter of 2 to 4 hr. Brattsten et al. (1977) concluded that polyphagous lepidopteran larvae are more quick to

respond to dietary chemicals. Their estimate of 30 min is surprisingly short, considering the nature of the events which must occur according to our present understanding of this phenomenon. These include uptake and transfer of the inducer to the cell, transport to the nucleus, interaction with gene regulation system(s) to produce additional protein (e.g. cytochrome P-450 and reductase), and incorporation of this new protein into the membrane. More research is warranted to determine whether the time parameter is a phenomenon that is tissue and/or species specific.

The stimulatory effects of induction appear to linger for several hours following the removal of the inducing agent. Capdevila et al. (1973) showed that PB-treated house flies (Fc strain) returned to control activity (P-450, P-450 reductase, and napthalene hydroxylase) 48 hr after treatment was stopped. Yu and Terriere (1973) demonstrated that PB treatment afforded R and S house flies an increased tolerance to propoxur and that this effect was absent 72 hr following the withdrawal of PB. The present data confirm these earlier observations, but also show that specific activities return much faster to control levels when the inducer is removed due to increases in microsomal protein.

A common hypothesis among workers studying the induction of the insect PSMO is that a particular agent, such as PB, causes the production of specific forms of P-450 each having different monooxygenase capability (Terriere, 1984). This theory receives support from very convincing lines of evidence generated in studies involving other higher organisms, principally mammals (reviewed by

Conney, 1982). We offer additional evidence in support of this hypothesis. PB treatment caused gains in heptachlor epoxidation and MROD activities that respectively exceeded gains for AE and MMCOD in both strains tested (Table IV-1). Furthermore, P-450 and P-450 reductase did not increase as much as monooxygenase activities. This is explainable by considering that PB induced P-450 enzymes specific for the oxidation of heptachlor and methoxyresorufin. This is corroborated by the recent work of Feyereisen et al. (in press), which indicated that the self-catalyzed destruction of microsomal P-450 by heme-alkylating substrates was greater when R and S flies were induced by PB.

In general, the highly resistant Rutgers strain responded to the inducer more or less like the susceptible NAIDM strain. As already noted, this strain seemed less sensitive to the inducer, as indicated by its ability to tolerate higher levels of the drug and by its higher median effective concentration (0.15%). This was probably due to the greater ability of the R strain to metabolize the drug.

There are reasons to believe that genetic differences in the expression of P-450 are present between R and S flies. Two lines of evidence support this concept. First, PB treatment conferred specific activities to the NAIDM strain for AE and MMCOD equal to those in Rutgers, even though the activities for the other two substrates remained 50% higher for the Rutgers strain (Table IV-1). Second, turnover numbers increased significantly during PB treatment in the NAIDM strain (Table IV-2), whereas such changes were not

evident in the R-fly (Table IV-3). The data suggest that although P-450 is inducible by PB in both strains, the changes in Rutgers are of a quantitative nature while those in NAIDM are both qualitative and quantitative. This supports previous findings (Moldenke and Terriere, 1981) based on substrate-binding (Type I) spectra.

Acknowledgements

This work was supported by grant number ES 00362-25 from the United States Public Health Service.

Fig. IV-1. Response of microsomal PSMO enzymes of NAIDM (A) and Rutgers (B) house flies to phenobarbital (PB). Means of values from 2 or more separate experiments expressed as the percent of the greatest response observed for each parameter. (○) P-450, (□) NADPH-cytochrome c (P-450) reductase, (●) AE, (■) HE, (✶) MMCOD, (✪) MROD. Treatment started 24 hr following eclosion (day 1) and microsomes were prepared 48 hr later (day 3). AE = aldrin epoxidation, HE = heptachlor epoxidation, MMCOD = 7-methoxy-4-methylcoumarin O-demethylation, MROD = methoxyresorufin O-demethylation.

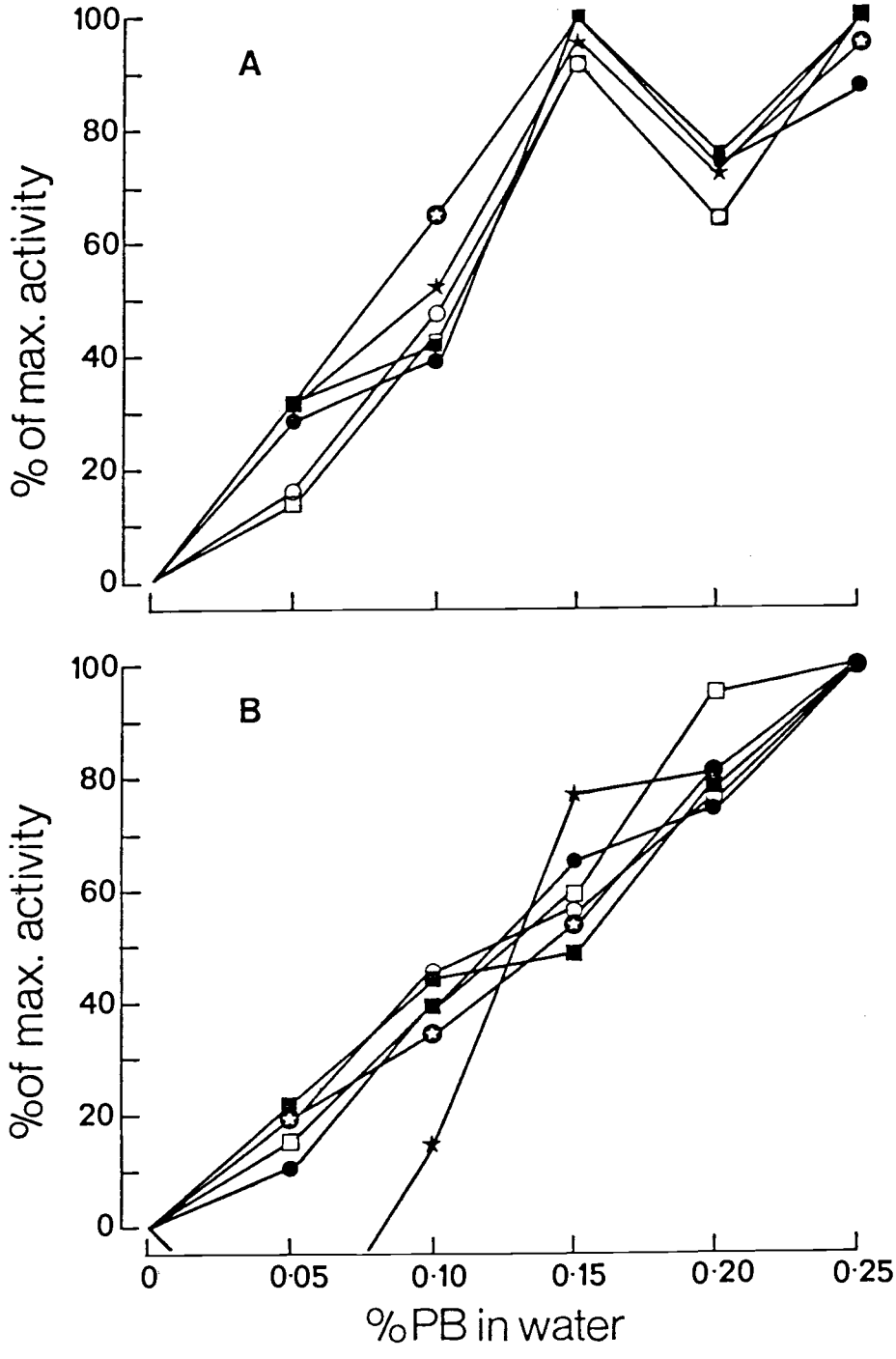
A
B
100
80
60
40
20
0
% of max. activity
%of max. activity
0
0·05
0·10
0·15
0·20
0·25
%PB in water

Fig. IV-1.

