AN ABSTRACT OF THE THESIS OF

Gerald Baldridge for the degree of Master of Science

in Entomology presented on June 7, 1984

Title: Cytochrome P-450 Monooxygenases in the Mosquito, Culex pipiens

Redacted for Privacy

Abstract approved: _____

Dr. René Feyereisen

A sucrose density-gradient ultracentrifugation technique for obtaining microsomes has been developed. This technique has advantages over the classic differential centrifugation technique, in that it is faster, is better suited to work with small quantities of tissue, and offers the opportunity to work with a defined microsomal sub-cellular fraction, rather than a pellet assumed to contain the desired cellular organelles. This procedure has been applied in conjunction with others to study changes in microsomal monooxygenase activities in adult female mosquitoes (Culex pipiens L.) of different ages and reproductive states. Evidence has been obtained that microsomal aldrin epoxidation is a cytochrome P-450 dependent activity in this species. Changes in microsomal aldrin epoxidation and NADPH-cytochrome c reductase activities in mosquitoes of different ages, both before and after a blood meal, have been determined and correlated with changes in whole-body ecdysteroid titers and growth of follicles. The periods of maximal enzymatic activity (second day after emergence and 48 to 96 hours after the blood meal) follow the peaks in ecdysteroid titers by 12 to 24 hours and coincide with

the periods of maximal follicle growth and the onset of oviposition. Ecdysteroids were detectable by radioimmunoassay in non-blood-fed mosquitoes on the first day after emergence (15.1 fmol 20-hydroxy-ecdysone equivalents per insect). Ecdysteroids rose to a peak (394 fmol 20-hydroxyecdysone equivalents per insect) at 36 hours after the blood meal. A critical period for release of a head factor which controls vitellogenic development of the follicles was determined by decapitation experiments to be 4 to 8 minutes after beginning but before completing the blood meal.

Cytochrome P-450 Monooxygenases in the Mosquito, *Culex pipiens*

by

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A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

Completed June 7, 1984

Commencement June 1985

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Date thesis is presented June 7, 1984

Typed by Cherri Lynn Spence for Gerald Baldridge

Acknowledgements

I would like to express my deep appreciation for the advice, help, and patience offered by my advisor, Dr. René Feyereisen, by my fellow laboratory workers, Drs. Alison Moldenke, Dan Vincent, and Ross Halliday, and by our laboratory technician, Dan Farnsworth.

I thank the members of my committee, Drs. Paul Shirk and Bruce Eldridge for their time and concern.

Ann Boerner deserves much credit for her competent and diligent maintenance of our mosquito colony.

To Cherri Spence, I extend my thanks for her long hours in front of the keyboard typing this manuscript.

Finally, I thank my wife, Pamela, for her support, concern, love, and assistance rendered when she, also, was busy preparing a thesis.

This work was supported by NIH grant AI 19192 to Dr. R. Feyereisen.

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INTRODUCTION

1.1. Historical Perspectives and Significance of Cytochrome P-450

Cytochrome P-450 is a membrane-bound monooxygenase enzyme system which is widely distributed in plants, animals, and micro-organisms (Hodgson, 1979). Klingenberg (1958) and Garfinkel (1958) first described cytochrome P-450 as a carbon monoxide binding pigment in microsomes obtained from rat and pig liver cells. These workers were unable to solubilize or further characterize this pigment and its nature remained undefined until 1962, when Omura and Sato published a preliminary account of their investigations which indicated that it was a hemoprotein and was probably a b-type cytochrome. Omura and Sato were able to solubilize the protein and study its spectral characteristics, publishing their findings on its basic features in 1964 (Omura and Sato, 1964A and B). Shortly thereafter, Estabrook and Cooper and coworkers provided a strong impetus for further work on cytochrome P-450 when they discovered its probable physiological function as the 'oxygen-activating terminal oxidase in several mixed-function oxidase reactions' occurring in liver microsomes (for review see Cooper, 1973). In subsequent years, many investigators working in a wide range of disciplines focused their attention on cytochrome P-450. Results of their work have given us the modern-day view of a membrane-bound enzyme system located in the endoplasmic reticulum, but in some cases in association with mitochondria or the

nuclear envelope, and occurring in multiple isozymic forms dependent on age and developmental status of the organism and the environmental factors affecting it. These isozymic forms have overlapping substrate specificities and catalyze a number of oxidative reaction types involving a wide array of exogenous and endogenous lipophilic substrates (Sato and Omura, 1978; Lu and West, 1980). Regardless of isozymic form, the general mechanism of cytochrome P-450 catalysis is thought to involve electron abstraction followed by oxygen transfer to an electropositive atomic center. The enzyme in the oxidized state binds the lipophilic substrate, accepts a reducing equivalent from NADPH through an associated flavoprotein reductase, binds O_2 followed by acceptance of a second reducing equivalent, and then monooxygenates the substrate accompanied by formation of H_2O (Ullrich, 1977; Guengerich and Macdonald, 1984).

Much of the research on possible physiological functions of cytochrome P-450 in vertebrates has focused on its role in xenobiotic metabolism. Induction of cytochrome P-450 in mammalian liver by various drugs and xenobiotics was noticed early on (Conney, 1967). This is by now a well-described phenomenon and research is beginning to turn toward elucidation of genetic mechanisms controlling induction of cytochrome P-450 isozymes (Nebert et al., 1981; Ohyama et al., 1984). Equally interesting has been the discovery that cytochrome P-450 isozymes in the liver are differentially expressed during growth and development (Conney et al., 1969; Neims et al., 1976; Botelho et al., 1979; Parandoosh and Franklin, 1983). This work has had

important implications for carcinogenesis theory and drug therapy decisions in general (Gelboin, 1983).

While ontogenetic expression of cytochrome P-450 isozymes has received considerable study in mammals, research with insects has only superficially addressed this topic. Following the demonstration by Ray (1967) that cytochrome P-450 occurred in house flies and apparently catalyzed oxidative epoxidation of aldrin to dieldrin, attention among insect toxicologists focused on the possible involvement of cytochrome P-450 in the oxidative metabolism of insecticides by insects, a field which had been under study for some time (see review by Casida, 1970). It became apparent that cytochrome P-450 probably played an important role in detoxication of insecticides in insects (see review by Wilkinson and Brattsten, 1972) and work on this enzyme system as it occurs in insects has continued to focus primarily on its role in insecticide toxicity to insects of agricultural and medical importance. It now appears that cytochrome P-450 is responsible for reactions that both activate and detoxify insecticides (Kulkarni and Hodgson, 1980).

In recent years, widespread resistance to insecticides has been noted among various insect populations being subjected to chemical control programs by man. Early observations that a monooxygenase type of activity might be involved in resistance (Eldefrawi et al., 1960; Hodgson and Casida, 1960; Sun and Johnson, 1960) were later confirmed (Schonbrod et al., 1968; Folsom et al., 1970; Hammock et al., 1977; Feyereisen, 1983). However, considerable disagreement exists in the literature concerning cytochrome P-450 monooxygenase involvement in

resistance and it is difficult to make generalizations. The relative importance of monooxygenase enzymes in resistance phenomena can only be characterized by correlation of *in vivo* studies of resistance with *in vitro* determinations of enzymatic activity (Wilkinson, 1983). Such complicating factors as penetration of the cuticle by the insecticide and presence of other enzymes such as carboxy-esterases must also be considered.

Despite the emphasis placed on an insecticide toxicity approach in research involving microsomal monooxygenases in insects, evidence has accumulated in recent years that monooxygenases are differentially expressed during insect development (Krieger and Wilkinson, 1969; Anderson, 1978; Gould and Hodgson, 1980; Hallstrom *et al.*, 1983) and may well play an important role in control of growth, metamorphosis, and reproduction. Yu and Terriere (1974A) suggested this possibility following experiments with house flies involving phenobarbital induction and piperonyl butoxide induction and inhibition of microsomal monooxygenase activity. They noted aberrations in growth and reproduction. An apparent correlation of cyclic changes in monooxygenase activities and nymphal ecdyses had been demonstrated previously in crickets and cockroaches (Benke and Wilkinson, 1971; Benke *et al.*, 1972). These results were interpreted as implying a connection between microsomal monooxygenase activity and the action and regulation of hormones controlling growth and reproduction. The involvement of cytochrome P-450 dependent reactions in the metabolism of juvenile hormone and ecdysone has since been demonstrated in several insects (for reviews see Koolman, 1982; Hodgson, 1983).

However, demonstration of a direct connection between variations of cytochrome P-450 dependent microsomal monooxygenase activity and ecdysteroid or juvenile hormone titers has been more difficult to attain. Feyereisen and Durst (1980), working with the last larval instar of Locusta migratoria L., were able to show that the peak of cytochrome P-450 dependent ecdysone 20-monooxygenase activity in malpighian tubules and fat body coincided with the peak in hemolymph 20-hydroxyecdysone titer seen on the fifth day of the instar and were further able to demonstrate a simultaneous peak in NADPH-cytochrome c reductase activity and a cytochrome P-450 linked lauric acid w-hydroxylase activity.

This work has important implications. If, as appears likely, there is an interrelationship between insect cytochrome P-450 dependent monooxygenases and metabolism of hormones controlling growth, metamorphosis, and reproduction, then elucidation of these interactions could have a profound effect on the design and use of insecticides and pest management strategies for control of agriculturally and medically important insects. It is of interest to note that Brattsten and Metcalf (1973) found that susceptibility of several species of flies to commonly used insecticidal chemicals was dependent on age and sex. In vitro experiments indicated that the differences in susceptibility were probably due to differences in oxidative metabolism.

Much remains to be learned concerning cytochrome P-450 in insects. It has been characterized in detail in only two species, Musca domestica L. and Spodoptera eridania Cramer. A homogenous

purified cytochrome P-450 has not yet been obtained from an insect, although Agosin and coworkers have been partially successful (Naquira et al., 1980; Agosin, 1982), while in recent years multiple isozymic forms of cytochrome P-450 have been purified and characterized from several vertebrate species and several laboratories are now engaged in cloning genes encoding vertebrate cytochrome P-450 (Chen et al., 1982; Hardwick et al., 1983; Mizukami et al., 1983).

1.2. Choice of Culex pipiens for study

Culex pipiens was chosen for study for two major reasons. Firstly, as will become apparent in the discussion of the literature below, very little is known about the possible role of cytochrome P-450 dependent monooxygenases in insecticide detoxication by mosquitoes. Secondly, as mentioned in the previous section, evidence has accumulated in recent years that cytochrome P-450 dependent monooxygenases are involved in metabolism of hormones regulating growth and reproduction in insects.

There is little doubt that the mosquito family, Culicidae, constitutes the chief threat to human health by insects on a world-wide basis (Harwood and James, 1979). This alone provides adequate reason to study enzymatic mechanisms in these insects which may play a role in insecticide toxicity. It is also true that mosquito populations throughout the world have begun to display increasing levels of resistance to chemical insecticides in recent years (WHO, 1981). Many in vivo studies of this resistance have been reported in the literature and, in general, have indicated that it

involves multiple metabolic mechanisms (as based on results obtained with various insecticide synergists and analysis of metabolites) and is broad spectrum in character, e.g., cross resistance to the various insecticides. (Plapp et al., 1966; Apperson and Georghiou, 1975; Georghiou et al., 1975; Herath and Davidson, 1981A, 1981B). However, as pointed out earlier, a fundamental understanding of resistance mechanisms requires correlation of in vivo data concerning insecticide toxicity and the nature of the final metabolites produced with in vitro biochemical data on the actual metabolic processes which may be occurring within the insect. In this regard it is surprising how little in vitro data concerning insecticide metabolism in mosquitoes has been reported in the literature and, in addition, most of the reported studies have involved larvae only.

Much of the early work is seriously flawed by the fact that crude tissue homogenates were utilized for incubations in vitro, apparently because it was simply easier than preparing defined sub-cellular fractions for study (Lang, 1959; Plapp et al., 1963; Stone, 1969). The relevance and reliability of results obtained by such procedures are in question. Shrivastava et al. (1970, 1971) studied metabolism of carbamate insecticides in several mosquito species with improved in vitro procedures, in some cases using microsome preparations, and attempted to correlate results of in vivo and in vitro analysis. They also included pupae and adult insects in some experiments. They concluded that a monooxygenase system (as based on requirement for NADPH in vitro, analysis of metabolites, and synergism studies with piperonyl butoxide) played a major role in detoxication of

carbamates in mosquitoes. They further noted apparent minor differences in the mosquito monooxygenase system from that reported for other insects in regard to pH optima, enzyme stability, and presence of endogenous inhibitors. Following a report by Quistad *et al.* (1975) that larvae of *Culex pipiens quinquefasciatus* Say could metabolize methoprene, Brown and Hooper (1979) investigated the possibility that oxidative detoxication played a role in methoprene resistance in a selected *Culex pipiens pipiens* L. strain. They were able to demonstrate metabolism of methoprene *in vivo* by larvae but were unable to demonstrate metabolism *in vitro*. Additional experiments involving microsomal incubations *in vitro* with aldrin failed to demonstrate formation of the epoxidized product, dieldrin.

Cocke *et al.* (1979) performed an ultrastructural study by electron microscopy on cuticular development of *Aedes aegypti* L. larvae exposed to methoprene or a fluorescent insect growth regulator, 5-[[[5-(dimethylamino)-1-naphthalenyl]-sulfonyl]amino]-1,3-benzodioxole (DNSAB). This compound has lipophilic characteristics and is a juvenile hormone (JH) analog containing the functional group of the methylenedioxyphenyl (MDP) insecticide synergist family. Mayer and Prough (1976) demonstrated that DNSAB formed a metabolite-cytochrome P-450 complex in rat liver microsome incubations with spectral characteristics typical for the MDP synergist family interaction with cytochrome P-450 (Hodgson and Philpot, 1974).

Gross morphological aberrations of cuticle were among the effects noted by Cocke and coworkers in *Aedes aegypti* larvae treated with both compounds. They also observed that DNSAB delayed new cuticle

formation. It was possible that this was due to changes in basic membrane structure and permeability characteristics induced by exposure to DNSAB, but the authors proposed an alternative hypothesis that DNSAB could bind to smooth endoplasmic reticulum and "interrupt normal oxidative degradation of endogenous JH by monooxygenases (cytochrome P-450)," thus allowing JH to accumulate and delay larval development. In a subsequent paper (Mayer et al., 1982), they reported work in which they indirectly investigated this hypothesis by first showing that DNSAB synergizes the activity of JH against Aedes aegypti larvae in vivo and then performing extensive characterization procedures of the interaction of DNSAB with house fly microsmal cytochrome P-450 in vitro. They characterized a DNSAB metabolite-cytochrome P-450 complex and then demonstrated that its formation could be competitively inhibited by addition of JH to the house fly microsome incubation medium. In view of earlier work that had established an interaction between JH and JH analogs with insect microsomal monoxygenase systems and inhibition of these interactions by the MDP compound piperonyl butoxide (Yu and Terriere, 1973, 1974B, 1978), they felt confident that their original hypothesis that DNSAB blocked normal oxidative degradation of JH by cytochrome P-450 monooxygenases was reinforced by their new data, and that it provided a possible explanation of the mechanism of action of DNSAB against Aedes aegypti larvae in vivo.

The indirect method resorted to by these investigators to study a possible mechanism of action of an insecticidal compound against Aedes aegypti larvae in vitro is symptomatic of the rarity of in vitro data

for mosquitoes in the literature. Furthermore, studies with adult mosquitoes *in vitro* are almost completely non-existent, a peculiar state of affairs given their importance to human health and the increasing levels of resistance among mosquitoes to chemical insecticides. We, therefore, considered it desirable to develop and put into use procedures for study of cytochrome P-450 dependent monooxygenase activities *in vitro* in the mosquito, *Culex pipiens*.

An additional reason to study monooxygenase activities in *Culex pipiens* *in vitro* involves mosquito reproduction. Endocrine control of reproduction in mosquitoes has been partially characterized recently, principally in the anautogenous species *Aedes aegypti*. In brief, juvenile hormone released from the corpora allata of the newly emerged mosquito regulates growth of the follicles in the two or three days following emergence (Gwadz and Spielman, 1973). The follicles grow to a previtellogenic "resting stage" (Clements, 1963) and then cease growth until the mosquito obtains a blood meal. The period of suspended growth is manifested by arrested development of nurse cells and other physiological phenomena within the follicles. The blood meal initiates release of an egg development neurosecretory hormone (EDNH) synthesized in the brain and stored in the corpora cardiaca (Lea, 1972). The EDNH is released into the hemolymph and is thought to initiate synthesis and release of a vitellogenin stimulating hormone (VSH) from the ovaries. VSH is believed to be ecdysone, however, this point is still in controversy (Hagedorn *et al.*, 1975; Borovsky and Van Handel, 1979; Fuchs and Kang, 1981). Assuming that VSH is ecdysone, it is thought to be converted to 20-hydroxyecdysone

(the physiologically active form) in the fat body which then responds by synthesizing and releasing vitellogenin into the hemolymph. Responsiveness of the fat body to 20-hydroxyecdysone may be mediated by prior exposure to juvenile hormone (Flanagan and Hagedorn, 1977). The follicles take up the vitellogenin from the hemolymph and resume growth (Roth and Porter, 1964; Yonge and Hagedorn, 1977).

This system offers the possibility of experimental manipulation by exogenous administration of hormones or removal of their endogenous sources. In view of the demonstrated involvement of microsomal and mitochondrial monooxygenases in hormone metabolism in other insects, the experimental possibilities offered by the endocrine control of reproduction in mosquitoes provides an attractive opportunity to investigate possible interrelationships between cytochrome P-450 dependent monooxygenase activities and the hormones regulating a physiological process which can readily be monitored with an ordinary microscope. The possible importance of such information in the design of pest management strategies for mosquito control should not be underestimated. Brown and Pal (1971) discuss evident increases in insecticide tolerance noted in several species of mosquitoes in the first 1 to 2 days after the blood meal.

1.3 Methods of Investigation

The goal of research described in this thesis was to study ecdysteroid titers and developmental expression of cytochrome P-450 monooxygenase and NADPH-cytochrome c reductase activities in adult female *Culex pipiens* before and after the blood meal. This

information was correlated with growth of follicles which provided an *in vivo* physiological marker of the reproductive developmental status of the mosquitoes. Ecdysteroid titers were determined on a whole-body basis by radioimunoassay while enzymatic activites were studied with microsomes obtained from abdominal tissue homogenates. Preliminary experiments indicated that Malphighian tubules had high enzymatic specific activities, but use of this organ as a routine source of tissue proved unfeasible due to its very small size. Midguts were removed from abdomens of both blood-fed and non-blood-fed mosquitoes before homogenization of abdomens because of technical difficulties encountered in cleanly and reproducibly removing blood from midguts of blood-fed mosquitoes.

In view of reports in the literature that cross-contamination of "microsomal" and "mitochondrial" pellets occurs during the classic differential centrifugation method of preparing microsomes from insects (Benke *et al.*, 1972; Wilkinson and Brattsten, 1972), that rough endoplasmic reticulum may occur in close association with mitochondria (Meier *et al.*, 1981), and that cytochrome P-450 can apparently be localized in rough or smooth endoplasmic reticulum in some organisms (Kuriyama *et al.*, 1979; Mercurio and Nemeth, 1983), it was considered desirable to develop a better method for preparing microsomes from *Culex pipiens*. A sucrose density-gradient procedure utilizing a vertical rotor and centrifugaton at high *g* forces was therefore developed for use in preparation of microsomes from abdominal tissue homogenates. During development of this technique, NADPH-cytochrome c reductase and succinate-cytochrome c reductase

activities were used as enzymatic markers to assess distribution of microsomes and mitochondria, respectively, in the gradients. This procedure for obtaining microsomes was used for study of developmental changes in enzymatic activities where it was considered most important to have reproducible preparations of microsomes between experiments. Microsomes were obtained in large quantity by differential centrifugation when necessary for certain characterization procedures requiring relatively large quantities of "microsomal" protein.