Fig. IV-2. Microsomal PSMO enzyme activities for the NAIDM house fly. Mean and s.e.m. of determinations from 2 separate experiments (closed circles day 4 and 5 are from a single experiment). Day 0 is immediately prior to eclosion. PB treatment (●) began 24 hr following eclosion (day 1), and was withdrawn (◑) 48 hr later (day 3) for part of that group. Constitutive values (○) also presented. Methods described in text.

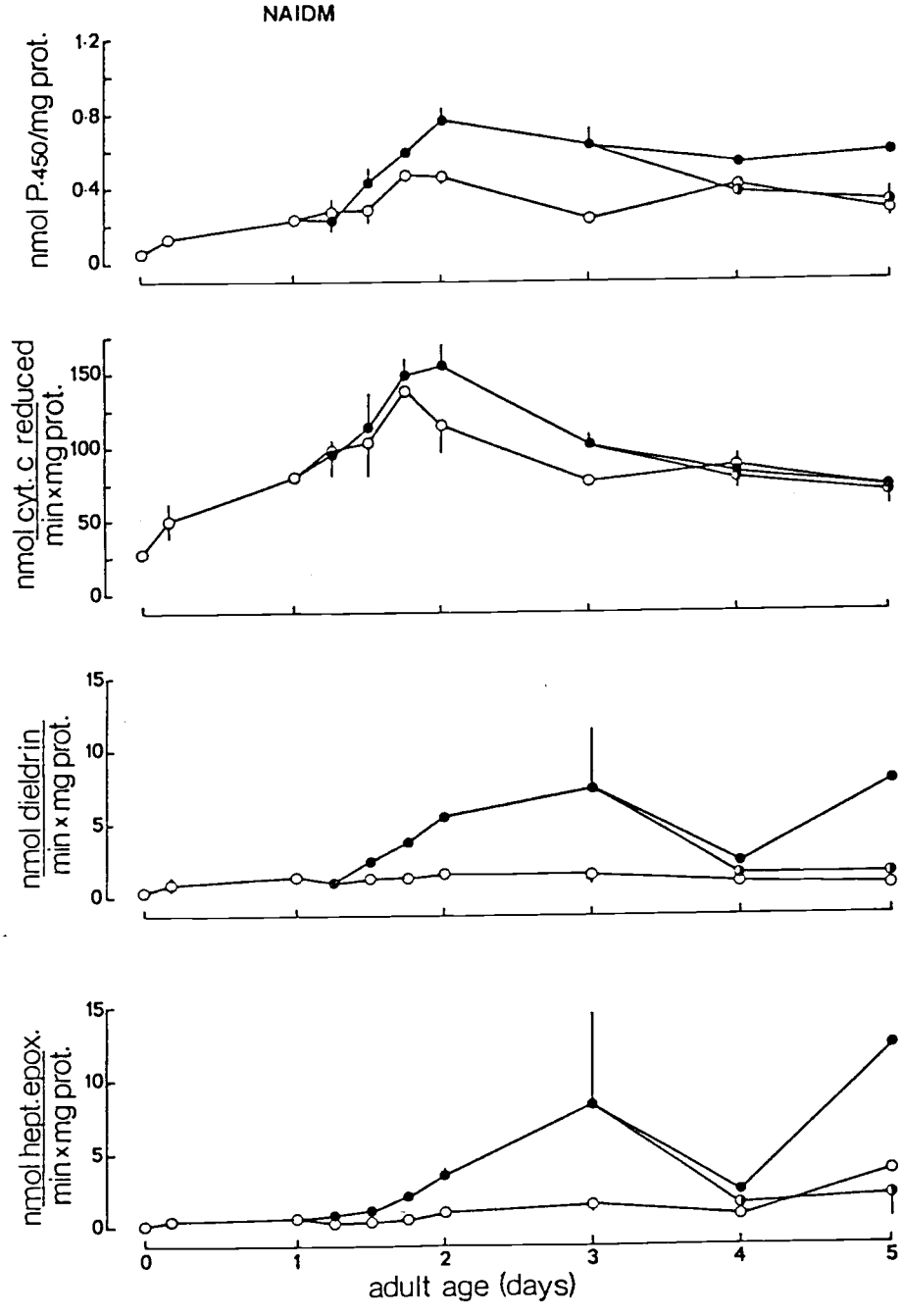
NAIDM
nmol P.450/mg prot.
1·2
0·8
0·4
0
nmol cyt. c reduced / min × mg prot.
150
100
50
0
nmol dieldrin / min × mg prot.
15
10
5
0
nmol hept.epox. / min × mg prot.
15
10
5
0
0
1
2
3
4
5
adult age (days)

Fig. IV-2.

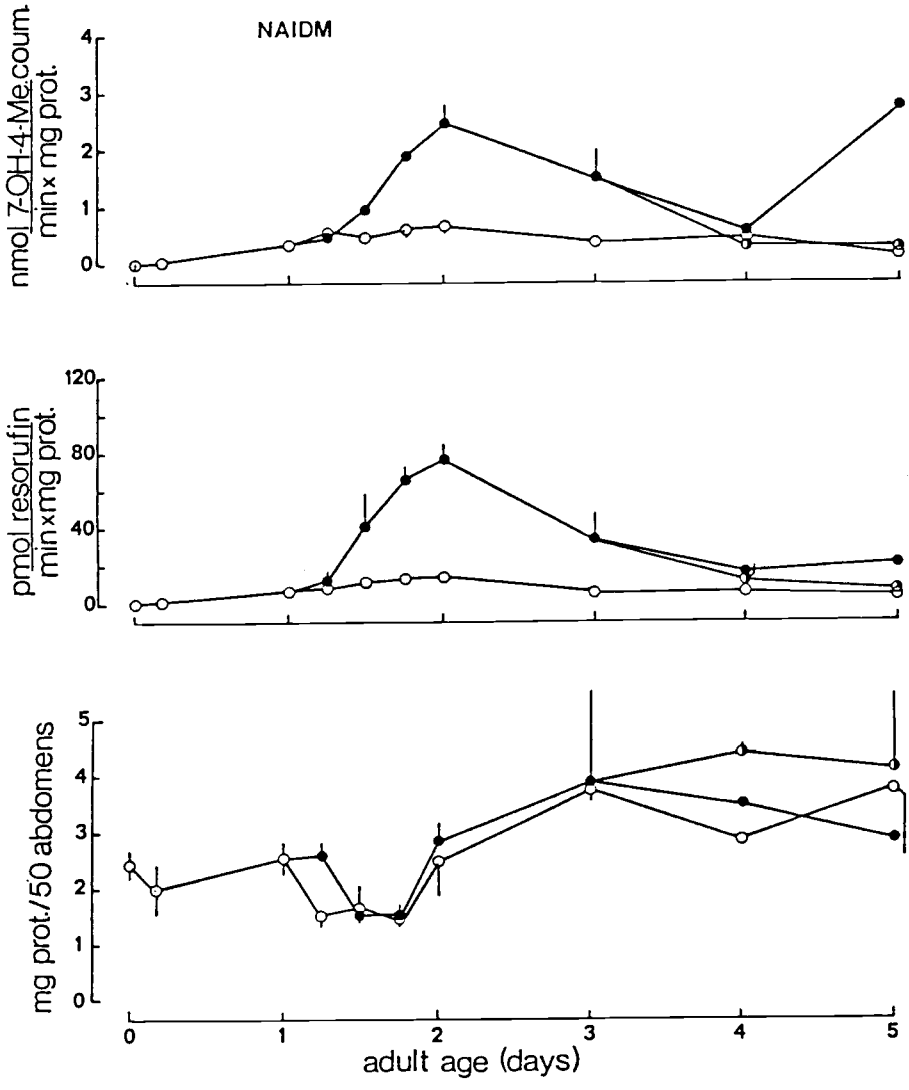
NAIDM
nmol 7-OH-4-Me.coum
min x mg prot.
4
3
2
1
0
pmol resorufin
min x mg prot.
120
80
40
0
mg prot./50 abdomens
5
4
3
2
1
0
0
1
2
3
4
5
adult age (days)

Fig. IV-2 (Cont.).