Microsomal aldrin epoxidation activity was characterized and optimal incubation conditions were established. This reaction is often assumed to be cytochrome P-450 dependent and many studies utilizing it are published in the literature without any attempt to demonstrate that it actually is dependent on cytochrome P-450. We have attempted to demonstrate P-450 character for this activity in Culex pipiens with inhibition experiments employing antibody to house fly NADPH-cytochrome P-450 reductase and carbon monoxide as inhibitors.

NADPH-cytochrome c reductase and aldrin epoxidation activities were followed at daily intervals in non-blood-fed mosquitoes from 1 to 6 days and at days 8, 10, and 12 after emergence. Activities were also followed in mosquitoes given a blood meal on the 6th day after emergence at 12 hour intervals through 72 hours after the blood meal and thereafter at 96, 120, and 144 hours after the blood meal. Microsomal activity for O-dealkylation of coumarin and resorufin aryl hydrocarbon ethers was also investigated by fluorometric assay and was partially characterized for methoxyresorufin. Decapitation

experiments were used to investigate the "critical period" for release of a hormonal stimulus from the brain and corpora cardiaca following a blood meal on which subsequent developmental physiological events and growth of follicles might be dependent.

1.4. Summary of Principal Results

A sucrose-density gradient centrifugation procedure was developed for obtaining microsomes. A 5 ml linear gradient prepared from equal volumes of 45, 35, 25, and 15% sucrose solutions centrifuged in a Beckman VTI-80 vertical rotor for 20 minutes at 65,000 RPM (w^2t = 3.70×10^{10}; 310,000 g max.) was found to give good reproducible separation of microsomal from mitochrondrial and cytosolic sub-cellular fractions of tissue homogenates. Integrity of separation declined as total protein loaded on the gradient was increased above 1 mg. The gradients were also sensitive to lipid.

Efforts to develop spectrofluorometric assays for microsomal O-dealkylation of 7-methoxycoumarin, 7-ethoxycoumarin and 7-methoxy-4-methylcoumarin were unsuccessful even with protein concentrations in incubation mixtures of 0.6 mg/ml. Microsomes obtained from mosquitoes were capable of inhibiting these activities in house fly microsome incubations. The inhibitor was heat stable. Efforts to develop a fluorometric assay for O-dealkylation of methoxyresorufin by mosquito microsomes were partially successful and allowed limited characterization of this activity. However, the enzyme was apparently not stable and possible differences in its activity in mosquitoes of various ages was not studied.

A modified aldrin epoxidation assay was developed and employed a final incubation volume of 350 µl, usually containing less than 20 µg of "microsomal" protein. The epoxidation product, dieldrin, was extracted with 100 µl of hexane and quantified by gas chromatography with endrin as an internal standard. Aldrin epoxidation in mosquito microsomes displayed an apparent K_M of 3.4×10^{-6} M and an apparent V_{max} of 38 pmoles dieldrin/min/mg protein. Aldrin epoxidation was inhibited in a dose-dependent manner by antibody to house fly NADPH- cytochrome P-450 reductase. Results of preliminary experiments indicated that carbon monoxide could also inhibit aldrin epoxidation. These results can be interpreted as evidence that aldrin epoxidation in Culex pipiens microsomes is cytochrome P-450 dependent.

NADPH-cytochrome c reductase and aldrin epoxidation activities were followed in non-blood-fed and blood-fed mosquitoes. In non-blood-fed mosquitoes activities showed a distinct peak on the 2nd day after emergence while activites in blood-fed mosquitoes were maximal at 48 to 96 hours after the blood meal. Ecdysteroid titers were detectable in non-blood-fed mosquitoes only on the first day after emergence (15.1 fmol 20-hydroxyecdysone equivalents/insect). In blood-fed insects ecdysteroid titers rose to a peak at 36 hours after the blood meal (394 fmol 20-hydroxyecdysone equivalents/insect) and declined rapidly thereafter. These data were correlated with growth of follicles. In non-blood-fed mosquitoes, follicles were approximately 20 µm in length immediately after emergence. From Day 1 to 3 after emergence, they grew to an average length of approximately 88 µm and then ceased growth until a blood meal was obtained by the

insect. The peak of enzymatic activities on the 2nd day after emergence coincided with the period of maximum follicle growth in non-blood-fed mosquitoes while the apparent peak of ecdysteroids in non-blood-fed mosquitoes immediately preceded the period of greatest follicle growth. In blood-fed mosquitoes, follicles grew to approximately 300 µm in length during the first 48 hours after the blood meal. In the next 12 hours, they grew rapidly to about 600 µm, followed by an apparent slight decline in length from 60 to 78 hours after the blood meal which coincided with chorionation and the onset of oviposition. Most mosquitoes oviposited from 72 to 96 hours after the blood meal. The peak in ecdysteroid titers at 36 hours after the blood meal was followed by the 12 hour period of greatest follicle growth from 48 to 60 hours after the blood meal. The peak in enzymatic activities from 48 to 96 hours after the blood meal coincided with the periods of greatest follicle growth and oviposition. No causal relationships between follicle growth, ecdysteroid titer, and enzyme activities were either inferred or implied by our studies.

An apparent critical period for presence of the head after the blood meal was found to be between 4 and 8 minutes after beginning the blood meal. Most mosquitoes fed for about 10 minutes before completing the blood meal. It was thus impossible to use decapitation as a means of investigating whether it was the blood meal itself or a secondary hormonal stimulus which was responsible for subsequent increases in enzymatic activities, ecdysteroid titers, and follicle growth.

MATERIALS AND METHODS

2.1 Rearing of Insects

An anautogenous strain of Culex pipiens collected in 1978 from a log-pond at Philomath, Oregon was used to establish a colony. A subcolony was established from this colony and was maintained in a 15 by 30 foot room at 26°C ± 1° under a 16 hr light:8 hr dark photoperiod. Adults were fed a 10% sucrose in water solution ad libitum. Live quail were used to provide blood meals. Larvae were reared in tap water on a diet of ground Tetra SM80, Purina Dog Chow, L-Ascorbic Acid, and lyophilized pork liver (49/49/1.5/0.5, w/w/w/w).

Pupae were transferred on a daily basis from larval rearing pans to 16 oz. glass jars containing tap water. The jars were placed in 12x10x13 inch screened rearing cages and were simultaneously moved to a new cage every morning. This procedure provided groups of adult insects which had all emerged from pupae during the same 24 hour period. These insects were used for subsequent experiments.

2.2 Handling of Insects for Experiments

2.2.1. Blood-feeding

Approximately 18 hours before the blood meal, the 10% sucrose solution was removed from rearing cages containing 6-day old adult insects and the insects were starved overnight. The next morning the cage was moved to a cool darkened room and live quail, restrained in nylon stockings and taped to overturned cottage cheese cartons, were placed in the cage for 1 to 2 hours. Following the feeding period,

blood-fed insects were removed with an aspirator and transferred to new rearing cages. The 10% sucrose solution was again provided ad libitum. Jars containing water for oviposition were placed in all cages of insects held more than 48 hrs after the blood meal.

2.2.2. Follicle Growth Curves

Follicle growth curves for non-blood-fed (NBF) and blood-fed (BF) mosquitoes were obtained by measuring and correlating increase in follicle length with increasing age of the insect (determined as days after emergence). The NBF follicle growth curve was obtained from mosquitoes measured at daily intervals from Day 0 (immediately after emergence) through 9 days after emergence. Mosquitoes were given blood meals on Day 6 as described in section 2.2.1. and follicles were measured at 6 hour intervals from 6 to 78 hours after the blood meal to obtain the BF follicle growth curve.

To measure follicles, appropriately aged mosquitoes were removed from rearing cages with an aspirator, anesthetized with a 5 second CO_2 exposure, and held on ice in a covered petri plate till dissection. Ovaries were removed intact by grasping the anterior abdominal segments with forceps and then pulling away the 2 posterior segments, accompanied by the ovaries, with a 2nd forceps. Ovaries were immersed in Lum's mosquito saline (1961) and teased apart with dissecting needles. Lengths of 10 follicles per insect were measured at a magnification of 125X with a Zeiss binocular microscope equipped with an ocular micrometer. The longest of the 10 lengths measured for each insect was recorded in accordance with procedures found in

recently published literature (Borovsky, 1982; Lea, 1982A). These experiments were continued over a period of 6 months and data were pooled to obtain follicle growth curves.

2.2.3. Decapitation Experiments: Dependence of Follicle Growth on a Head Factor

Decapitation experiments with blood-fed mosquitoes were used to determine the critical period for presence of the head following blood-feeding. The blood-feeding procedure described in section 2.2.1. was used as modified in the following description:

One restrained quail was placed in a cage containing a small number of 6-day old female mosquitoes. A stop watch was used to time length of blood-feeding by individual or small groups of insects. Mosquitoes were removed from the cage by aspirator after feeding to repletion or at specific times after beginning but before completing the blood meal. They were held in 1/2 inch inside diameter glass tubing till time of decapitation, when they were anesthetized with a 5 second CO_2 exposure, dropped into an iced petri plate, and decapitated at the juncture of neck and thorax with a methanol-washed razor blade.

Those insects receiving a full blood meal were decapitated at 5, 10, 15, 30, 60, 240, and 360 minutes after the blood meal. Insects interrupted 1 1/2 min after beginning the blood meal were used to obtain less than or equal to 2 minute interrupted-blood-meal (IBM) decapitees. The 30 second difference in times was accounted for by processing and decapitation procedures. Insects interrupted in

blood-feeding at 3 minutes and 7 minutes after beginning the blood meal provided less than or equal to 4 and 8 minute decapitees, respectively. Cessation of probing behavior was used as a marker for beginning of the blood-meal. Average length of time to obtain a full blood-meal was 10 minutes. Control insects were obtained by the same procedures but were not decapitated.

After decapitation, mosquitoes were attached by the wings to tape on the convex surface of a watch glass. The watch glass was inverted (convex surface facing down) and placed over a 4 oz. jar containing water-saturated surgical cotton. The insects were held in the rearing room and the watch glass was lifted at 12 hour intervals to provide air exchange.

Forty-eight hours after the blood meal, ovaries were removed from all mosquitoes and follicle growth was measured as described in section 2.2.2.

2.3 Chemicals and Reagents

Morpholinopropanesulfonic Acid (MOPS) was from Sigma, St. Louis, Mo., and was made up to 0.05 M, pH 7.2, with 1 mM EDTA, unless specifically stated otherwise.

NADPH-Regenerating System contained 10 units/ml glucose-6-phosphate dehydrogenase, 106 mM glucose-6-phosphate and 3.8 mM $NADP^+$ in MOPS buffer.

Dieldrin and Endrin were from Chem Service, West Chester, PA. Aldrin was from Shell Biosciences Laboratories, Sittingbourne, Kent ME9 8AG, England.

Phenylmethanesulfonyl fluoride (PMSF) and 7-methoxy-4-methylcouomarin were from Calbiochem, La Jolla, CA.

7-methoxycoumarin, 7-ethoxycoumarin, and 7-hydroxycoumarin were from Aldrich, Milwaukee, Wi.

Resorufin and methoxyresorufin were from Molecular Probes, Inc., Junction City, OR.

20-hydroxyecdysone was from Simes, Milan, Italy.

[23-24-^{3}H]ecdysone was from New England Nuclear, Boston, Ma.

Ethylene glycol monomethyl ether (methyl cellosolve) was from Sigma.

2.4. Preparation of Microsomes

Microsomes were prepared by two different techniques. The differential centrifugation method was used to prepare large-scale batch preparations of microsomes for characterization of aldrin epoxidation and methoxyresorufin O-demethylation activities and Cytochrome P-450 content determinations. A second method, involving separation of microsomes from tissue homogenates by centrifugation on sucrose density-gradients, was developed for use in investigating the developmental profile of aldrin epoxidation activity.

2.4.1. Preparation of Microsomes by Differential Centrifugation

2.4.1.1. For Aldrin Epoxidation Characterization and Cytochrome P-450 Content Determination

For aldrin epoxidation characterization, 100 to 200 abdomens from mosquitoes given a blood meal 72 hours previously were dissected

in Lum's mosquito saline. All procedures following dissection were performed at 0 to 4°C. The midguts and ovaries were removed and the abdomens were homogenized in 20 ml of MOPS buffer containing 400 µm PMSF as a protease inhibitor with 10 strokes of a motor-driven teflon pestle in a Thomas 50 ml glass tissue grinder. The homogenate and a 5 ml MOPS rinse of the grinding vessel and pestle were poured into a 25.5 ml centrifuge tube which was centrifuged in a Beckman Type 30 rotor at 1,000 g for 15 min. The supernatant was filtered through glass wool and centrifuged at 10,000 g for 15 minutes to pellet "mitochondria." The supernatant was again filtered through glass wool and centrifuged at 105,000 g for 65 minutes. The "microsomal" pellet was resuspended in 30% sucrose/MOPS.

2.4.1.2. For Methoxyresorufin O-demethylation Characterization

Approximately 500 to 1,000 mosquitoes (mixed 72 hour after the blood meal and Day 10 non-blood-fed) were placed in a 400 ml beaker containing about 100 glass boiling beads and 200 cc of crushed dry ice. The beaker was covered with aluminum foil and shaken vigorously by hand for 10 seconds to separate abdomens from the mosquitoes. The abdomens were removed from the mixture with forceps and dropped into a Thomas 50 ml glass tissue grinder held in a beaker of crushed dry ice. The grinding vessel and collected tissue were warmed to 0°C in an ice-water bath and microsomes were prepared as described in section 2.4.1.1. The "microsomal" pellet was resuspended in 30% sucrose/MOPS containing 400 µm PMSF.

2.4.2. Preparation of Microsomes by Sucrose Density-Gradient Ultracentrifugation

2.4.2.1. For Developmental Profile of Aldrin Epoxidation and NADPH-Cytochrome c Reductase Activities

Abdomens of appropriately aged mosquitoes were removed in Lum's mosquito saline and midguts were pulled out with forceps and discarded. Maturing ovaries were also removed from blood-fed mosquitoes. The abdomens were homogenized in 0.3 ml of MOPS buffer containing 400μm PMSF in a 2 ml Wheaton glass tissue homogenizer for 30 seconds with a motor-driven teflon pestle. The homogenate was centrifuged for 10 min at 1,000 g in a Beckman Microfuge II and the supernatant below the lipid layer was removed with a finely drawn-out pasteur pipet. After vortexing, three 5 μl aliquots of supernatant were removed for determination of protein concentration.

A sucrose gradient consisting of 1.25 ml volumes of 45, 35, 25, and 15% sucrose/MOPS solutions in a Beckman Quick-Seal 5.3 ml centrifuge tube was prepared. The gradient was linearized by horizontal reorientation for 2 hours at 4°C. A volume of tissue homogenate supernatant containing 1 mg or less protein was loaded on top of the gradient and the gradient was centrifuged at 65,000 RPM for 20 minutes (average $w^2t = 3.7 \times 10^{10}$) using a Beckman VTI-80 rotor in a Beckman L8-80 preparative ultracentrifuge. An 18 gauge syringe needle was inserted in the bottom of the centrifuge tube and the top of the tube was punctured with a 22 gauge needle. A 2,000 μl fraction of sucrose solution was collected and discarded as "mitochondria".

The next 2,150 µl of sucrose solution was collected and retained for assay as the "microsomal" fraction. The remaining portion of sucrose solution from the top of the gradient contained cytosolic proteins and was discarded. All procedures, except dissection of insects, were performed at 0 to 4°C.

2.4.3. Incorporation of ^{35}S-labelled Methionine Into Sub-Cellular Fractions as Followed by Sucrose Density-Gradient Fractionation of Tissue Homogenates

Approximately 50 mosquitoes (6 days after emergence) were micro-injected with 7 x 10^6 CPM of ^{35}S-labelled methionine and kept for 6 hours. Abdomens were then removed from the insects and homogenized in 0.25 ml of MOPS buffer for 30 seconds in a 2 ml Wheaton glass tissue homogenizer with a motor-driven teflon pestle. The homogenate was centrifuged at 1,000 g for 10 minutes and a 5 µl aliquot of the supernatant was removed for a liquid scintillation count of radioactivity. The remaining supernatant was loaded on a sucrose gradient and centrifuged at 65,000 RPM for 20 minutes in a Beckman VTI-80 rotor as described in section 2.4.2.1. A total of 15 fractions of 350 µl each were collected from the gradient and 5 µl aliquots of each fraction were counted for radioactivity in Aquasol (New England Nuclear) on a Searle Isocap/300 liquid scintillation counter. Data were plotted as CPM per fraction and were correlated with protein concentration and enzymatic activity data per fraction collected from gradients loaded with non-radioactive insect tissue homogenates.

2.5. Protein Concentration Assay

Protein concentration was determined by the method of Bradford (1976). Gamma-globulin in MOPS buffer was used as standard in concentrations of 2.5, 5.0, 7.5, 10.0, 15.0, 20.0, and 30.0 µg/100 µl. The assay utilizes binding of Coomasie Brilliant Blue G-250 dye to protein as monitored by increase in absorption at 595 nm. It is sensitive to protein concentrations as low as 10 to 20 µg per ml and is not affected by sucrose. A Beckman DB instrument was used for all determinations.

2.6. NADPH-Cytochrome c Reductase and Succinate-Cytochrome c Reductase Assays

2.6.1. NADPH-Cytochrome c Reductase Assay

NADPH-cytochrome c reductase activity was measured at 30°C with an Aminco DW-2a recording spectrophotometer in the double beam mode by the method of Williams and Kamin (1962) with the following modifications:

Cytochrome c was 50µM rather than 34 µM. Buffer was 0.05 M MOPS, pH 7.2, containing 1 mM EDTA. One ml of MOPS, 0.4 ml of NaCN/H_2O (5 mM), 0.4 ml of cytochrome c and 200 µl of microsomes in 30% sucrose/MOPS were mixed in a test tube and incubated in a 30°C water bath for one minute. Equal volumes of the solution were pipetted into reference and sample cuvettes and a baseline of difference in absorption at 550 nm was recorded. Reduction of cytochrome c was initiated in the sample cuvette by addition of 50 µl of NADPH-regenerating system (see section 2.3) and difference in absorption

between the cuvettes at 550 nm was recorded over an average period of 2 1/2 minutes.

2.6.2. Succinate-Cytochrome c Reductase Activity

Succinate-cytochrome c reductase activity was measured by the procedure of section 2.6.1., with the exception that reactions were initiated by addition of 10 µl of 1 M succinate rather than NADPH-regenerating system.

2.7. Aldrin Epoxidation Assay with Endrin as Internal Standard

2.7.1. Aldrin Epoxidation Incubations

Incubations were conducted in 1.5 ml vials at 30°C for 15 or 20 minutes in a shaker-type water bath. Incubation mixtures contained 28 µl NADPH-regenerating system, 50 to 200 µl of microsomes in sucrose/MOPS solution, 7 µl of 1 mM aldrin in methylcellosolve (final concentration - 20 µM) and MOPS buffer to a final volume of 350 µl. Reactions were initiated by addition of aldrin.

The reactions were stopped with 28 µl of 1 M HCl and the incubation mixtures were immediately placed on ice. A 35 µl volume of a 9% w/v $NaCl/H_2O$ solution was added to suppress emulsion formation in the next step. A glass capillary micropipet was used to add 100 µl of ice-cold hexane containing 0.5 µM endrin and the incubation vials were covered with parafilm and vortexed at medium speed for 20 seconds to extract dieldrin. The incubation mixtures were returned to the ice bath and approximately 10 mg of Na_2SO_4 was added and followed by a 5 second vortexing. The hexane phase (containing endrin and extracted

aldrin and dieldrin) and part of the aqueous phase were transferred to 0.6 ml glass tubes (4 mm inside diameter) with a clean capillary micropipet. The phases were separated by a 2 minute centrifugation at 4°C and the tubes were returned to the ice bath.