Fig. IV-3. Microsomal PSMO enzyme activities for the Rutgers house fly. Mean and s.e.m. of determinations from 2 separate experiments (closed circles day 4 and 5 are from a single experiment). Day 0 is immediately prior to eclosion. PB treatment ( ) began 24 hr following eclosion (day 1), and was withdrawn ( ) 48 hr later (day 3) for part of that group. Constitutive values ( ) also presented. Methods described in text.

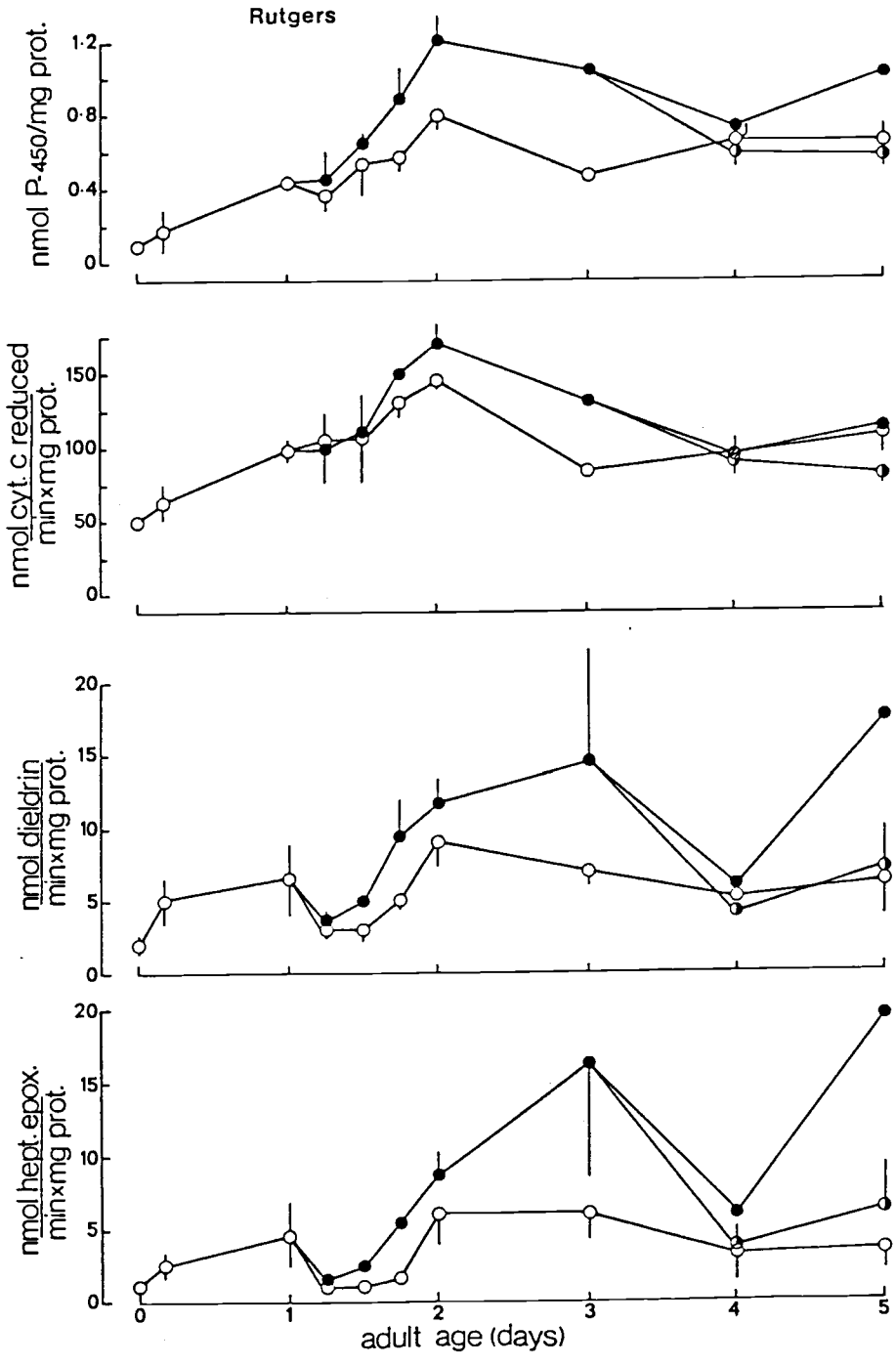
Rutgers
nmol P-450/mg prot.
1·2
0·8
0·4
0
nmol cyt. c reduced
min×mg prot.
150
100
50
0
nmol dieldrin
min×mg prot.
20
15
10
5
0
nmol hept. epox.
min×mg prot.
20
15
10
5
0
0
1
2
3
4
5
adult age (days)

Fig. IV-3.

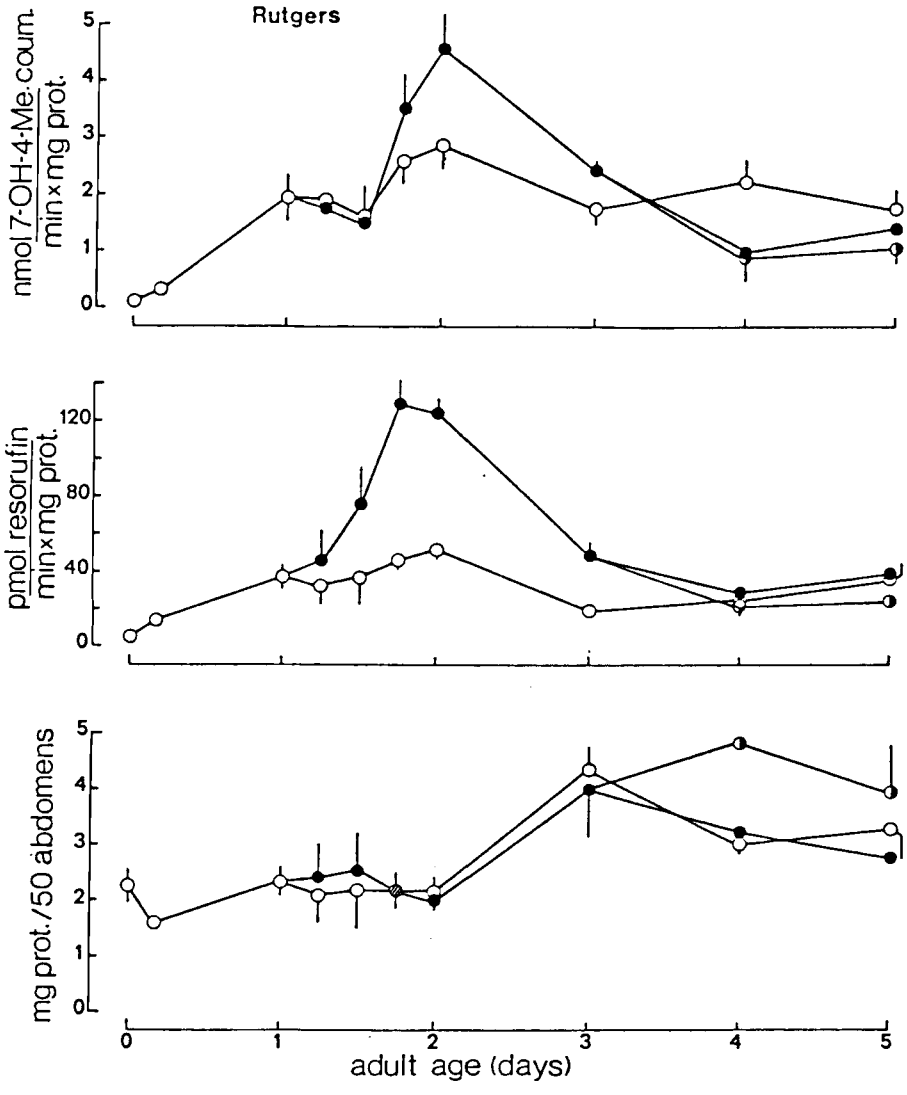
Rutgers
nmol 7-OH-4-Me-coum.
min×mg prot.
pmol resorufin
min×mg prot.
mg prot./50 abdomens
adult age (days)

Fig. IV-3 (Cont.).

Table IV-1. Maximal levels of PSMO enzymes and activities in house flies treated with phenobarbital.

Parameter	NAIDM*		Rutgers*	
	Max. Activity	% Control Activity	Max. Activity	% Control Activity
P-450 (nmol/mg)	1.04 ± 0.13	367 ± 55	1.49 ± 0.12	351 ± 83
Reductase (units/mg)	180 ± 30	238 ± 27	200 ± 40	232 ± 35
AE**	20.00 ± 0.85	1817 ± 762	19.52 ± 5.54	289 ± 17
HE**	23.12 ± 7.27	2325 ± 80	38.65 ± 1.85	601 ± 25
MMCOD**	2.85 ± 0.96	726 ± 383	2.17 ± 0.37	156 ± 21
MROD**	0.048 ± 0.007	1165 ± 140	0.078 ± 0.017	501 ± 83

* Maximum PB concentrations were 0.15% (NAIDM) and 0.25% (Rutgers).

** nmol product/min/mg protein from two or more separate determinations (mean and s.e.m.). AE = aldrin epoxidation, HE = heptachlor epoxidation, MMCOD = 7-methoxy-4-methylcoumarin O-demethylation, MROD = methoxyresorufin O-demethylation.

Table IV-2. Monooxygenase turnover numbers for the NAIDM strain of M. domestica during phenobarbital (PB) treatment.

Substrate	Group	Hours Exposure to PB*				
		12	24	48	72	96
Aldrin	Control	4.3 ± 0.9	3.3 ± 0.6	4.4 ± 1.1	2.9 ± 1.5	2.7 ± 0.0
	PB	5.1 ± 0.2	7.2 ± 0.4	11.0 ± 5.0	4.1	13.0
	PBW**	---	---	---	3.7 ± 0.2	4.4 ± 1.7
Heptachlor	Control	1.1 ± 0.2	2.4 ± 1.0	4.9 ± 1.8	2.0 ± 1.0	1.3 ± 0.8
	PB	1.6 ± 0.3	4.6 ± 0.9	12.2 ± 8.0	4.2	20.6
	PBW	---	---	---	3.7 ± 0.3	5.8 ± 3.3
7-methoxy-4-methylcoumarin	Control	1.6 ± 0.1	1.5 ± 0.3	1.2 ± 0.2	1.0 ± 0.2	0.5 ± 0.2
	PB	2.3 ± 0.4	3.2 ± 0.3	2.3 ± 0.5	1.0	4.6
	PBW	---	---	---	0.8 ± 0.2	0.7 ± 0.1
Methoxyresorufin	Control	0.04 ± 0.00	0.03 ± 0.00	0.02 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
	PB	0.09 ± 0.03	0.10 ± 0.01	0.05 ± 0.01	0.03	0.03
	PBW	---	---	---	0.03 ± 0.1	0.02 ± 0.00

* pmol product/min/pmol P-450. Mean and s.e.m. for determinations from 2 separate experiments (except for single experiments for PB at 72 and 96 hr). Treatment started 24 hr following eclosion.

** PBW - PB withdrawn at 48 hr exposure.

Table IV-3. Monooxygenase turnover numbers for the Rutgers strain of M. domestica during phenobarbital (PB) treatment.

Substrate	Group	Hours Exposure to PB*				
		12	24	48	72	96
Aldrin	Control	5.7 ± 1.8	11.6 ± 2.1	15.6 ± 2.8	7.7 ± 0.9	10.0 ± 3.5
	PB	7.9 ± 0.6	9.8 ± 0.6	13.8 ± 6.9	8.3	17.1
	PBW**	---	---	---	7.0 ± 0.0	12.2 ± 3.7
Heptachlor	Control	2.2 ± 1.0	7.7 ± 2.7	13.4 ± 4.2	4.5 ± 2.3	5.7 ± 2.0
	PB	4.1 ± 0.5	7.2 ± 0.6	15.3 ± 7.2	7.1	19.1
	PBW	---	---	---	6.1 ± 1.3	10.5 ± 3.3
7-methoxy-4-methylcoumarin	Control	3.1 ± 1.0	3.8 ± 0.6	3.6 ± 0.4	3.3 ± 0.5	2.5 ± 0.3
	PB	2.4 ± 0.1	3.8 ± 0.2	2.4 ± 0.1	1.4	1.6
	PBW	---	---	---	1.6 ± 0.4	1.8 ± 0.3
Methoxyresorufin	Control	0.07 ± 0.3	0.06 ± 0.01	0.04 ± 0.00	0.04 ± 0.00	0.05 ± 0.00
	PB	0.12 ± 0.03	0.11 ± 0.01	0.05 ± 0.01	0.04	0.04
	PBW	---	---	---	0.04 ± 0.00	0.04 ± 0.00

* pmol product/min/pmol P-450. Mean and s.e.m. for determinations from 2 separate experiments (except for single experiments for PB at 72 and 96 hr). Treatment started 24 hr following eclosion.

** PBW - PB withdrawn at 48 hr exposure.

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Cytochrome P-450 in Insects. 7. Age Dependency and Phenobarbital Induction of Cytochrome P-450, P-450 Reductase and Monooxygenase Activities in the Black Blow Fly (*Phormia regina*, Meigen).

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Abstract

Development and phenobarbital (PB) induction of cytochrome microsomal P-450, P-450 reductase, and two epoxidation and two O-demethylation activities were examined in chronologically timed populations of female black blow flies (Phormia regina, Meigen). Measurements of these enzymes started with the pharate adult stage and ended 5 days following eclosion. Induction occurred in all enzymes even at 0.005% PB, and was maximum at 0.15%. Dramatic induction of the O-demethylation of 7-methoxy-4-methylcoumarin was observed in flies dosed with the maximum concentration of the drug. This monooxygenase activity increased to nearly 1400 times the level in control flies, whereas the other O-demethylation (methoxyresorufin) and the two epoxidation reactions exhibited considerably less change. Induction of the structural enzymes of this enzyme system were 10-fold for P-450 and 5-fold for P-450 reductase. These data suggest that PB induces several P-450's in the blow fly, particularly one bearing a high degree of specificity for 7-methoxy- 4-methycoumarin.