2.7.2. Assay of Aldrin Epoxidation Incubations for Dieldrin

Two µl aliquots of the hexane phase were assayed for dieldrin by gas chromatography with a Varian series 2700 instrument equipped with a tritium electron capture detector. Operating parameters were as follows: Carrier Gas was N_2; Injection port 237°C; Column 197°C; Detector 217°C; Attenuation - 8X; the column (5' x 1/8") was packed with a 1:1 ratio of 5% DC11 and 5%QFL on 100-120 mesh High Performance Chromosorb W. Baseline separation of dieldrin and endrin (retention times - 7 and 8 minutes, respectively) was obtained with a gas pressure of 52 lbs/sq. inch. Peak heights of dieldrin and endrin were measured in mm and the ratio of dieldrin to endrin peak heights was referred to a standard curve in order to quantify dieldrin yield.

2.7.3. Standard Curve for Quantification of Dieldrin

The following procedure was used to develop a standard curve in order to quantify dieldrin yields by use of an endrin internal standard:

A dieldrin in methylcellosolve concentration series was prepared: 0.79, 1.30, 1.83, 2.62 µM. A hexane solution containing 0.50 µM endrin and 70.0 µM aldrin was also prepared.

Duplicate mock 350 µl aldrin epoxidation incubation mixtures were prepared for each dieldrin molarity described above. They contained 8.5 µg of gamma-globulin in 85 µl of MOPS (as a substitute for protein normally present in microsomes), 130 µl of 25% sucrose/MOPS, 100 µl of MOPS, 28 µl of NADPH-regenerating system and 7 µl of dieldrin in methylcellosolve. The mock incubation mixtures were incubated, extracted, and assayed as described in sections 2.7.1. and 2.7.2. This procedure was performed 4 times and, since endrin was present in the hexane extraction solution at a constant molarity of 0.5 µM while dieldrin was present in a range of 4 molarities, a linear series of dieldrin to endrin ratios was obtained after extraction of the mock incubation mixtures. The standard curve (Fig. 7) was plotted as the ratio of dieldrin to endrin peak heights versus molarity of dieldrin. In subsequent experimental work involving microsomal incubations, no dieldrin to endrin peak height ratios exceeding the highest ratio on the standard curve were obtained.

2.8. Alkoxycoumarin and Methoxyresorufin O-dealkylation Assays

2.8.1. Alkoxycoumarin O-dealkylation Assays

The procedure of Matsubara et al. (1983) was used for alkoxycoumarin O-dealkylation assays with some modifications. Incubation mixtures contained microsomes in sucrose/MOPS solution, 30 µl of NADPH-regenerating system, 10 µl of substrate in ethanol and MOPS buffer to a final volume of 0.5 ml. Final substrate concentrations in incubation mixtures of 7-methoxycoumarin, 7-ethoxycoumarin, and 7-methoxy-4- methylcoumarin were 40, 400, and

400 µM, respectively. Reactions were initiated by addition of substrate and incubations were performed in 1.5 ml Eppendorf tubes in a shaker-type water bath at 30°C for periods of 5 to 20 minutes. Reactions were stopped by addition of 330 µl of 4% (w/v) perchloric acid. After vortexing, 170 µl of 8.5% (w/v) K_2CO_3 was added and the mixture was vortexed again. Denatured proteins were precipitated by a 5 min 1,000 g centrifugation.

For assay of product, 7-hydroxycoumarin, a 200 µl aliquot of supernatant was added to 2.5 ml of 0.2 M carbonate-bicarbonate buffer (pH 10) and mixed. Fluorescence emission of this solution at 450 nm, excitation 370 nm, was measured with a Perkin Elmer 650-10S fluorescence spectrophotometer thermostatted at 30°C (slit widths: $3nm_{ex}$, $5\ nm_{em}$) and was recorded as millivolts output as registered by a Lutron Digital Multimeter.

Quantity of 7-hydroxycoumarin formed in incubation mixtures was calculated, after correction for dilution factors, from a standard curve of fluorescence emission of known quantities of 7-hydroxycoumarin.

2.8.2. Methoxyresorufin O-demethylation Assay

Methoxyresorufin O-demethylation activity was measured by the same procedure as the coumarin O-dealkylation assays but substrate in ethanol was 20 or 40 µM final and reactions were stopped with 50 µl 1 N HCl followed by 450 µl 8.5% (w/v) K_2CO_3. Excitation and emission wavelengths were 560 nm and 583 nm, respectively. These wavelengths were determined to be the optimum values based on analysis

of excitation and emission wavelength scans of resorufin over a range of 400 nm to 640 nm.

Quantity of resorufin formed in incubations was calculated, after correction for dilution factors, from a standard curve of fluorescence emission of known quantities of resorufin.

2.9. Radioimmunoassay of Ecdysteroids

2.9.1. Collection of Tissue

Mosquitoes were reared and blood-fed as described in sections 2.1 and 2.2.1, respectively, with the exception that all blood-fed mosquitoes in a particular sample received full blood-meals within 1 hour of each other. Samples of mosquitoes were removed from the rearing cages by aspirator, anesthetized with CO_2, sorted, and counted in an iced petri plate, and frozen at -80°C till homogenization and extraction of ecdysteroids. All handling procedures were performed with methanol-washed equipment.

Samples were collected for the following time points: non-blood-fed mosquitoes aged Days 1, 2, 3, 4, 5, and 6 after emergence and blood-fed mosquitoes at 6 hour intervals from 6 to 78 hours after the blood meal.

2.9.2. Extraction and Purification of Ecdysteroids

An adaptation of the method of Lafont et al. (1982) was used to extract and purify ecdysteroids from whole-body tissue homogenates of mosquitoes. Samples of mosquitoes were homogenized for 30 seconds in a solution of 5 ml H_2O/5 ml $CHCl_3$ with a PT10 Polytron tissue

homogenizer at the 7 setting. The probe, a PT10ST, was rinsed with 5 ml H_2O after homogenization of each sample.

Aqueous and organic phases in the homogenized samples were separated by centrifugation and the water phase, containing ecdysteroids, was saved. The 5 ml H_2O rinse of the polytron probe saved previously, was poured into the separated $CHCl_3$ phase and it was re-extracted for ecdysteroids by a 10 second vortexing. The phases were again separated by centrifugation and the two ecdysteroid-containing H_2O phases were pooled, heated to 65°C, and held at that temperature for 10 minutes. After cooling, they were centrifuged to sediment precipitate.

A crudely purified ecdysteroid preparation was obtained by use of Sep-Pak C_{18} cartridges (Waters Associates) that had been pre-conditioned by passage of 2 ml 100% CH_3OH followed by 5 ml H_2O. The H_2O extracts of tissue homogenates were loaded into 10 ml syringes and passed through the Sep-pak cartridges at a flow rate not exceeding 2 ml/minute. The eluant was discarded. Substances more polar than 20-hydroxecdysone were eluted from the Sep-paks with 5 ml 25% CH_3OH. Ecdysteroids were eluted from the Sep-paks with 5 ml 60% CH_3OH which was collected and stored in 10 ml screw-cap vials at -80°C.

2.9.3. Radioimmunoassay of Ecdysteroids

Aliquots of the 60% CH_3OH extract were lyophilzed in 1.5 ml Eppendorf tubes. The dried extract was taken up in 100 µl borate buffer (0.1 M, pH 8.4 with 0.075 M NaCl) containing approximately

8,500 cpm of [23,24-^{3}H] ecdysone (70 Ci/mmole). After vortexing, 100 µl of 1% ecdysone antiserum was added (Horn I-3, 16 week serum; gift of Dr. J. D. O'Connor, UCLA; diluted with borate buffer). The mixture was vortexed and incubated at 4°C for 24 hours. Following incubation, free and antiserum-bound ecdysteroids were separated by dextran-coated charcoal adsorption as described by Feyereisen (1980, 1984). A suspension of 1% charcoal, 0.1% dextran (in borate buffer) was added vol/vol and the mixture was vortexed for 5 seconds. Five minutes after addition of the charcoal suspension, dextran-coated charcoal was removed by a 5 minute 1,000 g centrifugation. A 300 µl aliquot of supernatant was assayed for radioactivity by liquid scintillation counting in 3 ml of Aquasol (NEN).

Fifty percent inhibition of antibody-binding to tracer, [23,24-^{3}H] ecdysone, required 0.48 pmole ecdysone or 1.65 pmole 20-hydroxyecdysone. The amount of ecdysteroids in the extract was calculated from a standard curve obtained with 20-hydroxyecdysone. The results are therefore expressed as 20-hydroxyecdysone equivalents.

2.10. Estimation of Microsomal Cytochrome P-450 Content

Microsomes were prepared by differential centrifugation as described in section 2.4.1.1., but the "microsomal" pellet was resuspended in 10% sucrose/MOPS solution. Cytochrome P-450 content was determined by the difference spectrum technique of Omura and Sato (1964) with an Aminco DW-2a recording spectrophotometer in the split beam mode and thermostatted at 12°C. One ml cuvettes were used.

2.11. Phenobarbital Induction Experiments

A cohort of mosquitoes was deprived of 10% sucrose/H_2O solution on the fourth day after emergence. Twenty-four hours later they were divided into two groups and one group was given 10% sucrose solution while the other group was given 0.1% (w/v) phenobarbital in 10% sucrose solution. At 12, 24, and 48 hour intervals following return of the sucrose solutions, 40 mosquitoes were removed from the control and phenobarbital-treated groups and microsomes were prepared from their abdomens with midguts intact by the sucrose density-gradient technique described in section 2.4.2.1.

Aldrin epoxidation activity of the microsomes was assayed by the procedure of Moldenke and Terriere (1981). NADPH-cytochrome c reductase activity was assayed as described in section 2.6.1.

RESULTS

3.1. Development of a Sucrose Density-Gradient Ultacentrifugation Technique for Preparation of Microsomes

In view of reports that cross-contamination of mitochondrial and microsomal pellets occurs in insect tissue homogenates subjected to sub-cellular fractionation by differential centrifugation (Brindley and Dahm, 1970; Benke and Wilkinson, 1971; Benke et al., 1972; Feyereisen and Durst, 1978) and a recent demonstration that mitochondria are associated with a morphologically and biochemically distinct portion of the endoplasmic reticulum in rat liver cells (Meier et al., 1981) experiments were undertaken to develop an improved method for preparation of microsomes.

3.1.1. Development of Technique

Development of a sucrose density-gradient ultracentrifugation technique for preparation of microsomes was initiated with experiments designed to discover amounts and concentrations of sucrose/MOPS solutions and centrifugation parameters which would form a linear gradient over the density range considered likely to separate microsomes from a tissue homogenate. After centrifugation, fractions were collected from gradients for analysis. Of the methods tested for fraction collection (including various combinations of syringe needles and micropipets inserted in top and bottom of centrifuge tubes and used with or without pressure or vacuum exerted by a peristaltic pump), insertion of an 18 or 20-gauge needle in the bottom of the tube

and collection of gravity-induced drops after puncturing the top of the tube with a 22-guage needle was chosen as the most efficient technique. Fifteen fractions, each containing approximately 350 µl, were collected from experimental gradients loaded with cockroach fat-body tissue homogenates, mosquito abdomen tissue homogenates or housfly "microsomes" prepared by differential centrifugation. These fractions were routinely analyzed for protein content and sucrose density. In addition, NADPH-cytochrome c reductase and succinate-cytochrome c reductase activities were assayed as enzymatic markers for presence of microsomes and mitochondria, respectively.

Gradients prepared from 1.25 ml volumes of 50, 40, 30, and 20% or 45, 35, 25, and 15% sucrose/MOPS solutions were centrifuged 20 minutes at speeds ranging from 30,000 to 75,000 RPM. Gradients prepared with the 50 to 20% solutions and centrifuged at 55,000 RPM (230,000 g; $w^2t = 2.76 \times 10^{10}$) or at 75,000 RPM (425,000 g; $w^2t = 4.70 \times 10^{10}$) and gradients prepared with the 45 to 15% sucrose solutions and centrifuged at 65,000 RPM (310,000 g; $w^2t = 3.70\ 10^{10}$) produced the best sub-cellular fractionations of tissue homogenates (Figs. 1, 2, 3). A comparison of the percent distribution of mitochondria and microsomes throughout the sub-cellular fractions of the data plotted in these figures is shown in Table 1. Analysis of Table 1 shows that the 45 to 15% sucrose solution gradient centrifuged at 65,000 RPM was clearly superior to the other two techniques for separating a microsomal fraction with as little mitochondrial contamination as possible from a tissue homogenate. While all 3 procedures focused about 50% of the NADPH-cytochrome c reductase

activity in the microsomal fraction (defined in Table 1 footnotes), only about 18% of the mitochondria as determined by succinate-cytochrome c reductase activity were present in the microsomal fraction of the 65,000 RPM gradient, while 50 and 45% of the mitochondria were present in the microsomal fractions of the gradients centrifuged at 55,000 and 75,000 RPM, respectively. An analysis of four 65,000 RPM gradients loaded with similar quantities of tissue homogenate protein showed that 53.6% ± 5.5 (S.E.M.) of the NADPH-cytochrome c reductase activity was focused in the microsomal fraction while 22.8% ± 7.5 of the succinate-cytochrome c reductase activity was present in this fraction. In two 65,000 RPM gradients in which aldrin epoxidation activity was used as a marker for presence of microsomes 84.1% ± 8.3 of the total epoxidation activity was present in the microsomal fraction while 20.0% ± 2.2 of the succinate-cytochrome c reductase activity was in this fraction. The 65,000 RPM gradient prepared with 45 to 15% sucrose solutions was therefore chosen for preparation of microsomes in all further work.

In order to further optimize microsomal separation, the effect of amount of protein loaded on the gradient on efficiency of microsomal separation was investigated. Protein loads above 1 mg caused the mitochondrial peak to "tail-off" in the "microsomal" fractions. In subsequent work, gradients were loaded with a volume of tissue homogenate containing 1 mg or less protein.

Tissue homogenates were subjected to a 1,000 *g* sedimentation of cell debris and supernatant only was loaded on gradients. Following sedimentation of cell debris, a thin layer of lipid was usually

present on top of the supernatant, and it was observed that contamination of supernatant by this lipid caused erratic distribution of "mitochondria" thoughout the gradient after centrifugation (Fig. 4). Lipid contamination was avoided by careful use of finely drawn-out pasteur pipets to remove supernatant from the 1,000 *g* sedimentation of cell debris.

Table 1. Percentage Distribution of Mitochondria and Microsomes in Sub-Cellular Fractions[1] of Mosquito Tissue Homogenates Fractionated by Sucrose Density-Gradient Ultracentrifugation.

	Percentage Distribution of Succinate-Cytochrome c Reductase Activity			Percentage Distribution of NADPH-Cytochrome c Reductase Activity		
RPM	"Mitochrondrial" Fraction	"Microsomal" Fraction	"Cytosolic" Fraction	"Mitochondrial" Fraction	"Microsomal" Fraction	"Cytosolic" Fraction
55,000[2]	41.4	50.2	8.4	7.5	50.2	42.3
65,000[3]	81.6	18.4	0	27.2	49.6	23.2
75,000[2]	52.7	45.4	1.9	13.4	53.7	32.9

1. The "mitochondrial" fraction consisted of the first 2,000 µl collected from the bottom of the gradient; the next 2,150 µl collected was designated the "microsomal" fraction and the remaining 1,150 µl from the top of the gradient was designated as the "cytosolic" fraction.

2. Linear gradient formed from equal volumes of 50, 40, 30, and 20% sucrose/MOPS.

3. Linear gradient formed from equal volumes of 45, 35, 25, and 15% sucrose/MOPS.

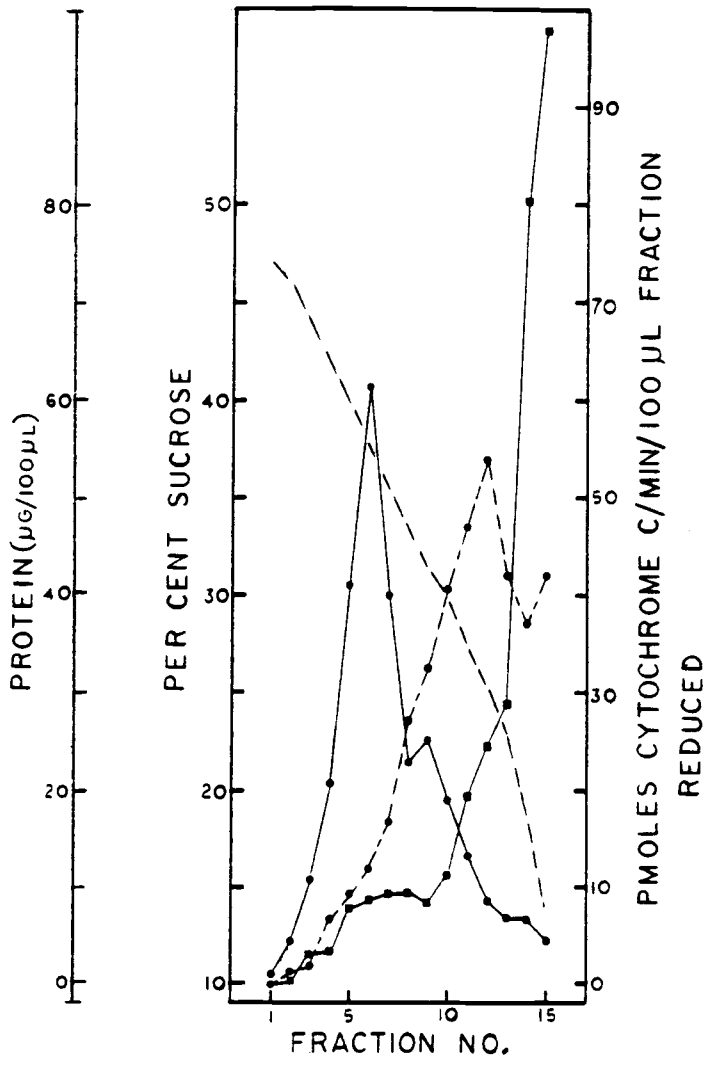

Fig. 1. Characterization of a 55,000 RPM ($w^2t = 2.76 \times 10^{10}$) sucrose density-gradient. Gradient was formed with 50, 40, 30, and 20% sucrose/MOPS solutions and loaded with 0.3 ml 1,000 g supernatant of mosquito abdomen tissue homogenate. (●—●) Succinate-cytochrome c reductase activity; (●--●) NADPH-cytochrome c reductase activity; (■—■) protein concentration; (- - -) relative density as per cent sucrose.