Introduction

The black blow fly (Phormia regina, Meigen) is closely related to the house fly (Musca domestica, L.), but the activities of the cytochrome P-450 - dependent polysubstrate monooxygenases (PSMO) are only one eighth to one-half those observed in house flies and its microsomal cytochrome P-450 content is correspondingly low (Schonbrod et al., 1965; Terriere et al., 1975; Terriere and Yu, 1976; Lu et al., 1978; Rose and Terriere, 1980). It is also of interest that this species seems to have little tendency to become resistant to insecticides (Brown and Pal, 1971). In spite of these apparent differences from the house fly, Phormia is easily inducible with the barbiturate phenobarbital (PB), responding even more dramatically in terms of the amount of enhancement of cytochrome P-450 and associated oxidase activities which have been measured (Terriere and Yu, 1976; Rose and Terriere, 1980). It also appears that the P-450 of Phormia occurs in multiple forms (Terriere and Yu, 1979).

Although the responsiveness of the blow fly PSMO system to phenobarbital has been known for some time, the "window" in adult development at which the inductive response might be greatest has never been determined, nor has the dose-response curve been established for the method of phenobarbital administration which we now employ. In the course of related studies on the reductase of the blow fly PSMO system (Vincent and Terriere, ms. in prep.) it became apparent that reductase and O-demethylation activity were particularly sensitive to phenobarbital administration. We

therefore set out to establish the optimal time- and dose-related conditions for induction and to examine the substrate specificities of the cytochrome P450(s) induced by phenobarbital treatment. We also measured the levels of the structural enzymes, cytochrome P-450 and P-450 reductase, not previously examined throughout the developmental period covered by this study.

Materials and Methods

Reagents. Chemicals used in this study were as follows: phenobarbital (U.S.P grade), J. T. Baker, Phillipsburg, PA; phenylmethylsulfonyl fluoride (PMSF) and 7-hydroxy-4-methylcoumarin, Calbiochem, La Jolla, CA; 1-phenyl-2-thiourea (PTU), Eastman Chemicals, Rochester, NY; aldrin and dieldrin, Shell Biosciences Laboratories, Sittingbourne, England; heptachlor and heptachlor epoxide, Chem Services, West Chester, PA; methoxyresorufin and resorufin, Molecular Probes, Junction City, OR; and 7-hydroxy-4-methylcoumarin, Aldrich Chemical Co., Milwaukee, WI. All other analytical reagents were of the highest grade commercially available. Buffers used in this study were filtered through a Millipore^{R} type HA, 0.45 micron filter disk prior to use.

Maintenance of insects. Blow flies were reared as described by Terriere and Yu (1979). An emergence window of 3 hr was used to establish chronological age of the insects. Treated flies received phenobarbital in their drinking water beginning 24 hr post-eclosion (eclosion is defined as the midpoint of the 3 hr window) following a

water fast of 2 hr. In the extended time course experiment (Fig. V-2), the PB solution was refreshed after 48 hr.

Preparation of microsomes and analytical methods. Microsomes were prepared from fresh-cut abdomens of female flies (50/group), using the methods described in the accompanying paper (Vincent et al., ms. in prep.). For each preparation, the following determinations were made: P-450; NADPH-cytochrome c (P-450) reductase; aldrin (AE) and heptachlor (HE) epoxidation; and 7-methoxy-4-methylcoumarin (MMCOD and methoxyresorufin (MROD) O-demethylation. Cytochrome P-450 levels were determined from single assays at 20°C by the method of Omura and Sato (1964). P-450 reductase activity was measured in duplicate at 30°C by the reduction of the artificial substrate cytochrome c (50μM)(Williams and Kamin, 1962); one unit of reductase activity is defined as the amount of enzyme required to reduce 1 nmole of cytochrome c in one minute. Protein determinations were made in triplicate according to the method of Bradford (1976), using bovine serum albumin as the protein standard. Epoxidation assays were as previously described (Moldenke and Terriere, 1981; Feyereisen, 1983). O-demethylation activity was determined from single assays, using conditions and substrates as described in the accompanying paper.

Results

Dose response. All parameters responded to the lowest dose of phenobarbital (0.005%, Fig. V-1). Maximum responses were obtained in all cases at 0.15% (Fig. V-1, Table V-1). Mortality increased

and PSMO activities declined at a concentration of 0.2%, indicating toxicity of the compound at this level. Microsomal protein also declined at concentrations exceeding 0.10%, indicating that normal physiological processes in the insects had been disrupted.

MMCOD was strongly induced by phenobarbital to levels nearly 1400-times those of controls. In contrast, MROD was increased only 15-fold at maximum levels (Table V-1). Both AE and HE were elevated to levels about 75-times greater than those in controls.

Phenobarbital-dependent increases in specific content (nmol P-450/mg protein, or units reductase/mg protein) were not so marked as increases in monooxygenation (pmol product/min/mg protein), a pattern which has been observed in several previous studies of insect PSMO induction (reviewed by Terriere, 1984), as well as in our companion paper on induction in house flies (Vincent et al., ms. in prep.).

Age-dependent changes in constitutive PSMO activity. The constitutive levels of cytochrome P-450 remained essentially constant from the pharate period to the fifth day post-emergence, with a slight elevation between 4 and 48 hr post-emergence (Fig. V-2). A similar pattern was observed for the P-450 reductase, with a more marked peak between 4 and 48 hr post emergence. Both AE and HE declined slightly between 24 and 48 hr after emergence, while MMCOD and MROD fluctuated. Microsomal P-420 content averaged 30% in control flies and 25% in PB-treated flies in all of the experiments of this study.

Rates of PSMO induction and decay. For these experiments, a median response concentration of phenobarbital (0.05%) was chosen, which was well below the toxic level (as indicated by decreases in total protein and PSMO activity and by increased mortality). This concentration was high enough to permit reliable assay. Under these conditions, significant increases over control levels for AE, HE and MMCOD were detectable 2 to 6 hr after initial exposure of the flies to PB (Table V-2). Increases in the specific content of P-450 and P-450 reductase, on the other hand, were not apparent until 18 hr exposure to PB and MROD did not increase over control levels until 24 hr of exposure. The turnover numbers of AE, HE, and MMCOD also increased with induction, but that for MROD did not (Table V-3), indicating both a qualitative and quantitative change in the P-450(s) responsible for metabolism of the first three substrates, but only a quantitative change in the P-450 involved in MROD.

All parameters exhibited maximum levels after 48 hr of PB treatment, with the exception of MMCOD (Fig. V-2). This activity did not peak until 72 hr after the introduction of PB and had declined 24 hr later, even in the continued presence of the drug. The activity of preparations from PB-treated insects remained higher, however, than that from microsomes of PB-withdrawn or control insects. The levels of the structural enzymes (P-450 and reductase) and of MROD remained elevated and essentially constant to the end of the experimental period in the continued presence of phenobarbital. If phenobarbital was withdrawn, the levels of P-450

and MROD began to decline within 24 hr and the P-450 reductase within 48 hr after.

A more complex situation was observed in the case of AE and HE. Both activities dropped at day 4, regardless of the presence or absence of PB in the diet. Twenty four hr later, AE of the PB treated flies had rebounded to the levels present on day 3, while that of flies from which PB had been withdrawn continued to decline, as expected. HE, on the other hand, rose again on day 5 whether or not PB was withdrawn and indeed its value in PB-withdrawn flies was slightly higher than that of flies which continued to receive the drug. By the termination of the experiment, the enzymes in flies from which PB had been withdrawn were approaching the levels of the control flies.

Effect of induction on microsomal protein content. Because both specific activity and specific content are functions of the quantity of total protein present in the microsomes, we examined the effect of induction on microsomal protein content (Fig. V-2). Protein content remained comparable to the control until PB concentrations exceeded 0.1% (data not shown). The protein levels of contol and phenobarbital- treated flies were also similar through most of the early post-emergence period (Fig. V-1), except in 36 hr flies (12 hr following the start of PB administration), when values were slightly higher. By 96 hr post-emergence, the protein levels of both control and PB-withdrawn flies were higher than those of PB-treated flies. This is probably attributable to factors involved with the

maturation of eggs, a process which is inhibited by PB in the house fly (Yu and Terriere, 1974), and probably also in the blow fly.

Discussion

The most remarkable feature of phenobarbital induction in the blow fly is the dramatic increase in the O-demethylation of 7-methoxy-4-methylcoumarin. At its maximum, this increase is 100-times greater than that observed for MROD and nearly 20-times the percent increase seen in AE or HE. This contrasts with the pattern observed in our parallel studies on houseflies, where the most strongly induced activity was HE. This activity was 23 times the control activity in the susceptible strain (NAIDM) and 6 times in the resistant strain (Rutgers) (Vincent et al., ms. in prep.).

This marked increase is indicative of a specific induction of a P-450 isozyme with a high affinity and/or Vmax for 7-methoxy-4 methylcoumarin but not methoxyresorufin. This induction also appears to be under different temporal control than the other monooxygenations studied. Significant increases in turnover number (pmol product/min/pmol P-450) and specific activity (pmol product/min/mg protein) for 7-methoxy-4-methylcoumarin occurred 18 hr after the introduction of phenobarbital, while significant changes appear at 6 hr for aldrin and heptachlor or 24 hr for methoxyresorufin.

Although MMCOD is induced greatly by 0.15% PB, this activity remains 50% lower than epoxidation activities at similar doses. The same result is evident in comparison of turnover numbers. Thus

the major portion of P-450 dependent monooxygenation activities in these insects appears to be devoted to epoxidation.

It is apparent from these studies that phenobarbital is handled somewhat differently in the blow fly than in the house fly. The median effective dose for induction was one-half that required for NAIDM and one-third that required for Rutgers. Marked mortality also occurred at a lower dose than that for either house fly strain. After withdrawal of phenobarbital the levels of structural enzymes and of enzyme activities remained above control levels within the time period of this study, while those of the house fly reached control levels. The blow fly showed a decline in epoxidation activity even in the continued presence of PB but the activities in PB-treated blow flies did not reach control levels, as they did in house flies. Furthermore, no decrease in structural enzymes or in O-demethylation activity was observed in blow flies which received PB continuously, whereas such decreases were observed in house flies. We do not yet know if these differences result from different patterns of uptake, sequestration or metabolism of the drug; differences in mechanisms of genomic regulation; or differences in rates of protein synthesis and turnover in the two genera.

Our data are consistent with previous studies (Yu and Terriere, 1979; Terriere and Yu, 1979) showing constitutive levels of both structural enzymes and enzyme activities in blow flies to be lower than those of house flies at comparable times. Upon induction, however, the maximum levels in the blow fly approached or (in the case of the structural enzymes and MMCOD) exceeded those attainable

even in the resistant house fly. The 5-fold induction of reductase activity by 0.15% PB is also larger than has been reported for this enzyme from other insects. This induction results in an enzyme level comparable to microsomes prepared from single tissues of induced insects (Brattsten et al, 1980) or mammals (Dignam and Strobel, 1977). This constitutively low but highly responsive system presents a fertile ground for exploration of the genetic and regulatory structure underlying the PSMO-dependent capabilities of this insect.

In the case of both constitutive and induced enzymes, the activities observed in these studies are several times higher than those in previously published reports (Terriere and Yu, 1976; Terriere and Yu, 1979; Rose and Terriere, 1980). On the basis of our similar experience with the house flies, we attribute these differences to three improvements in technique: the use of fresh rather than frozen abdomens; the inclusion of the protease inhibitor PMSF in the preparation of microsomes; and the administration of phenobarbital in the drinking water rather than in the food. This last procedure is particularly effective, requiring less effort to administer and producing similar response at much lower concentration.

As noted above, percent increase of monooxygenase activities was generally greater than that for P-450 reductase. Induction was evident earlier in the case of the monooxygenase activities, although the cytochrome P-450 showed a temporary increase as early as 2 hr after the initiation of PB treatment (Table V-2). These

data indicate that as early as 2 to 6 hr after the initiation of drug treatment the substrate specificity of the cytochrome P-450 is beginning to change, and it is even possible that newly induced P-450's have "first call" on available reductase, resulting in enhanced activities. However, virtually nothing is known about the factors determining reductase P-450 interactions *in vivo*, particularly in insects, and information on this must await further study.

Even though enzymes were maximally induced at 0.15% PB and the dose response curves were similar in shape, several lines of evidence indicate that the expression of the individual monooxygenase activities (particularly in PB-treated blow flies) is at least partially under separate control. HE activity rebounded after PB withdrawal to levels slightly above those obtained when PB was maintained, which was not observed for any other enzyme activity or for the structural enzymes. MMCOD was very strongly induced, while MROD was not. Peak levels of the former activity appeared 24 to 48 hr after the peaks for all other parameters and this was the only activity to fall off at 5 days in the continued presence of PB. O-demethylase activity also differed from epoxidase activity in that maximum levels of both MMCOD and MROD were retained at a threshold level of toxicity, whereas AE and HE declined at this concentration.

Because of differences in the life cycle, and particularly in the reproductive cycle (Maa and Terriere, 1983a,b), it is difficult to pinpoint possible differences in the mechanisms controlling the expression of oxidase capabilities in *P. regina* and *M. domestica*.

These studies present some evidence to indicate that control of both developmental and inductive expression do differ in the two genera. With one exception, maximum constitutive levels for the parameters examined here peaked within 4 to 24 hr after adult eclosion, while constitutive peaks in the house fly were attained 48 to 72 hr after eclosion. Secondly, induction of monooxygenase activities was detectable in 6 hr in the blow fly, whereas 12 hr was required for house fly. Finally, no enzyme activity in the house fly showed a "rebound" effect after phenobarbital withdrawal, while HE did so in the blow fly.

Acknowledgements

This work was supported by grant no. ES 00362-25 from the United States Public Health Service.

Fig. V-1. Response of blow fly microsomal PSMO enzymes to phenobarbital (PB). Means of values from 2 or more separate experiments expressed as the percent of the average greatest response observed for each parameter. ( ) P-450, ( ) NADPH-cytochrome c (P-450) reductase, ( ) AE, ( ) HE, ( ) MMCOD, ( ) MROD. Treatment started 24 hr following eclosion (day 1) and microsomes were prepared 48 hr later (day 3). AE = aldrin epoxidation, HE = heptachlor epoxidation, MMCOD = 7-methoxy-4-methylcoumarin O-demethylation, MROD = methoxyresorufin O-demethylation.