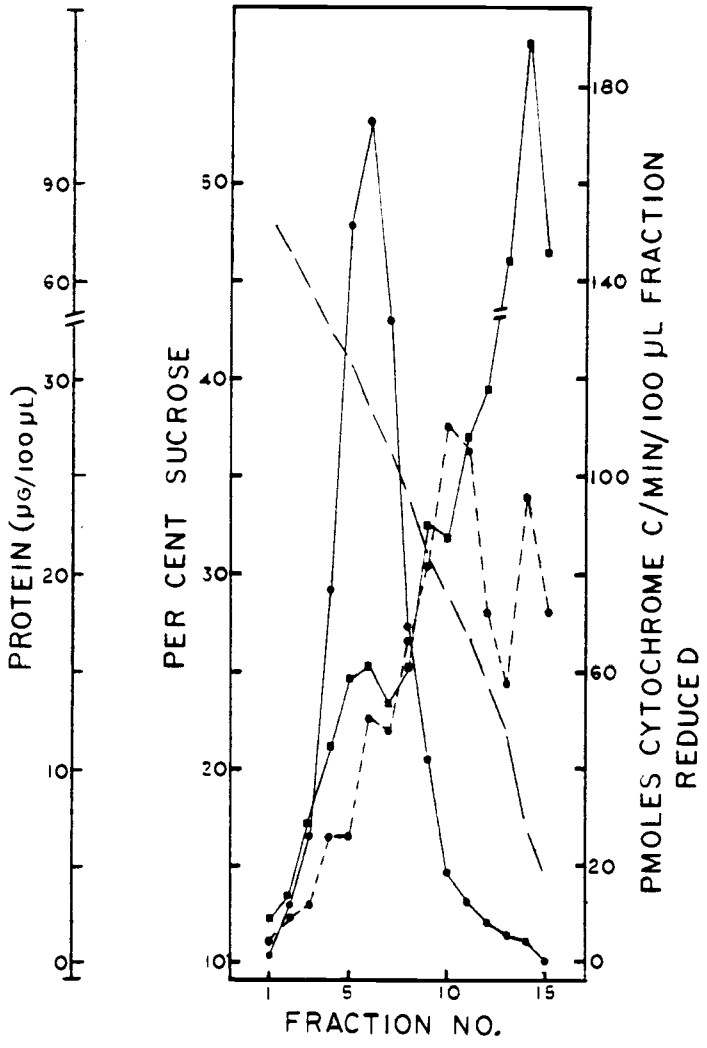

Fig. 2. Characterization of a 75,000 RPM ($w^2t = 4.7 \times 10^{10}$) sucrose density-gradient. Gradient was formed with 50, 40, 30, and 20% sucrose/MOPS solutions and loaded with 0.3 ml 1,000 g supernatant of mosquito abdomen tissue homogenate. (●—●) Succinate-cytochrome c reductase activity; (●--●) NADPH-cytochrome c reductase activity; (■—■) protein concentration; (- - -) relative density as per cent sucrose.

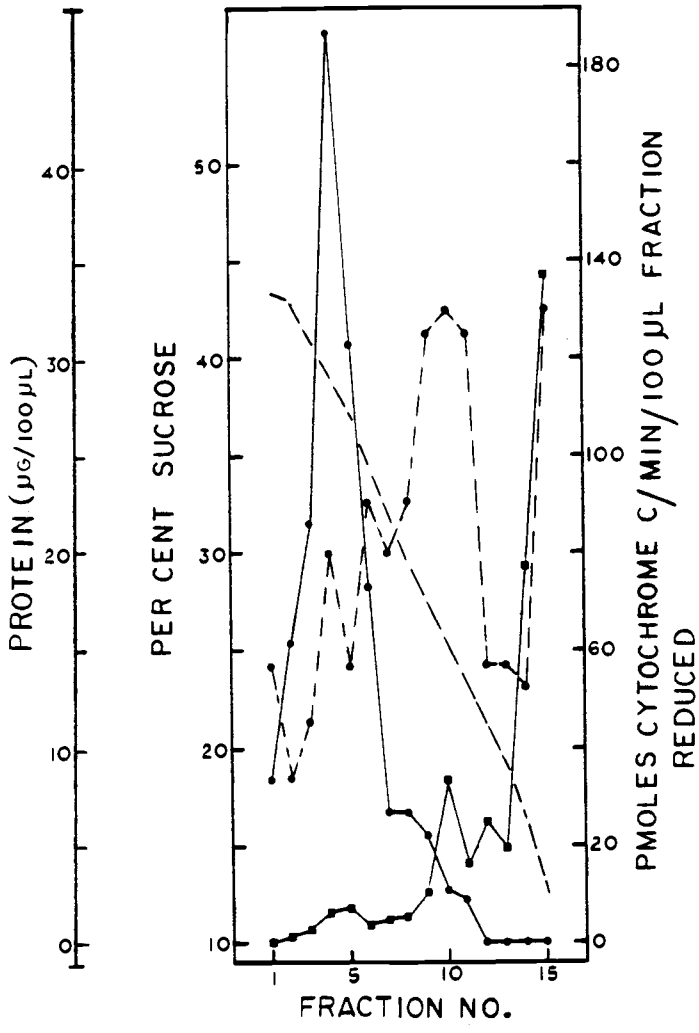

Fig. 3. Characterization of a 65,000 RPM ($w^2t = 3.7 \times 10^{10}$) sucrose density-gradient. Gradient was formed with 45, 35, 25, and 15% sucrose/MOPS solution and loaded with 0.3 ml 1,000 g supernatant of mosquito abdomen tissue homogenate. (●—●) Succinate-cytochrome c reductase activity; (●-·-●) NADPH-cytochrome c reductase activity; (●—●) protein concentration; (- - -) relative density as per cent sucrose.

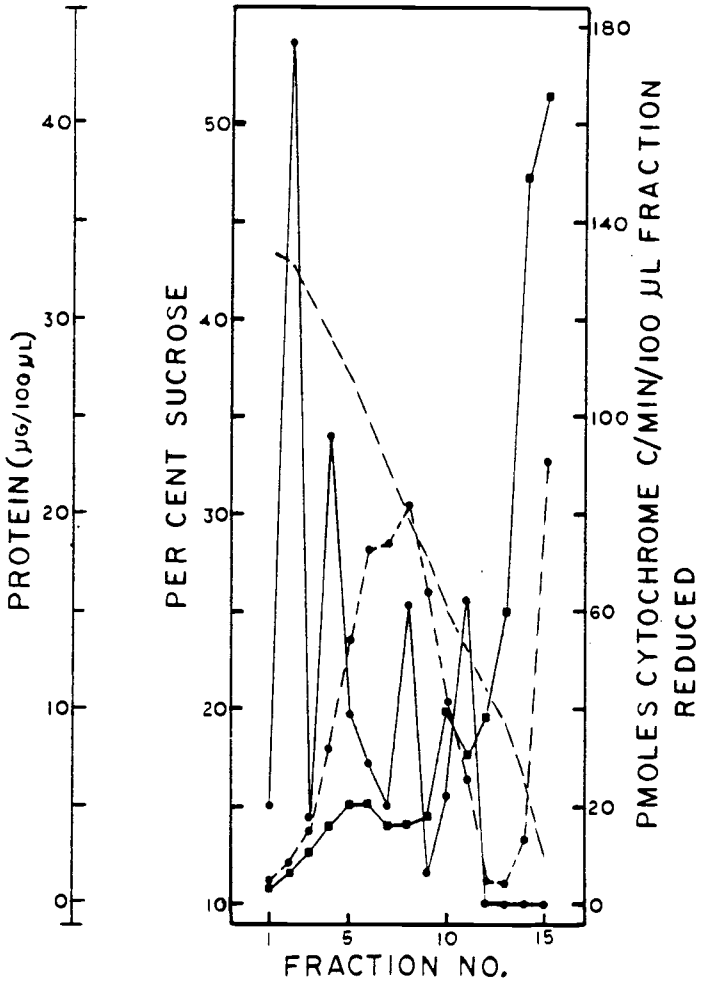

Fig. 4. Effect of lipid contamination in tissue homogenate supernatant on separation of sub-cellular fractions by a 65,000 RPM ($w^2t = 3.7 \times 10^{10}$) sucrose density-gradient centrifugation. Gradient was loaded with 200 µl of 1,000 g tissue homogenate supernatant (600 µg protein) and 30 µl of lipid-layer supernatant. (●—●) Succinate-cytochrome c reductase activity; (●--●) 2.5 x NADPH-cytochrome c reductase activity; (●—●) protein concentration; (---) relative density as per cent sucrose.

3.1.2. Incorporation of ^{35}S-Labelled Methionine Into Sub-Cellular Fractions as Followed by Sucrose Density-Gradient Fractionation of Tissue Homogenates

Mosquitoes were injected with ^{35}S-methionine and tissue homogenates were prepared and centrifuged as described (2.4.3.). Following collection of fifteen 350 µl fractions from the centrifuged gradients, aliquots were removed for a liquid scintillation count of radioactivity. Figure 5 shows a plot of data from one of four experiments. A comparison of Fig. 5 with Fig. 3, demonstrates a close correlation of counts incorporated in each fraction with distribution of sub-cellular fractions in the gradients as determined by enzymatic marker and protein assays. Fractions 12-15 were presumed to contain cytosolic proteins and non-incorporated ^{35}S-methionine.

These experiments were initiated in conjunction with *Culex pipiens* mRNA *in vitro* translation experiments. Aliquots of the separated sub-cellular fractions were incubated with house fly NADPH-cytochrome P-450 reductase antibody before separation of proteins by gel electrophoresis. The *in vitro* mRNA translation mixtures were similarly treated with antibodies and electrophoresed and results of the two procedures were compared for presence of identical protein bands on the gels. However, data obtained from the electrophoresis gels were ambiguous and the work was not continued.

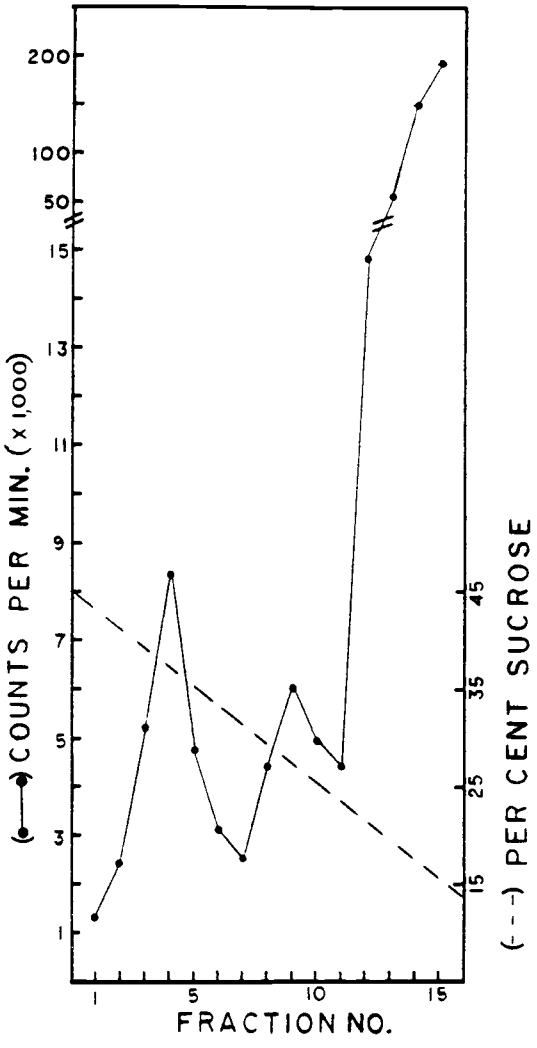

Fig. 5. Incorporation of ^{35}S-labelled methionine into subcellular fractions as followed by sucrose density-gradient fractionation of a mosquito abdomen tissue homogenate. Mosquitoes were homogenized 6 hours after injection of 6×10^6 cpm of ^{35}S-methionine.

3.2. Characterization of Aldrin Epoxidation Activity

Several types of experiments were done to characterize aldrin epoxidation activity by mosquito microsomes. The goal of these experiments was several-fold. It was of obvious importance to determine the optimum conditions in aldrin epoxidation incubation mixtures for aldrin, substrate, and "microsomal" protein concentrations, incubation times, and pH, as well as demonstrating that dieldrin formed in incubation mixtures was due to enzymatic activity and was not an artifact of reagent conditions. A second goal was to obtain estimates of apparent K_M for aldrin and pH dependency of aldrin epoxidation which could be used to make comparisons with data published in the literature for this activity in microsomes prepared from other organisms.

A third goal was to obtain evidence in support of the supposition that aldrin epoxidation in mosquito microsomes is in fact a cytochrome P-450 dependent activity. This goal was partially met by inhibition experiments with antibody to house fly NADPH-cytochrome P-450 reductase. A pilot experiment in which carbon monoxide was used as an epoxidation inhibitor was less successful but gave preliminary evidence of CO inhibition and held promise of success for further work with a better incubation apparatus.

Dieldrin produced in aldrin epoxidation incubations was assayed by gas chromatography with endrin as an internal standard. A representative chromatogram is shown in Fig. 6. To quantitate dieldrin, the ratio of dieldrin to endrin peak heights was referred to a standard curve shown in Fig. 7.

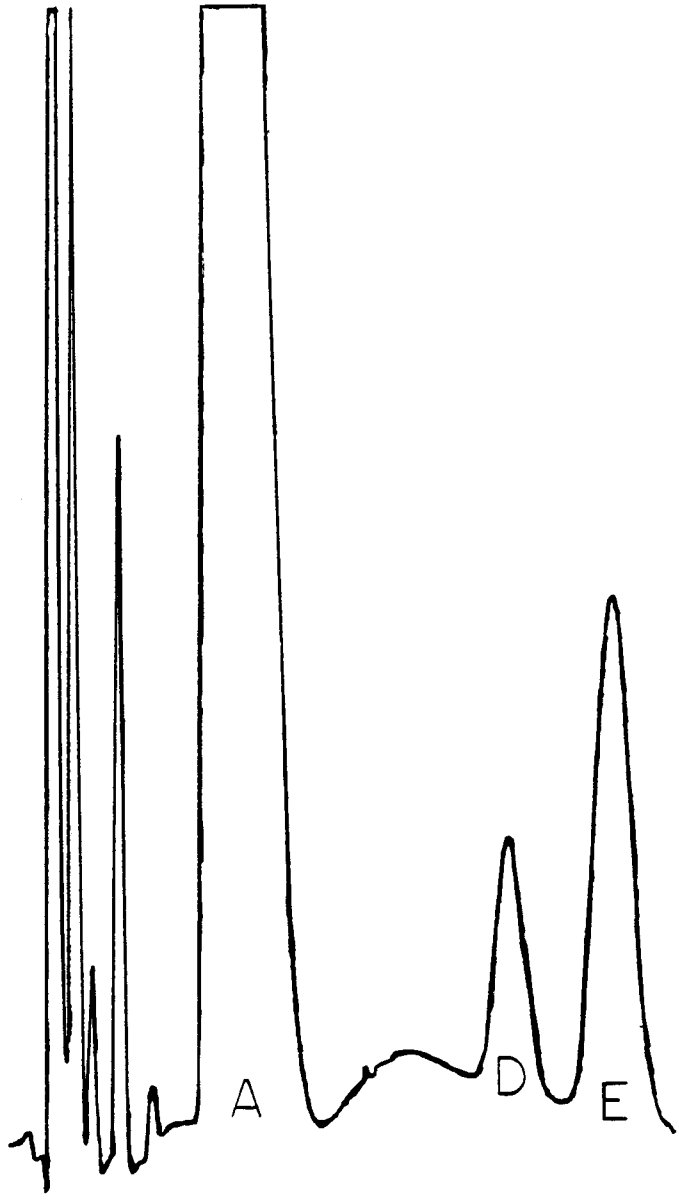

Fig. 6. Representative gas chromatogram of hexane-extracted aldrin epoxidation incubation mixture. See section 2.7.2. for instrument operating parameters. A - aldrin; D - dieldrin; E - endrin.

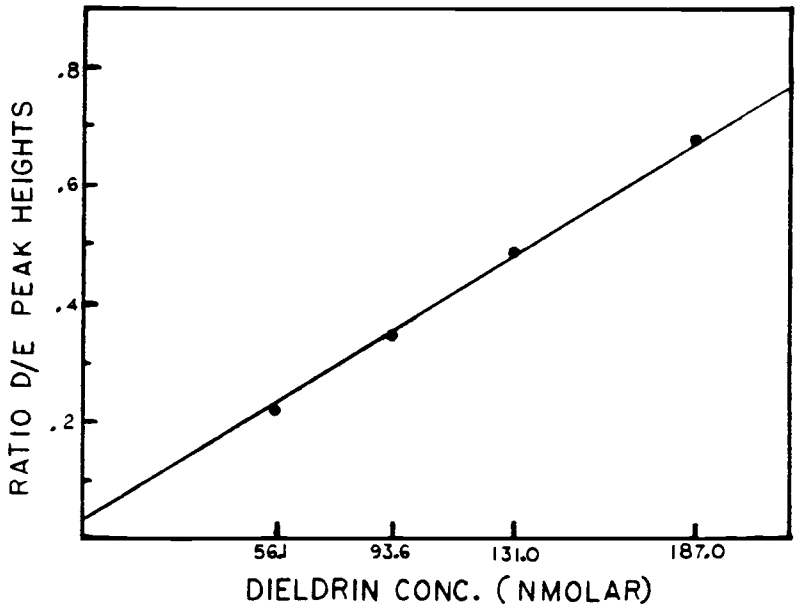

Fig. 7. Standard curve for quantification of dieldrin in aldrin epoxidation incubations. Endrin concentration was held constant at 0.5 μM and calculated dieldrin concentrations in hexane extracts of mock incubation mixtures were as shown. Each point is mean of 4 duplicate determinations. Standard errors of the means were toosmall to plot. A regression line through the points gave y = .0341266 + .003427X with an r^2 of 0.99620.

3.2.1. Effect of Microsomal Protein Concentration

Effect of "microsomal" protein concentration (as mg/ml) on quantity of dieldrin produced in aldrin epoxidation mixtures was determined with microsomes produced by two centrifugation techniques. "Microsomal" protein concentration in incubation mixtures containing microsomes produced by sucrose density-gradient centrifugation ranged from .0140 to .0560 mg/ml and from .0143 to .0857 mg/ml for incubation mixtures containing microsomes produced by differential centrifugation. Incubations were stopped after 20 minutes. An essentially linear increase in amount of product formed as "microsomal" protein concentraton was increased in incubation mixtures was observed (Fig. 8). In most subsequent experiments "microsomal" protein concentration in incubation mixtures was kept below .06 mg/ml.

3.2.2. Effect of Incubation Time

Effect of incubation time on quantity of dieldrin produced in aldrin epoxidation incubations was again investigated with microsomes produced by sucrose density-gradient and differential centrifugation. Incubation mixtures contained 16.6 µg of sucrose gradient "microsomal" protein or 9.8 µg of differential centrifugation "microsomal" protein. As seen in Fig. 9, dieldrin produced in the incubation mixtures increased with increasing time of incubation in an essentially linear fashion for the first 20 to 30 minutes with microsomes produced by both techniques. Most incubations in subsequent experiments were stopped after 20 minutes.

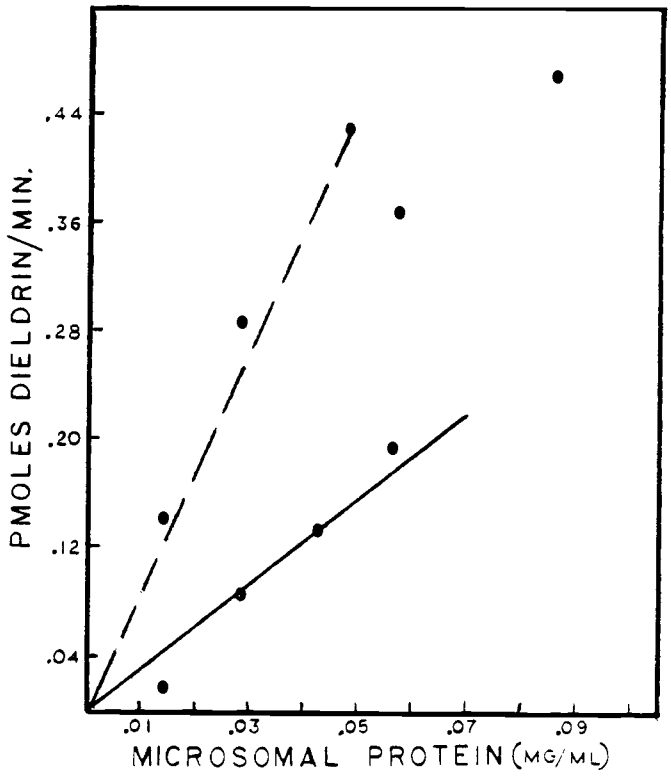

Fig. 8. Effect of "microsomal" protein concentration on aldrin epoxidation activity. Points are mean of duplicate samples. (•—•) microsomes obtained by sucrose density-gradient centrifugation; (•---•) microsomes obtained by differential centrifugation. Apparent difference in specific activity is accounted for by use of mosquitoes of different age in preparation of tissue homogenates for the two centrifugation procedures.