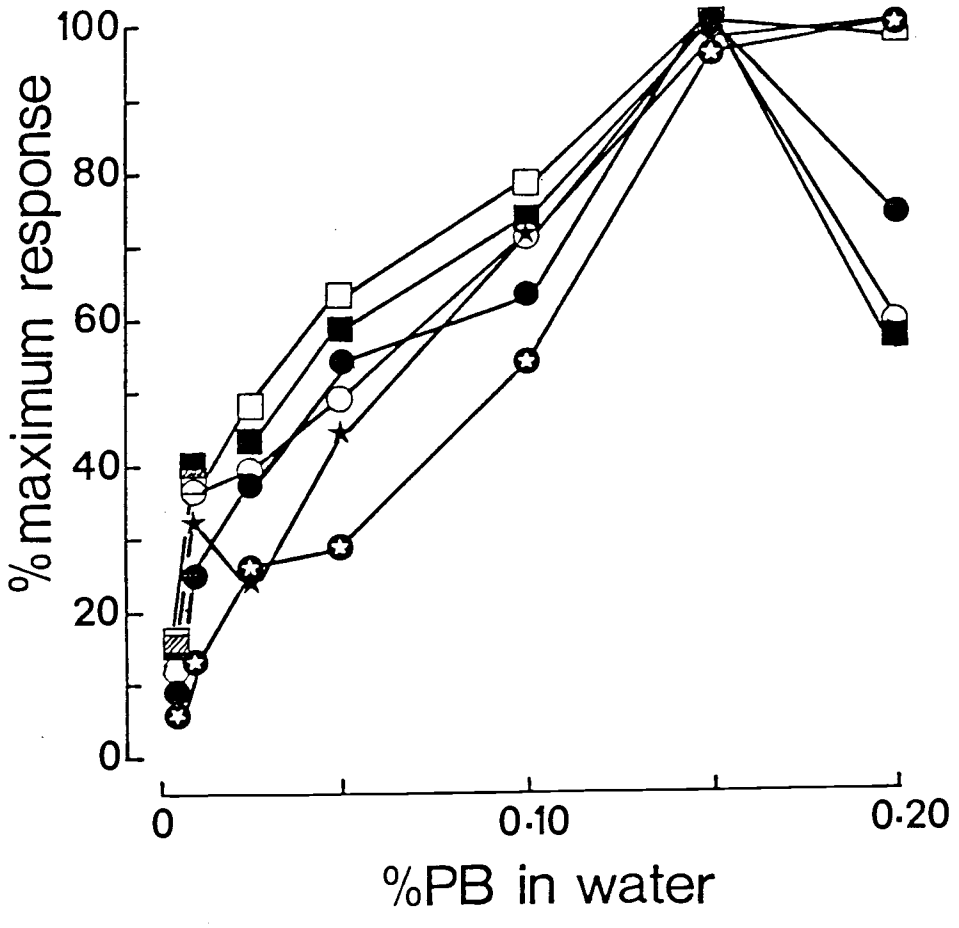

Fig. V-1.

Fig. V-2. Microsomal PSMO enzyme activities for the black blow fly. Mean and s.e.m. of determinations from 2 separate experiments (closed circles days 4 and 5 are from a single experiment). Day 0 is immediately prior to eclosion. PB treatment ( ) began 24 hr following eclosion (day 1), and was withdrawn ( ) 48 hr later (days) for part of that group. Constitutive values ( ) also presented. Methods described in text.

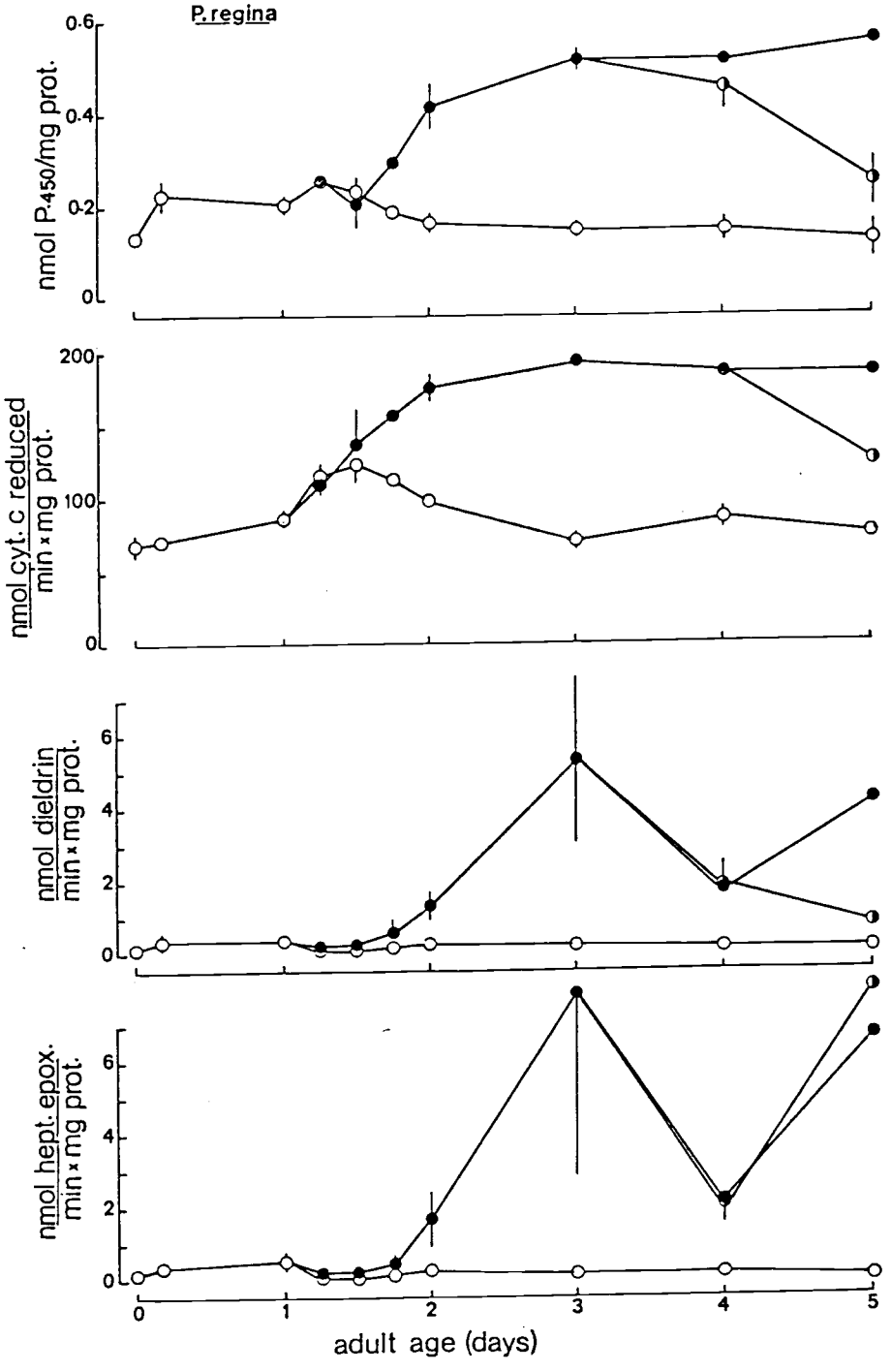
P. regina
nmol P-450/mg prot.
0.6
0.4
0.2
0
nmol cyt. c reduced / min × mg prot.
200
100
0
nmol dieldrin / min × mg prot.
6
4
2
0
nmol hept. epox. / min × mg prot.
6
4
2
0
0
1
2
3
4
5
adult age (days)

Fig. V-2.