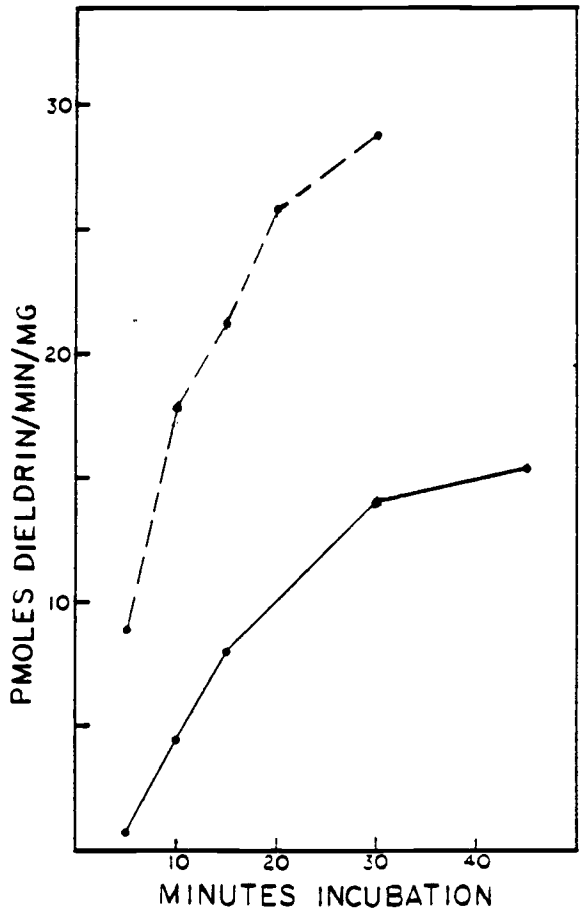

Fig. 9. Effect of incubation time on aldrin epoxidation activity. Points are mean of duplicate samples (●—●) microsomes obtained by sucrose density-gradient centrifugation; (●--●) microsomes obtained by differential centrifugation. Apparent difference in specific activity is accounted for by use of mosquitoes of different age in preparation of tissue homogenates for the two centrifugation procedures.

3.2.3. pH Dependency

The pH dependency of aldrin epoxidation activity was studied over a pH range of 4.50 to 8.45, with microsomes produced by differential centrifugation. All incubation mixtures contained 13.2 µg of "microsomal" protein pipetted in 25 µl of sucrose/MOPS soln. (pH 7.2). Final pH values of incubation mixtures were adjusted by addition of 290 µl of 0.10 M MOPS "pH modulating buffer" solutions. Titration experiments were performed to determine pH values of the modulating buffer solutions necessary to obtain the final desired pH of the incubation mixtures.

Aldrin epoxidation activity was maximal at pH 7.20 (Fig. 10). Activity fell off sharply below this pH, and was at the threshold of detection at pH 5.70. The decline in activity was not as great in alkaline incubation mixtures where it fell to about 65% of the maximum pH at 8.45. Subsequent experiments were run at pH 7.20.

The pH optimum of 7.20 for aldrin epoxidation in mosquito microsomes and the only slight decline in activity observed when the pH was raised to 7.70 is in broad agreement with pH optima for this activity found in many other insects. Shrivastava *et al.* (1971) found an optimum of about pH 7.00 for hydroxylation of N-methyl groups or N-dealkylation in larval *Culex pipiens fatigans* Wiedemann microsomal metabolism of ^{14}C-labelled propoxur, a carbamate insecticide. In general, the pH optimum for aldrin epoxidation in most insects has been found to lie in the range of pH 7.00 to 8.20 with activity declining slowly as the pH is raised (for review see Wilkinson and Brattsten, 1972).

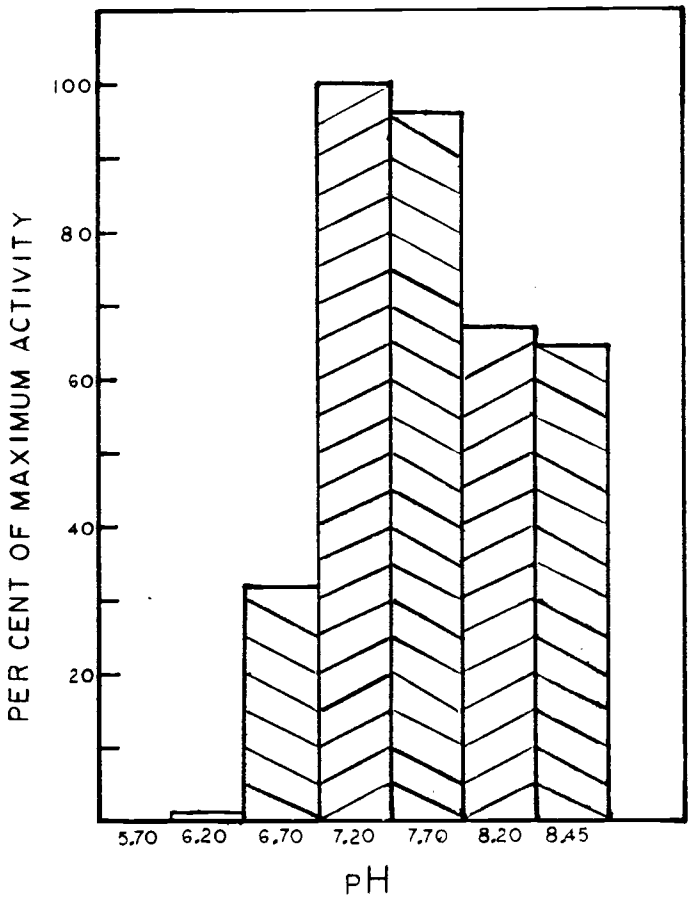

Fig. 10. Effect of pH on aldrin epoxidation activity.
Histogram bars represent mean of duplicate samples.

3.2.4. Effect of Substrate (Aldrin) Concentration

Microsomes produced by differential centrifugation were used to determine an apparent K_M for aldrin epoxidation activity. Duplicate incubation mixtures were prepared for each of 9 substrate concentrations ranging from 1.11 to 10 µM and contained 13.7 µg of "microsomal" protein. Reactions were initiated by addition of substrate and were stopped after 20 minutes. Results were plotted as a Lineweaver-Burk double reciprocal plot and as a Wolf-Augustinsson Hofstee plot wich are shown in Figs. 11 and 12. Apparent K_M values obtained from these plots were in close agreement, 3.4 and 3.1 x 10^{-6} M, respectively. From Figs. 10 and 11, apparent V_{max} values were 38 and 36 pmoles/ min/mg protein, respectively.

In an early study of aldrin epoxidation activity in microsomes prepared from the house fly (Ray, 1967), an apparent K_M for aldrin of about 2.4-2.5 x 10^{-5} M was observed when data was plotted as a Lineweaver-Burk plot. The author considered the linearity of the regression line in his Lineweaver-Burk plot surprising, because even the lowest concentration of aldrin (5.5 µM) that he used exceeded the solubility of aldrin in an aqueous incubation mixture. In a subsequent publication (Lewis *et al.*, 1967), experiments were described which indicated that the amount of dieldrin produced in an aqueous incubation mixture was dependent on the absolute amount of aldrin present and was independent of the total volume of the incubation mixture. He concluded that the true concentration of aldrin at the enzyme active site was probably dependent on its

solubility characteristics in the lipid phase of the microsomal suspension present in the incubation mixture.

In view of this possiblity, interpretation of the K_M and V_{max} values for aldrin epoxidation in mosquito microsomes and for other insects must be done with caution. The apparent K_M of 3.4×10^{-6} M for aldrin in mosquito microsomes reported in this thesis is lower than apparent K_M values of 3.5 and 5.8×10^{-6} M reported for microsome preparations from cockroach midgut caeca (Benke et al, 1972) and house cricket Malpighian tubules (Benke et al., 1971a), respectively. An apparent K_M of 5.9×10^{-5} M was reported by Krieger and Wilkinson (1969) for midgut microsomes prepared from the southern armyworm. However, midguts were excluded from tissue homogenates used to prepare microsomes in our experiments. Feyereisen (1983) has shown that "K_M" may also be dependent on the strain of insect used.

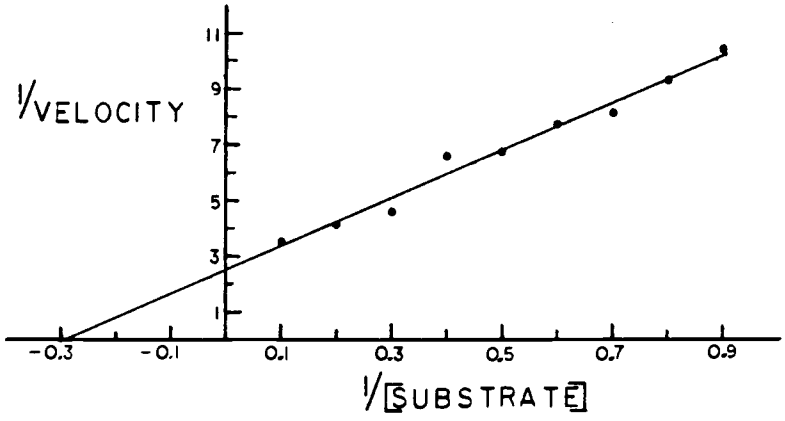

Fig. 11. Effect of substrate concentraton on aldrin epoxidation activity shown as a Lineweaver-Burk double reciprocal plot. Nine substrate concentrations ranging from 1.1 to 10.0 μM were used. Velocity is plotted as the reciprocal of the ratio of dieldrin to endrin peak heights. Points are means of 2 samples. Apparent K_M = 3.4 x 10^{-6} M; apparent V_{max} = 38 pmoles/min/mg protein; y = 2.52 + 8.65X; r^2 = 0.98.

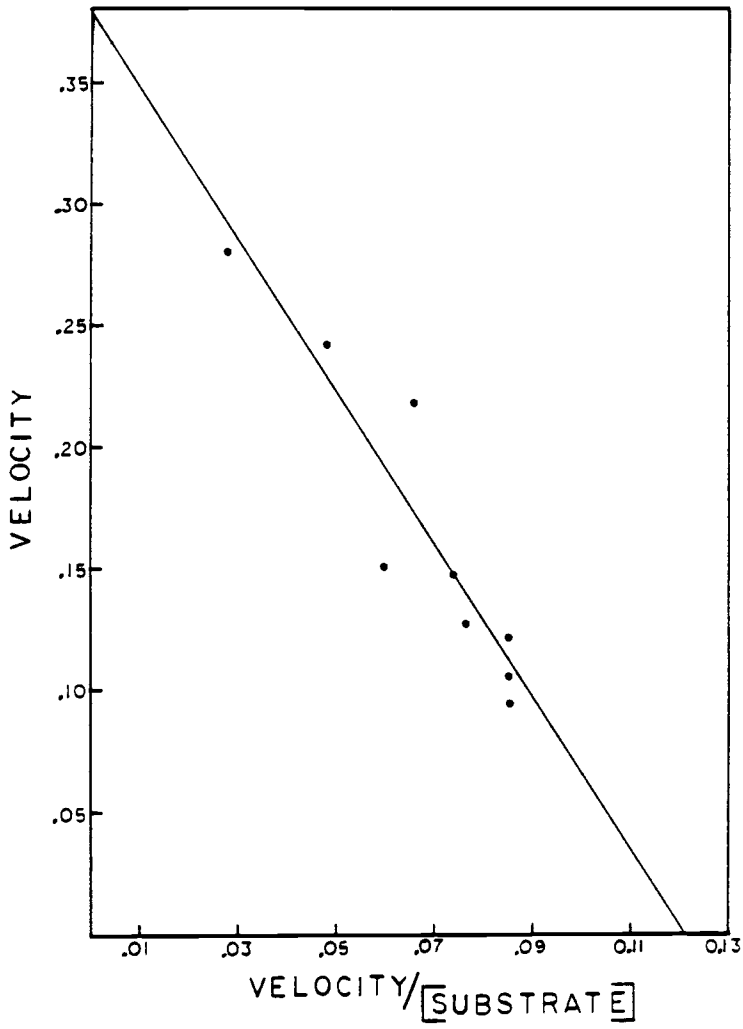

Fig. 12. Effect of substrate concentration on aldrin epoxidation activity shown as a Wolf-Augustinsson-Hofstee plot. Nine substrate concentrations ranging from 1.1 to 10.0 μM were used. Velocity is plotted as the ratio of dieldrin to endrin peak heights. Apparent K_M = 3.1 x 10^{-6} M; apparent V_{max} = 36 pmoles/min/mg protein; y = 0.3765 - 3.117X; r^2 = 0.87.

3.2.5. Inhibition of Aldrin Epoxidation Activity by Antibody to House Fly NADPH-Cytochrome P-450 Reductase

The characterization procedures for aldrin epoxidation activity discussed above had demonstrated that dieldrin formed in the incubation mixtures was, in fact, due to an enzymatic activity. It remained to obtain evidence that this activity was cytochrome P-450 dependent. Literature describing inhibition of cytochrome P-450 activities in microsomes prepared from mammalian liver and from house flies by antibodies specific for NADPH-cytochrome c or P-450 reductase suggested a possible experimental approach to demonstrate cytochrome P-450 dependency of aldrin epoxidation in Culex pipiens microsomes (Masters et al., 1971; Mayer et al., 1982).

NADPH-cytochrome P-450 reductase had been previously purified (Vincent et al., 1984) in our lab. A rabbit antibody to this purified enzyme was obtained and characterized (Feyereisen and Vincent, 1984). These workers demonstrated the specificity of the immune rabbit antibody for NADPH-cytochrome P-450 reductase on the basis that it did not inhibit the NADPH-dependent reduction of cytochrome c, dichloroindophenol, or ferricyanide. They further demonstrated its ability to inhibit 5 house fly microsomal reactions, which are probably NADPH-dependent cytochrome P-450 reactions.

Microsomes prepared from Culex pipiens by differential centrifugation, were incubated in varying ratios on a µg protein basis with immune and pre-immune rabbit IgG fraction to house fly NADPH-cytochrome P-450 reductase. Duplicate pre-incubation mixtures for

each ratio, containing IgG fraction, microsomes, and MOPS buffer were held at room temperature for 15 minutes. The epoxidation reaction was then initiated by addition of NADPH-regenerating system and aldrin substrate and was stopped after 20 minutes.

Inhibition of aldrin epoxidation activity in a dose-dependent manner was observed as the ratio of immune IgG fraction to microsomes was increased (Fig. 13). A 1.0/1.0 ratio of non-immune IgG fraction to microsomes inhibited epoxidation activity by about 30% and, therefore, demonstrated some non-specific inhibition of the reaction by rabbit serum. However, this degree of non-immune inhibition was not sufficient to account for the dose-dependent inhibition observed with the immune IgG fraction (compare with inhibition by 1.0/1.0 ratio of immune IgG fraction). These results indicate that Culex pipiens microsomal aldrin epoxidation activity is dependent on NADPH-cytochrome P-450 reductase and, therefore, constitute indirect evidence of the cytochrome P-450 character of the enzymatic activity.

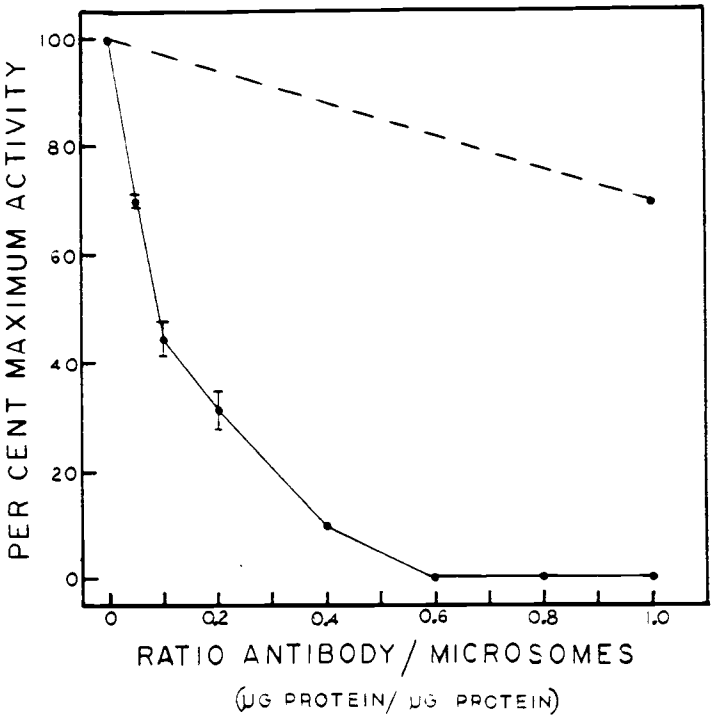

Fig. 13. Inhibition of aldrin epoxidation by antibody to house fly NADPH-cytochrome P-450 reductase. Points are means of duplicate samples (●—●) incubations with immune antibody or no antibody (at 0 on X axis); (●--●) incubation with non-immune antibody.

3.2.6. Inhibition of Aldrin Epoxidation Activity by Carbon Monoxide

Carbon monoxide is thought to compete with O_2 for the oxygen binding site at the heme moiety in the cytochrome P-450 catalytic site (Cooper et al., 1965). This phenomenon has been exploited as a technique for demonstrating cytochrome P-450 character of an enzymatic activity under study (Ray, 1967; Mayer et al., 1977; Feyereisen et al., 1981), Therefore, an experiment employing aldrin epoxidation incubations under a carbon monoxide-containing atmosphere was done in order to try to obtain further evidence of cytochrome P-450 dependent character for aldrin epoxidation activity in mosquito microsomes.

Microsomes were prepared by differential centrifugation as described (2.4.1.1.) with the exception that the 30% sucrose/MOPS resuspension solution contained 400 µM PMSF. Two series of triplicate incubation mixtures in sealed incubation vials connected by plastic tubing to allow gas transfer between vials were prepared. The incubation mixtures contained microsomes and all reagents except aldrin substrate. Atmospheric gas was flushed from the vials with pressurized N_2. One liter of gas solution was then passed through each series of incubation vials over a period of 5 minutes. Control incubations received a 90% N_2, 10% O_2 mixture and the second incubation series a 70% N_2, 10% O_2, 20% CO mixture. The vials were vortexed for 5 seconds and a syringe was used to add aldrin substrate, initiating the epoxidation reaction. After a 20 minute incubation, the reaction was stopped.

The control incubations produced 4.31 ± 0.63 (S.E.M.) picomoles dieldrin/min/mg protein. The incubations with 20% CO in the gas mixture had no detectable activity. Enough data to demonstrate a dose-dependent type of inhibition as CO content of the incubation atmosphere was increased was not obtained. However, the preliminary results demonstrated inhibition of aldrin epoxidation activity by CO.

3.3. Characterization of Methoxyresorufin O-Demethylation Activity

Methoxyresorufin O-demethylation activity was not detectable in incubation mixtures prepared with microsomes produced by sucrose density-gradient centrifugation. Low activity could be detected when microsomes were prepared by differential centrifugation (2.4.1.2.) and "microsomal" protein content of incubations was several-fold higher than could be attained with density-gradient-produced microsomes. However, microsomes prepared by this proceduere (which was necessary in order to obtain the mg quantities of protein needed for a series of incubation mixtures with detectable activities) were found to have endogenous fluorescence at the 583 nm emission wavelength used to detect resorufin, the demethylation product. This may have been due to contamination with a few mosquito heads in the tissue homogenates which could have introduced eye pigments. These problems combined to make use of the assay difficult and no attempt was made to characterize methoxyresorufin O-demethylation activity in different developmental states of the mosquitoes.

3.3.1. Effect of Microsomal Protein Concentration

Duplicate incubation mixtures contained 50 to 300 µg of "microsomal" protein and were stopped after 15 minutes. Amount of resorufin produced versus increase in protein content of incubations was approximately linear (Fig. 14A).

3.3.2. Effect of Incubation Time

A time course of 5, 10, 15, 20, and 25 minutes was run in duplicate with incubation mixtures containing 250 µg of "microsomal" protein. Results are shown in Fig. 14B. This plot does not show a linear increase in product as incubation time increases and a line through the points does not approach the 0 intercept on the y axis. These results can be interpreted as indicating that the enzyme is not stable.

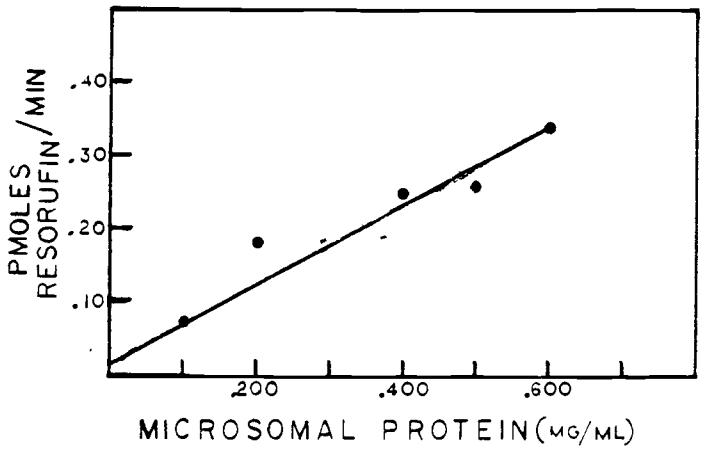

Fig. 14A. Effect of "microsomal" protein concentration on methoxyresorufin O-demethylation activity. Points are means of duplicate samples.

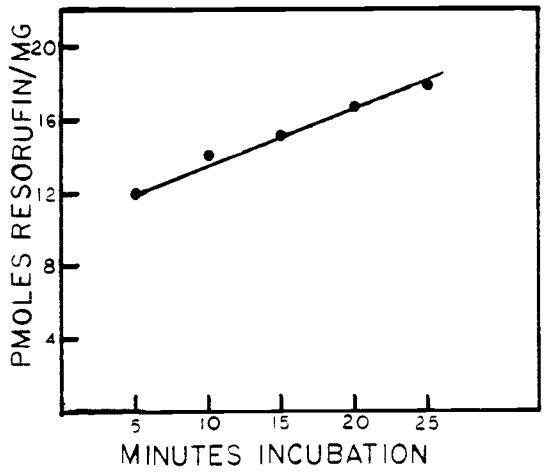

Fig. 14B. Effect of incubation time on methoxyresorufin O-demethylation activity. Points are means of duplicate samples.

3.3.3. pH Dependency

The pH dependency of methoxyresorufin O-demethylation activity was studied by procedures similar to those described for aldrin epoxidation (3.3.4.). Duplicate incubation mixtures contained 250 µg "microsomal" protein in a final volume of 0.5 ml and were adjusted to final pH values (5.70, 6.20, 6.70, 7.20, 7.70, 8.00, 8.50) with 435 µl of 0.1 M MOPS "pH modulating buffer." Incubations were stopped after 20 minutes. Maximum activity was obtained at pH 7.70 and 8.00. Activity was 80% of the maximum at pH 8.50 and 84% of maximum at pH 7.20. Below pH 7.20 activity could not be detected. Mayer et al. (1977) have reported a pH optimum of 8.00 for this activity in house fly microsome incubations.

3.4. Alkoxycoumarin O-Dealkylation Assays

Alkoxycoumarin O-dealkylation activity in mosquito microsomes was not detectable. Three coumarin derivatives were tested: 7-methoxycoumarin, 7-ethoxycoumarin, and 7-methoxy-4-methylcoumarin. A house fly microsome preparation with high activity for 7-methoxy-4-methylcoumarin O-demethylation could be inhibited as much as 90% by addition to the incubations of fresh or heat-killed mosquito microsome preparations containing only 10 to 20% as much protein as the volume of house fly microsome preparations present in the incubation mixture. This suggested the possible presence of a compound in the mosquito microsomes which quenched fluorescence at the 455 nm wavelength used to monitor 7-hydroxycoumarin formation or removed 7-hydroxycoumarin as

it was formed by some unknown mechanism. Other possibilities are noted in the discussion section. The presence of endogenous inhibitors of microsomal enzymatic activities in insect microsomes has been well-known for some time and has been reviewed by Wilkinson and Brattsten (1972).

3.5. Determination of Cytochrome P-450 Content of Microsomes

Microsomes prepared as described (2.4.1.1.) from blood-fed mosquitoes 96 hours after the blood meal, were used to estimate specific content of cytochrome P-450 by the procedure of Omura and Sato (1964). The carbon monoxide difference spectrum of the reduced microsomes is shown in Fig. 15. It should be noted that this procedure is based on a single extinction coefficient of 91 $cm^{-1}mM^{-1}$ for cytochrome P-450 in rabbit liver microsomes and, in view of the now well-demonstrated multiplicity of cytochrome P-450 isozymes in mammals (for review see Lu and West, 1980), results obtained by use of the procedure for insect microsome preparations should be interpreted with caution.

The specific content of cytochrome P-450 was estimated to be 71.2 pmoles/mg protein. This value is compared with recent estimations made for other adult Diptera in Table 2. The specific content of 71.2 pmoles/mg estimated for Culex pipiens ranks among the lowest values reported in the literature for adult Diptera.

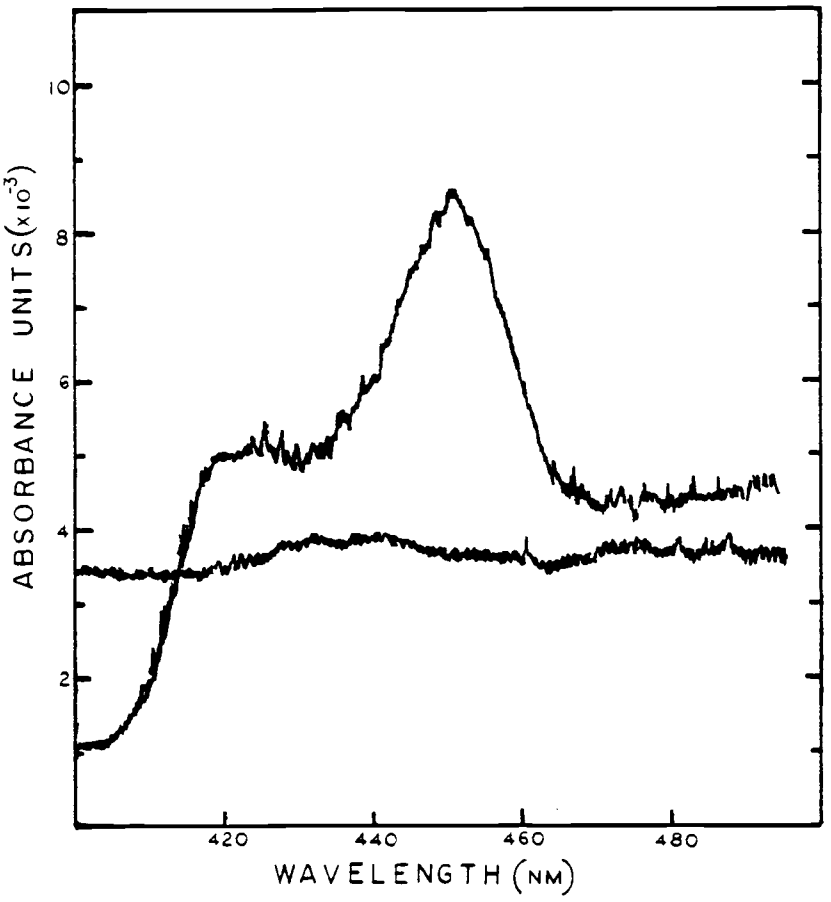

Fig. 15. Carbon monoxide difference spectrum of dithionite reduced Culex pipiens microsomes. The absorbance peak is at 450.4 nm and was maximal (A = .0038) at 20 minutes after carbon monoxide was bubbled through the sample cuvette. Baseline is shown.

Table 2. Specific Content of Cytochrome P-450 in Microsomes Obtained from Representative Adult Diptera.

Species Strain	Specific Content Cytochrome P-450 pmoles/mg Protein	Age as Days After Emergence	Literature Source
House Flies (Muscidae)			
Musca domestica			
Rutgers[1]	440	7 to 10	Yu and Terriere (1979)
Rutgers	280	6 to 9	Moldenke and Terriere (1981)
NAIDM[2]	160	6 to 9	Moldenke and Terriere (1981)
NAIDM	33	------	Devries and Georghiou (1981)
NAIDM	280	7 to 10	Yu and Terriere (1979)
147-R[3]	79	------	DeVries and Georghiou (1981)
CSMA[2]	140	6 to 9	Moldenke and Terriere (1981)
Blow Flies (Calliphoridae)			
Phormia regina	48	5 to 6	Rose and Terriere (1980)
Lucilia illustris	149	5 to 6	Rose and Terriere (1980)
Eucalliphora lilica	107	5 to 6	Rose and Terriere (1980)
Flesh Flies (Sarcophagidae)			
Sarcophaga bullata	50	8 to 9	Terriere and Yu (1979)

Table 2 Continued

Species Strain	Specific Content Cytochrome P-450 pmoles/mg Protein	Age as Days After Emergence	Literature Source
Fruit Flies (Drosophilidae)			
Drosophila melanogaster			
Karsnas 60_w	190	2 to 5	Hallstrom et al. (1983)
Berlin K	170	2 to 5	Hallstrom et al. (1983)
Oregon R^1	330	2 to 5	Hallstrom et al. (1983)
Eye Gnats (Chloropidae)			
Hippelates bishoppi	210	7	Kulkarni et al. (1976)
Hippelates pallipes	151	7	Kulkarni et al. (1976)
Hippelates pusio	164	7	Kulkarni et al. (1976)
Mosquitoes (Culicidae)			
Culex pipiens	71	10^4	this study

1. Insecticide resistant.
2. Insecticide susceptible.
3. Permethrin selected.
4. 96 hours after blood meal.

3.6. Phenobarbital Induction of Aldrin Epoxidation and NADPH-Cytochrome c Reductase Activities

Aldrin epoxidation and NADPH-cytochrome c reductase activities were measured at 12, 24, and 48 hours after exposure of 4 day-old mosquitoes to 0.1% phenobarbital in the 10% sucrose solutions used to feed the insects.

No induction of either activity was detected at 12 hours exposure (Table 3). At 24 hours exposure, NADPH-cytochrome c reductase activity was induced 1.7-fold above activity of control insects and aldrin epoxidation activity was induced 1.9-fold. At 48 hours exposure reductase and aldrin epoxidation activities were induced 1.5-fold and 2.0-fold, respectively. Phenobarbital is a known inducing agent for microsomal mixed-function oxidase activities in the house fly (Perry et al., 1971) and in Drosophila (Hallstrom et al., 1983). Moldenke and Terriere (1981) found that induction of aldrin and heptachlor epoxidation activities in house flies by phenobarbital could not be explained solely on the basis of increased cytochrome P-450 content. They interpreted their results as evidence for multiple forms of cytochrome P-450.

Although our data demonstrate induction of aldrin epoxidation and NADPH-cytochrome c reductase activities by phenobarbital, it is of a preliminary nature only. Inclusion of heptachlor epoxidation assays (an initial experiment indicated an apparent activity for this assay of about 1/2 that of aldrin epoxidation) and cytochrome P-450 content determinations in further experiments might form the basis for

Table 3. Induction of Aldrin Epoxidation and NADPH-Cytochrome c Reductase Activities by Phenobarbital.

Treatment and Time	n	Aldrin Epoxidation Activity[1] pmoles/Dieldrin/min/insect	Fold Induction	NADPH-Cytochrome-c Reductase Activity nmoles/cyt c reduced/ min/insect	Fold Induction
Controls no phenobarbital	6	0.35 ± .011		.105 ± .008	
Phenobarbital 12 hours	2	0.33 ± .030	0	.109 ± .005	0
Phenobarbital 24 hours	4	0.65 ± 0.122	1.9	.177 ± .003	1.7
Phenobarbital 48 hours	2	0.70 ± .035	2.0	.158 ± .004	1.5

1. Aldrin epoxidation activity was measured by the procedure of Moldenke and Terriere (1981).

partial demonstration of multiplicity of cytochrome P-450 in Culex pipiens.

3.7. Developmental Profile of Aldrin Epoxidation and NADPH-Cytochrome c Reductase Activities

A developmental profile of changes in aldrin epoxidation and NADPH-cytochrome c reductase activities was determined for adult female mosquitoes before and after the blood meal. Since there is an almost complete lack of this type of information from in vitro assays for adult mosquitoes in the literature, it was felt that such information could be valuable in gaining a better understanding of oxidative metabolic mechanisms in adult mosquitoes which might play a role in xenobiotic metabolism and/or hormonal control of reproduction.

Aldrin epoxidation and NADPH-cytochrome c reductase activities were determined on a per mg protein basis and per insect basis in non-blood-fed mosquitoes aged Days 1-6, 8, 10, and 12 after emergence and in blood-fed mosquitoes at 12, 24, 36, 48, 60, 72, 96, 120, and 144 hours after the blood meal. Microsomes were obtained by sucrose density-gradient centrifugation (2.4.2.1.) for these experiments. From 12 to 25 mosquito abdomens were homogenized for each blood-fed sample and 25 to 35 abdomens for each non-blood-fed sample.

Aldrin epoxidation results are shown in Figs. 16A and B. A distinct peak in activity occurred on Day 2 in non-blood-fed mosquitoes. Activity returned to a "basal" level on Day 3 and was maintained through Day 12. Activity in blood-fed mosquitoes increased during the 48 hours immediately following the blood meal to a

"plateau" which was maintained through 96 hours after the blood meal and then declined, becoming roughly equivalent to the activity of the Day 12 non-blood-fed insects by 144 hours after the blood meal. Activity on a per insect basis in blood-fed mosquitoes during the 48 to 96 hours after the blood meal "plateau period" was approximately twice that of the peak activity in non-blood-fed insects seen on Day 2. However, this difference was not apparent on a per mg protein basis.

NADPH-cytochrome c reductase activity (Figs. 17A and B) displayed a developmental profile similar to that of aldrin epoxidation activity. In both profiles, activity on a per mg protein basis during the early hours following the blood meal was lower than in comparably aged non-blood-fed insects but was higher when calculated on a per insect basis. This may reflect a general proliferation of tissue in those mosquitoes which have obtained a blood meal.

The peak in aldrin epoxidation and NADPH-cytochrome c reductase activities in 2 day-old non-blood-fed mosquitoes coincided with the period of greatest follicle growth in young mosquitoes (Fig. 19). At 36 hours after the blood meal, these enzymatic activities increased rapidly to a "plateau" which continued from 48 to 96 hours after the blood meal. The "plateau" of high activity coincided with the period of greatest follicle growth in blood-fed mosquitoes and the period marked by the onset of oviposition (Fig. 20). The rapid increase in enzymatic activity from 36 to 48 hours after the blood meal to the "plateau" of high activity occurred in the 12 hour period immediately following the peak in ecdysteroid titers (Fig. 18.).

While conclusions based on these apparent correlations must be drawn with great caution, it should be pointed out that developmental differences in expression of apparent cytochrome P-450 activities have been demonstrated in both mammals (Atlas et al., 1977; Guenthner and Nebert, 1978) and insects (Benke and Wilkinson, 1971B; Wilkinson and Brattsten, 1972; Hallstrom et al., 1983). Since the suggestion by Yu and Terriere (1974) of a possible role for "microsomal oxidases" in house fly metamorphosis and reproduction, considerable evidence has accumulated that cytochrome P-450 mediated reactions are involved in synthesis and degradation of hormones known to be involved in regulation of development and reproduction in insects (for review see Hodgson, 1983). Our data are not extensive enough to support specific conclusions concerning cytochrome P-450 involvement in hormonal regulation of mosquito development and reproduction. However, the apparent correlations discussed above are intriguing and enough basic data on reproductive development and procedures for measuring microsomal enzymatic activities in Culex pipiens has been accumulated to provide a firm basis of support for further work.

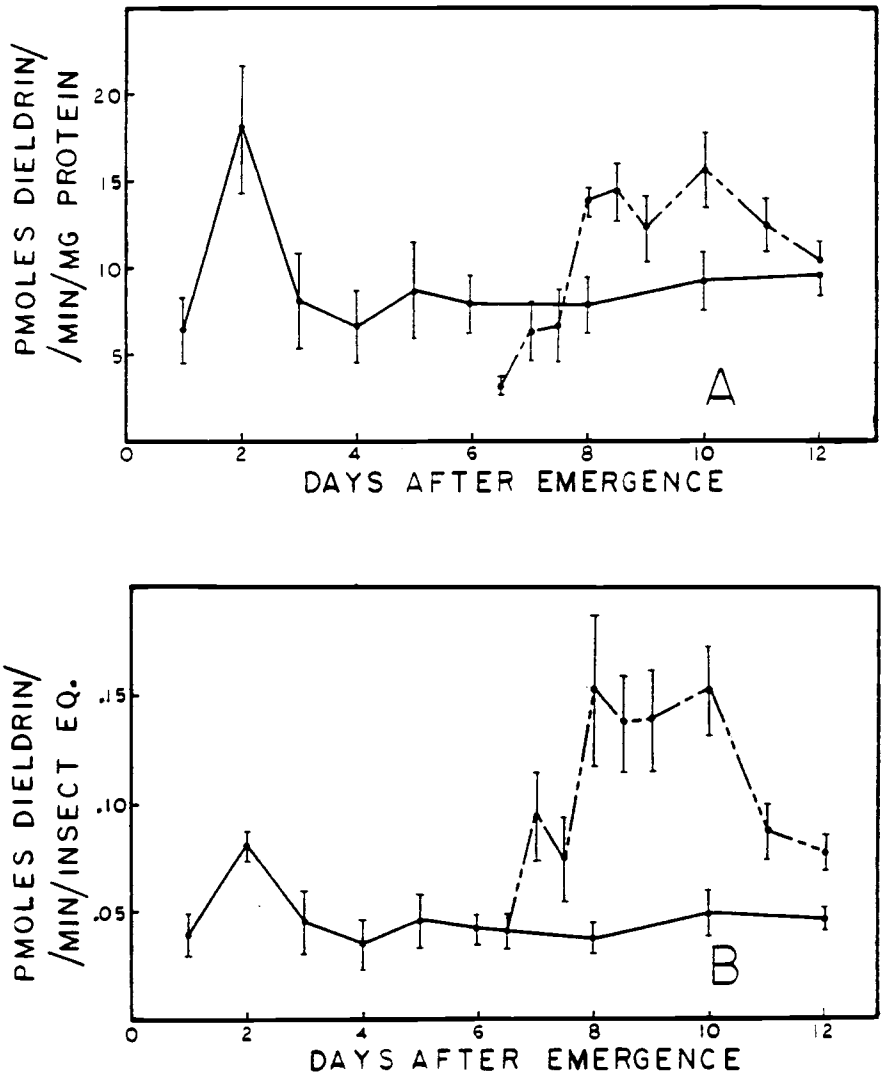

Fig. 16. Developmental profile of aldrin epoxidation activity in adult female Culex pipiens. A - as pmoles dieldrin/min/mg protein; B - as pmoles dieldrin/min/insect equivalent; (●—●) non-blood-fed mosquitoes; (●--●) blood-fed mosquitoes. Points represent mean of 4 to 7 duplicate samples ± S.E.M. Microsomes were obtained from tissue homogenates prepared from abdomens with midguts removed and with ovaries removed from blood-fed mosquitoes. Mosquitoes were given a blood meal on the 6th day after emergence.