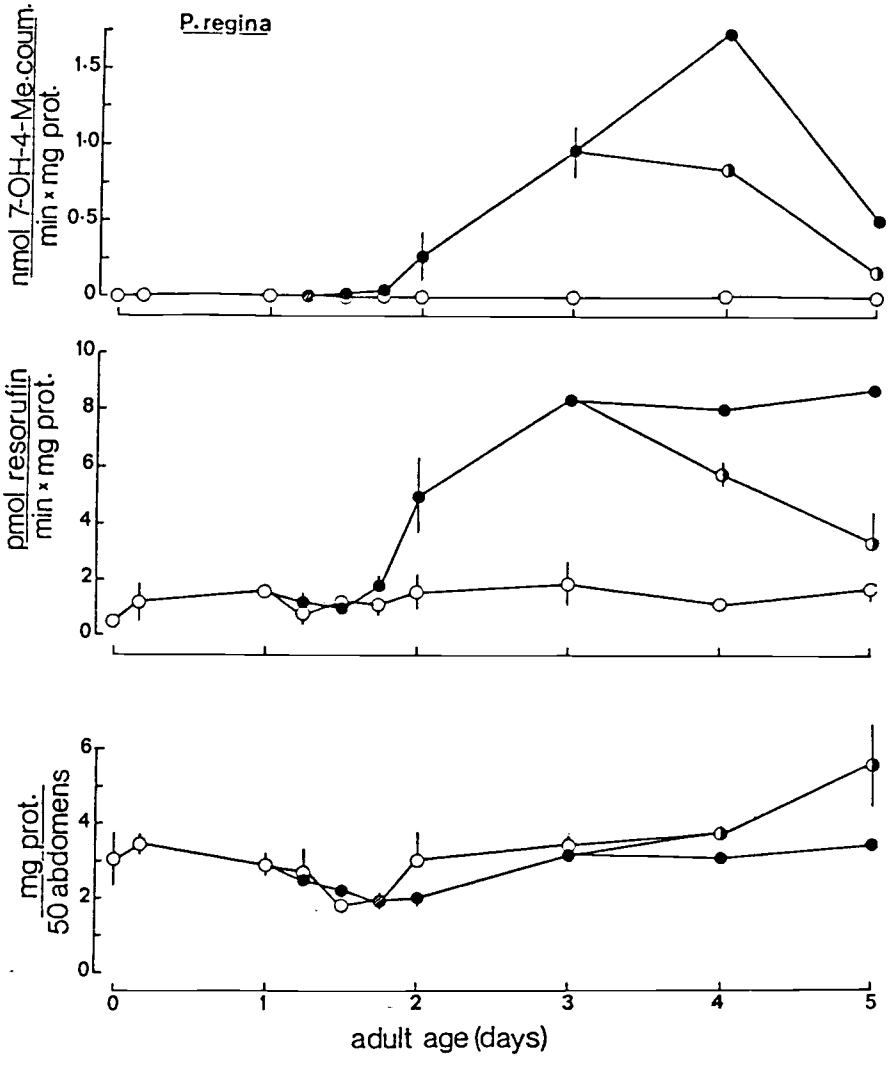
P. regina
nmol 7-OH-4-Me.coum. / min × mg prot.
1·5
1·0
0·5
0
pmol resorufin / min × mg prot.
10
8
6
4
2
0
mg prot. / 50 abdomens
6
4
2
0
0
1
2
3
4
5
adult age (days)

Fig. V-2 (Cont.).

Table V-1. Maximal levels of PSMO enzymes and activities in the black blow fly following treatment with phenobarbital.

Parameter	Maximum Activity	-Fold Control* Activity
P-450 (nmol/mg)	1.25 ± 0.22	10.1 ± 5.1
Reductase (units/mg)	330 ± 3	5.1 ± 0.1
AE**	12.3 ± 3.1	71.4 ± 16.2
HE**	14.3 ± 4.7	77.2 ± 1.4
MMCOD**	7.2 ± 1.6	1383.6 ± 7.8
MROD**	.04 ± .01	14.5 ± 4.9

* at 0.15% PB

** nmol product/min/mg protein. Mean ± s.e.m. of two or more separate determinations. AE = aldrin epoxidation, HE = heptachlor epoxidation, MMCOD = 7-methoxy-4-methylcoumarin O-demethylation, MROD = methoxyresorufin O-demethylation.

Table V-2. Rate of blow fly PSMO induction by phenobarbital (PB).

Hours Treatment	Group	Activity*					
		P-450	Reductase	AE	HE	MMCOD	MROD
2	Control	246 ± 27	121 ± 13	98.1 ± 48.5	66.2 ± 35.3	7.6 ± 3.5	1.0 ± 0.4
	PB**	305 ± 4	120 ± 3	106 ± 31.6	83.7 ± 33.6	8.4 ± 1.3	1.2 ± 0.2
6	Control	248 ± 19	115 ± 8	66 ± 21	80 ± 25	4.8 ± 0.2	0.8 ± 0.3
	PB**	249 ± 77	111 ± 9	127 ± 53	93 ± 37	5.4 ± 0.4	1.1 ± 0.2
12	Control	228 ± 41	124 ± 13	110 ± 1.4	61 ± 21	5.3 ± 1.7	1.2 ± 0.1
	PB**	198 ± 62	136 ± 23	219 ± 83	192 ± 80	9.5 ± 4.5	1.1 ± 0.1
18	Control	175 ± 14	111 ± 2	143 ± 22	90 ± 20	5.0 ± 1.0	1.1 ± 0.4
	PB**	287 ± 10	154 ± 0	586 ± 344	465 ± 226	36.9 ± 12.9	1.8 ± 0.4

* P-450, pmol/mg; NADPH-cytochrome c reductase, units/mg; AE and HE, nmol product/min/mg; MMCOD and MROD, pmol product/min/mg. Mean and s.e.m. for two separate experiments.

** 0.05% PB in drinking water following 2 hr water fast. Treatment started 24 hr following eclosion.

Table V-3. Monooxygenase turnover numbers for the blow fly during phenobarbital (PB) treatment.

Activity	Group	Hours Exposure to PB*				
		12	24	48	72	96
AE	Control**	0.5 ± 0.1	1.55 ± 0.39	1.43 ± .028	0.95 ± .08	0.76 ± .17
	PB	1.09 ± 0.08	3.12 ± .070	10.34 ± 4.02	3.42	7.57
	PBW	---	---	---	4.07 ± 1.06	3.32 ± 0.60
HE	Control	0.26 ± 0.04	1.60 ± 0.39	1.13 ± 0.09	0.84 ± .12	0.48 ± .05
	PB	0.94 ± 0.11	3.72 ± 1.45	15.18 ± 9.32	4.07	12.02
	PBW	---	---	---	4.27 ± 0.76	3.58 ± 0.75
MMCOD	Control	.025 ± .012	.038 ± .008	.034 ± .004	.039 ± .010	.023 ± .006
	PB	.046 ± .009	.600 ± .330	2.15 ± 0.17	3.42	2.72
	PBW	---	---	---	1.92 ± 0.16	0.75 ± 0.14
MROD	Control	.006 ± .001	.008 ± .002	.011 ± .003	.010 ± .004	.014 ± .014
	PB	---	---	---	.016	.016
	PBW	---	---	---	.013 ± .000	.014 ± .001

* pmol product/min/pmol P-450. Mean and s.e.m. two or more determinations from 2 separate experiments (except for single experiment for PB at 72 and 96 hr), PB days 4 and 5 are single determinations. Treatment started 24 hr after eclosion.

** PBW = PB withdrawn at 48 hr exposure.

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