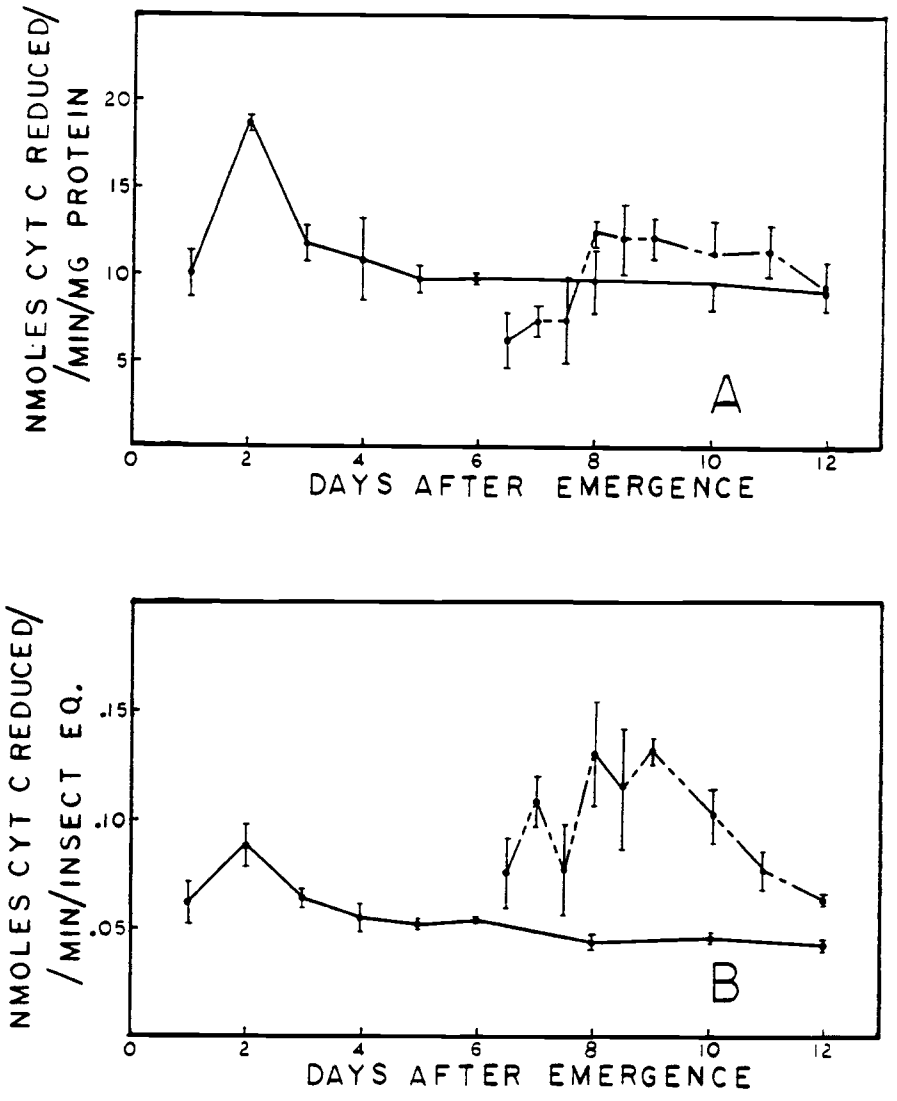

Fig. 17. Developmental profile of NADPH-cytochrome-c reductase activity in adult female Culex pipiens. A - as nmole cytochrome-c reductase reduced/min/mg protein; B - as nmoles cytochrome-c reduced/min/insect equivalent; (●—●) non-blood-fed mosquitoes; (●--●) blood-fed mosquitoes. Points represent mean of 4 to 7 duplicate samples ± S.E.M. Microsomes were obtained from tissue homogenates prepared from abdomens with midguts removed and with ovaries removed from blood-fed mosquitoes. Mosquitoes were given a blood meal on the 6th day after emergence.

3.8 Radioimmunoassay of Ecdysteroid Titers

Ecdysteroid titers were determined as pmoles 20-hydroxyecdysone equivalents per insect and results are shown in Fig. 18. Except for the 1st day after emergence, ecdysteroids were below the limit of detection by the assay in non-blood-fed mosquitoes even when as many as 300 insects were extracted for a sample. Ecdysteroid titers of blood-fed mosquitoes rose to a peak at 36 hours after the blood meal and then declined rapidly.

It should be pointed out that the ecdysteroid peak at 36 hours after the blood meal immediately preceeds the 12 hour period of greatest follicle growth following the blood-meal (Fig. 20). Hagedorn (1974) and Hagedorn et al. (1975) have proposed that the ovaries secrete ecdysone in response to some stimulus from the blood-meal and that this ecdysone initiates vitellogenin synthesis by the fat body, ultimately resulting in follicular growth. However, the possible involvement of ovarian ecdysteroids in mosquito vitellogenesis is not fully understood by investigators working in this field (for review see Fuchs and Kang, 1981).

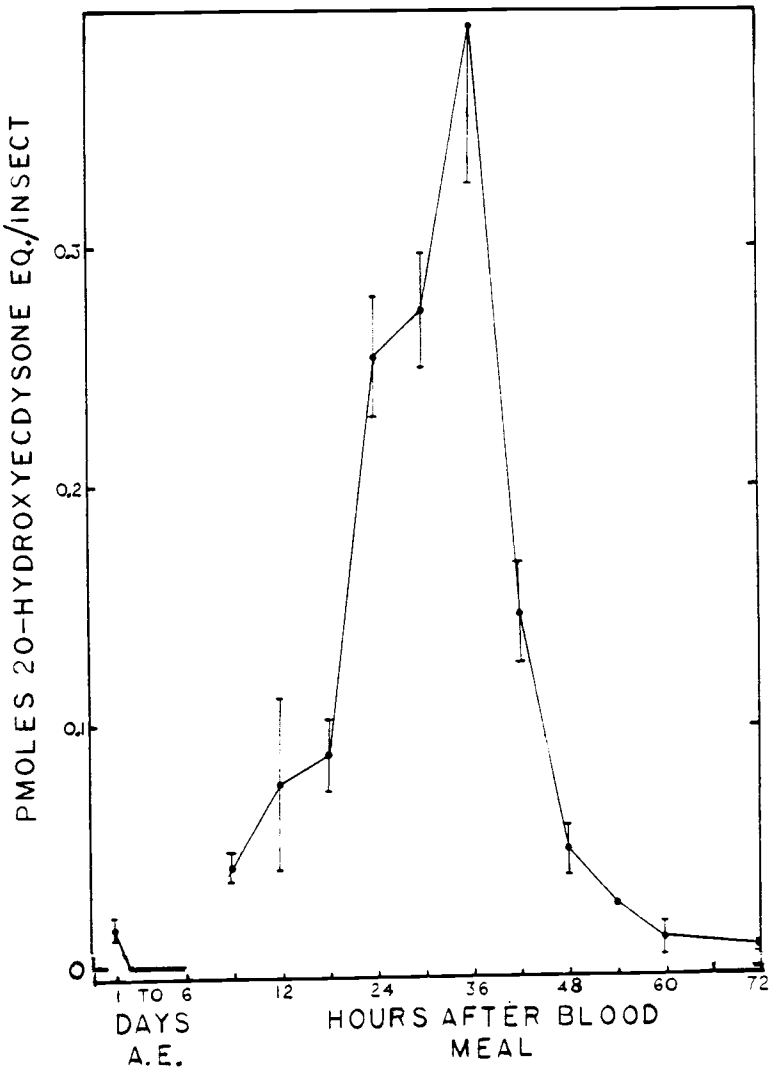

Fig. 18. Ecdysteroid titers in adult female Culex pipiens. Ecdysteroid titers were determined by radio-immunoassay in mosquitoes aged 1 to 6 days after emergence (A.E.) and at 6 hour intervals from 6 to 72 hours after a blood meal given on the 6th day after emergence. Points represent means of 1 to 7 samples ± S.E.M.

3.9 Growth of Primary Follicles

3.9.1. Non-Blood-Fed and Blood-Fed Mosquito Follicle Growth Curves

Primary follicle growth curves were obtained for non-blood-fed and blood-fed mosquitoes and were used to provide a physiological marker of the reproductive developmental status of mosquitoes with which *in vitro* data collected in other experiments could be correlated.

Growth of primary follicles was followed as described (2.2.2.) from Day 0 (immediately after emergence) through Day 9 in non-blood-fed mosquitoes. Follicles increased approximately four-fold in length from Day 0 to Day 3 to an average length of about 88 µm and then ceased to grow (Fig. 19). Growth resumed only after mosquitoes obtained a blood meal.

The follicle growth curve was extended to blood-fed mosquitoes (fed on Day 6 after emergence) by measuring follicles at 6 hour intervals from 0 to 78 hours after the blood meal (Fig. 20). Follicles increased from 88 to about 600 µm in the 60 hours following the blood meal and then showed an apparent slight decline in length from 60 to 78 hours after the blood meal. This period coincided with the beginning of oviposition. Earliest observation of oviposition was 66 hours after blood-feeding. More than 50% of mosquitoes in any group under observation oviposited between 72 and 96 hours after blood-feeding and more than 90% had oviposited by 120 hours after blood-feeding.

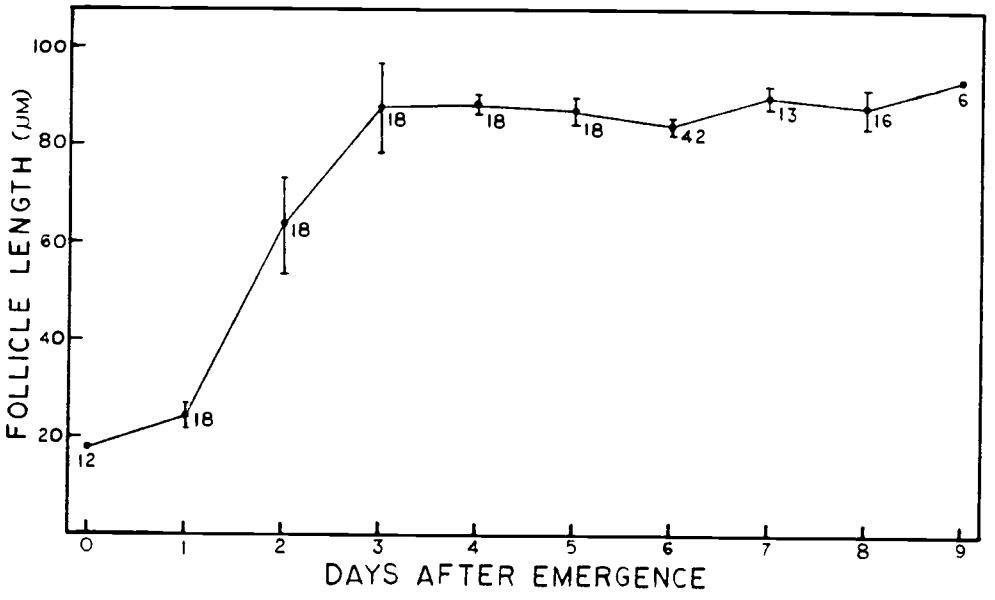

Fig. 19. Follicle growth in non-blood-fed Culex pipiens. Follicle lengths were measured at daily intervals after emergence (Day 0) to 9 days after emergence. Points represent means of 6 to 42 determinations ± S.E.M.

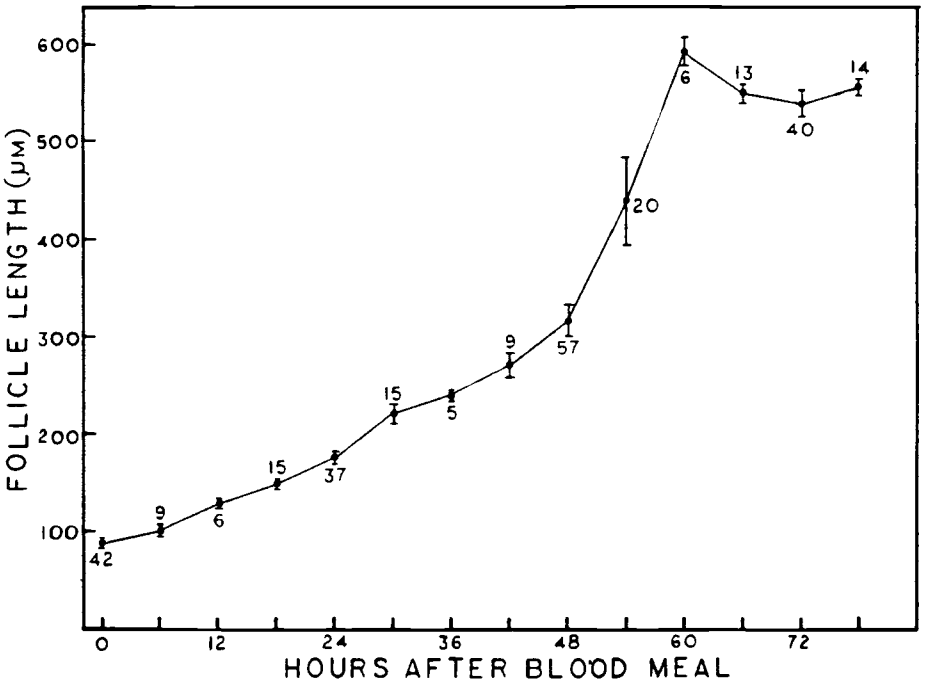

Fig. 20. Follicle growth in blood-fed Culex pipiens. Mosquitoes were given a blood meal on the 6th day after emergence and follicle lengths were measured at 6 hour intervals from 0 to 78 hours after the blood meal. Points are means of 5 to 57 determinations ± S.E.M.

3.9.2. Dependence of Follicle Growth on a Head Factor

Since the early experiments of Wigglesworth in the 1930's with the blood-sucking Hemipteran Rhodnius, decapitation after the blood meal has been a recognized procedure for attempting to determine a "critical period" for presence of the head after the blood meal during which a humoral influence from the head accumulates in the hemolymph and initiates subsequent developmental events. We reasoned that if the critical period occurred after the blood meal in Culex pipiens, decapitation would then offer an experimental approach to the problem of determining whether the increases in ecdysteroid titers, microsomal enzymatic activities, and follicle growth observed after the blood meal were caused by the blood meal itself or by a secondary hormonal influence released in response to the blood meal. Decapitation experiments, as described (2.2.3.), were used to determine the critical period and results are described below. Initial experiments gave poor results due to high mortality among decapitated mosquitoes. Mortality dropped to about 35% after procedures described in 2.2.3. were developed.

In mosquitoes allowed to obtain a full blood-meal before decapitation follicles grew to about 80% of the length attained by follicles in non-decapitated controls regardless of time of decapitation after the blood-meal (Fig. 21). If mosquitoes were decapitated about 7 to 8 minutes after beginning the blood-meal but before completing it, follicles attained lengths slightly less than those attained by follicles in mosquitoes decapitated after a full

blood-meal. However, the difference was not statistically significant. Mosquitoes decapitated from 3 to 4 minutes after beginning the blood-meal had average increases in follicle length of about 20 μm over "resting stage" length while non-decapitated controls increased their follicle lengths by more than 200 μm over "resting stage" length. This difference was statistically significant ($p < .01$). Mosquitoes decapitated within 2 minutes after beginning the blood-meal had little or no follicle growth.

These results indicated a critical period of 4 to 8 minutes after beginning the blood meal, but before completing it. We had hoped that a longer critical period would be found, such as that in Aedes aegypti, where many workers have determined that the critical period is measured in hours after the blood meal. Such a longer critical period would offer an experimental approach to the problem of determining whether the increases in ecdysteroid titers and aldrin epoxidation and NADPH-cytochrome c reductase activities seen after the blood meal were initiated by the blood meal itself or by humoral influences from the head neurosecretory cells and the corpus cardiacum in response to the blood meal.

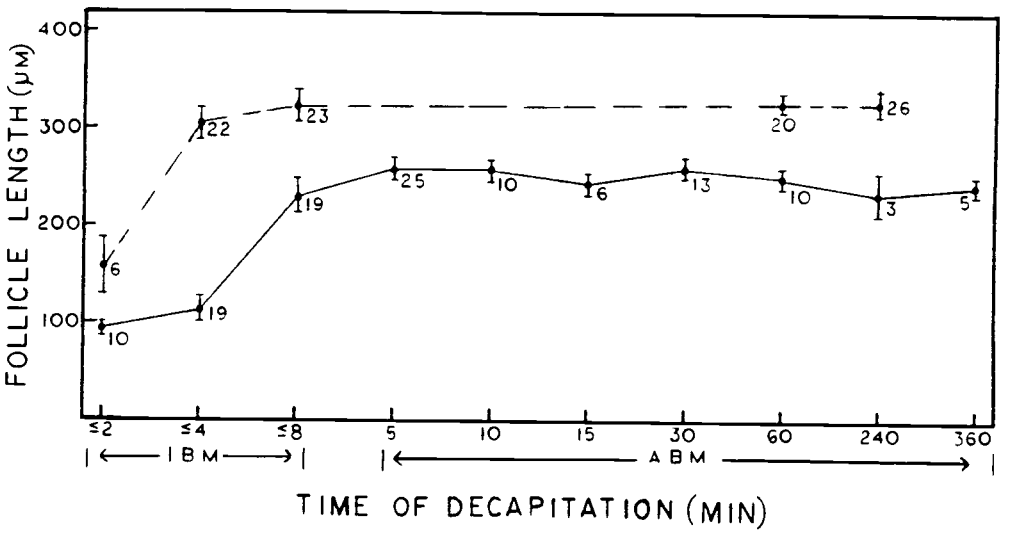

Fig. 21. Determination of Culex pipiens "critical period" for presence of head following a blood meal. IBM = interrupted blood meal; ABM = after blood meal. Mosquitoes were given blood meals on the 6th day after emergence and were decapitated at specific time points indicated either before or after completing the blood meal. Control insects, obtained by the same procedures, were not decapitated. The mosquitoes were then held in the rearing room for 48 hours after the blood meal prior to measurement of follicle lengths. Points are means of 3 to 26 samples ± S.E.M. (●- -●) control (●——●) decapitated

DISCUSSION

4.1. Preparation of Microsomes by Sucrose Density-Gradient Ultracentrifugation

The utility of a sucrose density-gradient ultracentrifugation technique for obtaining microsomes from tissue homogenates has been demonstrated. Microsomes, suspended in a solution suitable for use in enzymatic assays, can be obtained from a tissue homogenate in less than 45 minutes with this technique. It, therefore, represents a considerable savings in time and labor over the classic differential centrifugation procedure for obtaining microsomes which usually requires 3 to 4 hours of work. In addition, it is better suited for work with small quantities of tissuethan the differential centrifugation procedure and, therefore, represents an advance in the techniques necessary for study of microsomal monooxygenase activities in those insects where the small quantities of tissue available are a serious technical problem, or where it is desirable to study a single tissue from a larger insect.

The differential centrifugation procedures for obtaining microsomes currently used in most laboratories are similar to the original technique described by Siekevitz (1963, 1965). This author described the microsomal sub-cellular fraction as the "high speed pellet resulting when the supernatant fluid from the mitochondrial fracton is sedimented." The "high speed pellet" is generally obtained by a 100,000 to 200,000 g centrifugation for 1 to 2 hours. Many investigators working with insects have apparently accepted this

definition, although it is based on work with mammalian liver. However, as discussed by Wilkinson and Brattsten (1972), an examination of the literature indicates that this may not always be true. Nakatsugawa and Dahm (1965) found that a 30 minute centrifugation at 50,000 g could sediment all of the microsomal parathion activating activity in homogenates of Periplaneta americana L. Similarly, a 15 minute centrifugation at 12,000 g sedimented almost all of the microsomal epoxidation activity in tissue homogenates prepared from Acheta domesticus L. or Gromphadorina portentosa Schaum (Benke and Wilkinson, 1971A; Benke et al., 1972). These are no doubt extreme examples, but they serve to illustrate the advantage of defined separation of sub-cellular fractions offered by the sucrose density-gradient procedure reported in this thesis. Use of this technique enables an investigator to work with a defined microsomal sub-cellular fraction rather than a "high speed pellet" which is assumed to contain the desired cellular organelles and to exclude those not desired. The accuracy and reliability of enzymatic activity reported as specific activity will therefore be improved, an advantage with important implications for comparative studies.

Investigators of insect microsomal monooxygenase activities have previously used sucrose gradient centrifugation procedures in efforts to obtain better-defined sub-cellular fractions of tissue homogenates. Benke and Wilkinson (1971) encountered problems involving sedimentation of house cricket microsomal monooxygenase activities by low g forces during differential centrifugation procedures and again with the cockroach, Gromphadorhina protentosa (Benke et al., 1972).

Gilbert and Wilkinson (1973) encountered similar problems in their work with the honey bee. All of these investigators addressed the problem by centrifuging their tissue homogenates on 1.6 M sucrose for 30 minutes at 150,000 g. They obtained two fractions consisting of a "dense" layer of tissue constituents at the bottom of the centrifuge tube and a second layer near or at the top of the sucrose. Electron microscopy studies revealed that the upper layer contained predominantly microsomes while most of the mitochondria were in the bottom fraction. The "microsomal" fraction contained the majority of the monooxygenase activities and was employed in subsequent characterization procedures. However, use of this sucrose-gradient centrifugation procedure for obtaining microsomes is flawed by the fact that the "microsomal" fraction also contains the cytosolic cellular constituents which can introduce inhibiting compounds and undesired proteins.

Feyereisen and Durst (1978) and Feyereisen (1983) employed discontinuous sucrose density-gradients centrifuged at 22,000 and 26,000 RPM, respectively, in an SW 27 rotor for 4 hours. They employed succinate and NADPH-cytochrome c reductase assays as enzymatic activity markers for presence of mitochondria and microsomes and obtained distributions of these activities in their gradients qualitatively similar to those we have obtained with our linear sucrose density-gradient procedure. However, our procedure offers a considerable savings in preparation time.

The advantages of savings in time and labor, better suitability for work involving small quantities of tissue, and defined separation

of sub-cellular fractions offered by our sucrose density-gradient ultracentrifugation technique for preparation of microsomes should make it worthy of serious consideration for use in laboratories currently relying on the differential centrifugation procedure.

4.2. Development of Assays for Microsomal Monooxygenase Activities

A modified aldrin epoxidation assay procedure has been developed that offers greater reliability for quantification of small epoxidation product yields. The use of endrin as an internal standard during gas chromatographic analysis for quantity of dieldrin formed in microsomal aldrin epoxidation incubations minimizes sources of error inherent in the widely used procedure of using a dieldrin solution of known concentration as an external standard. These sources of error include small variations between samples in volumes of standard solution and incubation extraction solution contained in the microsyringe used to inject samples into the gas chromatograph, and changes in response of the detector and recorder from hour to hour as a series of samples is injected. With use of the internal standard and peak height ratio method for quantifying product, errors due to variation in sample volume are eliminated as long as care is taken to stay well within the limits of linearity of detector response. Error due to changes in detector or recorder response since time of injection of the last external standard sample series are also eliminated. In addition, the use of a small incubation volume (350 µl) with extraction of the product by a 100 µl volume of solvent allows use of minimal (20 µg or less) quantities of "microsomal"

protein while still giving extracted yields of dieldrin product sufficent to allow reliable detection by gas chromatography. Most aldrin epoxidation assays reported in the literature involve use of considerably greater quantities of protein in incubation mixtures and extraction of product with milliliter quantities of solvent which in our opinion results in unnecessary dilution of the product and consequent decreased sensitivity of the assay. Our procedure for assay of aldrin epoxidation activity may represent an improvement over methods currently in use in some laboratories and is particularly well suited for use with tissue preparations containing small quantities of enzymatic protein and/or having low specific activity.

Attempts to develop assays for O-dealkylation of coumarin derivatives were not as successful as the aldrin epoxidation work. Feyereisen and Vincent (1984) reported specific activities for O-demethylation of 7-methoxycoumarin of 433 pmole/min/mg and 1.277 nmole/min/mg for 7-methoxy-4-methylcoumarin by microsomes prepared form the Rutgers strain of adult house flies. Hallstrom et al. (1983) reported 67 pmole/min/mg for O-dealkylation of 7-ethoxycoumarin by microsomes prepared from the Karnasas 60_W strain of adult Drosophila. It is, therefore, of interest that no detectable activity towards any of these substrates by microsomes prepared from Culex pipiens was observed even with protein concentrations of 0.6 mg/ml in incubation mixtures, a value exceeding that used by the investigators cited above. It is possible that adult female Culex pipiens simply do not possess O-dealkylation activity for aryl hydrocarbon ether compounds. However, the low but demonstrable enzymatic activity for

demethylation of methoxyresorufin, and the observation that a 1/5 ratio of mosquito microsomal protein to house fly microsomal protein in an incubation mixture resulted in up to 90% inhibition of the activity for O-demethylation of 7-methoxy-4-methylcoumarin as compared to a house fly microsome control argues against the hypothesis of a complete lack of activity for these substrates and implies the presence of an endogenous inhibitor in the mosquito microsomes. The fact that approximately equivalent inhibition of house fly microsomal O-demethylation activity occurs with viable or heat-killed mosquito microsomes suggests that the inhibition mechanism does not depend on intact protein structure. Heat stable inhibitors of microsomal monooxygenation activities have been reported from locust fat body (Hook et al., 1968) and house flies (Schonbrod and Terriere, 1971; Wilson and Hodgson, 1972). The house fly inhibitor has been characterized as xanthommatin, an ommochrome pigment, which is believed to act as an "electron sink" and blocks flow of reducing equivalents from NADPH through the reductase to cytochrome P-450. The principle source of xanthommatin in house flies is believed to be the head and thorax, but neither of these tissues was used to prepare mosquito microsomes. However, in a single experiment it was observed that a 2 or 3 fold increase in the amount of NADPH present in an incubation mixture could partially relieve inhibition of house fly microsomal 7-methoxy-4-methylcoumarin O-demethylation activity by mosquito microsomes. In view of this observation, it is possible that microsomes prepared from Culex pipiens contain an endogenous inhibitor which acts as an electron sink. An additional possibility is

suggested by the work of Gilbert and Wilkinson (1973) who were able to partially characterize an inhibitor from honey bees and concluded that it seemed to be closely associated with RNA.

Mayer et al. (1977) reported development of spectrofluorometric assay procedures for O-demethylation of methoxyresorufin by housefly microsome preparations and suggested that this substrate was well suited for "direct" measurement of insect microsomal monooxygenase activity on the basis of their results with house flies, the fluorescence characteristics of substrate and product, and the sensitivity offered by spectrofluorometry in general. They suggested that use of 0.3 mg "microsomal" protein per ml of incubation mixture would probably give good results for most insects. However, in the case of Culex pipiens, use of as much as 0.6 mg of "microsomal" protein/ml provided product yields which were barely within the limit of reliable detection. Enough activity was present to allow limited characterization procedures but not enough to warrant use of this substrate in investigation of differences in monooxygenase activities among mosquitoes of different age and reproductive status.

An overall analysis of the problems encountered in developing assays for Culex pipiens microsomal monooxygenase activities points out the need for development of radioactive assays. The limited amount of tissue available from such a small insect, the relatively low specific content of cytochrome P-450 (71.2 pmoles/mg), and the apparent low specific activities found in those assays which were used are all problems which can reasonably be expected to occur in work with other mosquito species or with other small Diptera of medical and

veterinary importance, e.g., Simulidae, Phlebotominae, Ceratopogonidae. The sensitivity of product detection offered by radioactive assays could be instrumental in studying *in vitro* enzymatic activities in small Diptera.

4.3. Demonstration of Cytochrome P-450 Dependent Character of Aldrin Epoxidation

Evidence has been obtained that aldrin epoxidation activity in microsomes prepared from *Culex pipiens* is cytochrome P-450 dependent. This enzymatic activity has been widely assumed to be cytochrome P-450 dependent since the original demonstration by Ray (1967) that it could be inhibited by carbon monoxide in house fly microsome incubations. Our preliminary results indicated that this activity could also be inhibited by carbon monoxide in *Culex pipiens* microsomes.

A dose-dependent type inhibition of aldrin epoxidation activity by increasing ratios of house fly NADPH-cytochrome P-450 reductase antibody to mosquito microsomes in incubation mixtures was also observed. This antibody, characterized by Feyereisen and Vincent (1984), was demonstrated to inhibit microsomal NADPH-cytochrome c reductase activity in other dipteran species (including *Culex pipiens*) and was an effective inhibitor of 5 different monooxygenase activities in house fly microsome incubations, indicating that an unhindered NADPH-cytochrome P-450 reductase was necessary for monooxygenase activity. The fact that this antibody also inhibited aldrin epoxidation activity by mosquito microsomes in a similar manner is indirect evidence that the epoxidation is a cytochrome P-450 dependent

activity in Culex pipiens. Use of such a procedure for demonstration of cytochrome P-450 dependence has precedence (Masters et al., 1971; Burke and Mayer, 1974; Fisher and Mayer, 1982; Mayer et al. 1982).

4.4 Developmental Profile of NADPH-Cytochrome c Reductase and Aldrin Epoxidation Activities

Changes related to age and reproductive status have been demonstrated for NADPH-cytochrome c reductase and aldrin epoxidation activities in adult female Culex pipiens. Microsomal monooxygenase activities have been previously demonstrated to vary with age and developmental stage in several insects (Hook et al., 1968; Krieger and Wilkinson, 1969; Benke and Wilkinson, 1971A; Benke et al., 1972; Feyereisen and Durst, 1978; Gould and Hodgson, 1979; Tate et al., 1982). However, these studies and others have dealt with hemimetabolous insects or larvae of holometabolous species. Study of changes in monooxygenase activities in adult holometabolous insects has generally been limited to the families Muscidae, Calliphoridae, and Sarcophagidae (Khan, 1970; Perry and Buckner, 1970; Hansen and Hodgson, 1971; Brattsten and Metcalf, 1973; Capdevila et al., 1973; Yu and Terriere, 1974). Gilbert and Wilkinson (1973) studied monooxygenase activities in adult drone and worker honeybees during the first 4 days after emergence.

Generally, monooxygenase activities in holometabolous species a rise gradually during the successive instars, attaining a maximum in the ultimate instar. A striking decrease in activity occurs immediately before pupation and activity remains low during the pupal

period. Following emergence, activity increases in the adult during the first few days, usually reaching a peak between the second and fourth days after emergence and then declines thereafter to a relatively constant level, or in some cases, continuing to slowly decline as the organism ages.

Our results have demonstrated a similar pattern for Culex pipiens in which the peak activities of NADPH-cytochrome c reductase and aldrin epoxidation occur on the second day after emergence and then decline to a "basal" level maintained through 12 days after emergence. In blood-fed mosquitoes, these activities rose to peak values approximately twice that of comparably aged non-blood-fed mosquitoes during the period of 48 to 96 hours after the blood meal. The increase in these enzymatic activities shown to occur after a blood meal represents the first demonstration in vitro of induction of cytochrome P-450 associated enzymatic activities in a blood-feeding insect.

It is interesting that the peaks in activity in both non-blood-fed and blood-fed mosquitoes occur 12 to 24 hours after the peak ecdysteroid titers are seen and coincide with the periods of maximum follicle growth. Feyereisen and Durst (1980A, 1980B) have demonstrated apparent regulation of developmental expression of cytochrome P-450 monooxygenase activities by ecdysone in the fifth instar of Locusta migratoria. In this instar, which lasts about 8 days, hemolymph ecdysteroid titers, cytochrome P-450 dependent ecdysone 20-monooxygenase and lauric acid w-hydroxylase activities, and NADPH-cytochrome c reductase activity were all shown to reach a

simultaneous peak on the 5th day of the instar. When the prothoracic glands (which synthesize ecdysone) were removed prior to the 5th day of the instar, hemolymph ecdysteroid titers were approximately 100 times lower on the 5th day of the instar than in control insects. In the gland-deprived insects, ecdysone 20-monooxygenase activity on the 5th day of the instar was similar to that of insects in the early days of the instar and the increases in cytochrome P-450 content, lauric acid w-hydroxylase and NADPH- cytochrome c reductase activities normally seen on the 5th day of the instar were prevented. When a 1 µg dose of ecdysone was injected into 2 day old last instar larvae the enzymatic activities described above were induced.

These data provide a strong argument for ecdysone-mediated control of developmental expression of cytochrome P-450 monooxygenases in Locusta migratoria. While the present data concerning Culex pipiens do not provide such an argument, the correlation of increases in aldrin epoxidation and NADPH-cytochrome c reductase activities shortly after the ecdysteroid titer peaks provides reason to further investigate the possiblity of an interrelationship between these parameters. It is interesting to note the recent demonstrations that aldrin epoxidation activity in rat liver, lung and kidney microsomes can be induced by prior treatment of the rats with cyanopregnenolone and progesterone (Newman and Guzelian, 1983; Van Cantfort et al., 1983). Wolf et al. (1979) have shown that the ontogenetic appearance of aldrin epoxidation in rat liver coincides with that of ethylmorphine demethylase, a monooxygenase activity that can be induced by pregnenolone-16α-carbonitrile.

The increases in ecdysteroid titers and aldrin epoxidation and NADPH-cytochrome c reductase activities following the blood meal are of considerable interest. The question arises as to whether either or both are a response to the blood meal itself or are dependent on release and accumulation of a secondary hormonal influence from the brain and corpora cardiaca in response to the blood meal. An experimental approach to investigating this question would involve decapitation of fully blood-fed mosquitoes before the critical period for release of a head humoral factor. Ecdysteroid titers and enzymatic activities in these decapitated mosquitoes could then be determined at various times after the blood meal and compared to those of non-decapitated control mosquitoes. However, the fact that the critical period in Culex pipiens was 4 to 8 minutes after beginning but before completing the blood meal did not allow us to use this approach. Even if differences could be demonstrated on such a basis, we would not be able to rule out the possible explanation that they were merely a manifestation of the fact that the decapitated mosquitoes obtained smaller blood meals than the non-decapitated controls.

4.5. Ecdysteroid Titers

Spielman et al. (1971) demonstrated that egg maturation in non-blood-fed Aedes aegypti could be initiated by injection of µg quantities of 20-hydroxyecdysone. This finding was confirmed by Schlaeger et al. (1974) who further demonstrated, by use of a radioimmunoassay, that the titer of ecdysteroids in blood-fed

mosquitoes was higher than in non-blood-fed mosquitoes. Hagedorn and Judson (1972), employing an *in vitro* organ culture assay, observed that *Aedes aegypti* fat body synthezised vitellogenin after a blood meal. Subsequent experiments by Hagedorn and Fallon (1973) and Hagedorn (1974) indicated that fat bodies removed from blood-fed females that had been ovariectomized prior to the blood-meal were not capable of synthesis of vitellogenin *in vitro*. In addition, ovaries from blood-fed females which were cultured *in vitro* for 12 hours with fat bodies from non-blood-fed females initiated synthesis of vitellogenin by these fat bodies.

These observations led Hagedorn *et al.* (1975) to perform a radioimmunoassay for whole-body ecdysteroids in *Aedes aegypti*. In blood-fed *Aedes aegypti*, Hagedorn found an ecdysteroid titer profile which was qualitatively similar to that we have found for *Culex pipiens* with the exception that the peak in titers occured 20 hours after the blood meal, whereas it occurs 36 hours after the blood meal in *Culex pipiens*. In a radioimmunoassay of whole-body ecdysteroids in the autogenous species *Aedes atropalpus* Coquillett, Fuchs *et al.*(1981), found a similar profile with the peak titer occurring 32 hours after emergence. A hybrid resulting from a mating of male *Aedes atropalpus* and females of anautogenous *Aedes epactius* was also found to have a qualitatively similar ecdysteroid titer profile with a major peak at 24 hours after emergence. It should be pointed out that an EDNH-like factor is thought to be released form the corpora cardiaca in autogenous mosquitoes shortly after emergence (Lea, 1970, 1972; Masler *et al.*, 1980). Redfern (1982), also using the radioimmunoassay

technique, found that ecdysteroid titers in the anautogenous species Anopheles stephansi Liston followed the same general pattern and peaked about 30 hours after the blood meal.

While the qualitative nature of the ecdysteroid titer profiles for Culex pipiens, Anopheles stephensi, Aedes aegypti, Aedes atropalpus, and the hybrid of Aedes atropalpus and epactius are similar, our quantitative determination for 1 day old non-blood-fed Culex pipiens (15.1 fmol 20-hydroxyecdysone/insect; average live weight 2.5 mg/insect) is approximately 10 to 15 times lower than that reported for the Aedes species (non-blood-fed Aedes aegypti with age not specified or immediately after emergence for the autogenous Aedes species). Peak titers in the blood-fed profiles are approximately twice that of Culex pipiens (394 fmol 20-hydroxyecdysone/insect) in the case of Aedes aegypti and Aedes atropalpus while the hybrid Aedes value is about the same as that for Culex pipiens. The peak titer in blood-fed Anopheles stephensi is about 6-15 times that of blood-fed Culex pipiens.

The demonstration of increases in ecdysteroid titers in vitellogenic female Aedes species by Hagedorn and Fuchs and in Anopheles stephensi by Redfern, have now been extended to Culex by our work. Despite the fact that there is no clear consensus over the concept of a direct physiological role for ecdysteroids in control of vitellogenin synthesis in mosquitoes (for review, see Fuchs and Kang, 1981), our results are not in disagreement with Hagedorn's theory (1975) that ecdysteroids may play a role in control of egg maturation in mosquitoes (recall that the peak in ecdysteroid titers at 36 hours

after the blood meal in Culex pipiens was followed by a 12 hour period of rapid follicle growth). However, an unequivocal demonstration of a role for ecdysteroids in mosquito vitellogenesis and follicle maturation other than separation of the secondary follicles from the germarium following the blood meal (Beckemeyer and Lea, 1980) has not yet been attained. Hagedorn's demonstrations of fat body synthesis of vitellogenins in response to ecdysone in vitro were not confirmed by Borovsky and Handel (1979). Specifically these workers found that culture in vitro of ovaries from blood-fed Aedes aegypti with fat bodies from unfed females did not cause vitellogenin synthesis by the fat bodies as previously reported by Hagedorn and Fallon (1973), that fat bodies removed from unfed female Aedes aegypti 18 hours after they had been injected with 5 µg of 20-hydroxyecdysone synthesized 13% of the vitellogenin produced by control fat bodies removed from females 18 hours after a blood meal whereas Fallon et al. (1974) had reported an 80% value, that in an in vitro culture system fat bodies removed from unfed Culex nigripalpus Theobald or Aedes aegypti and incubated for 18 hours with 10^{-3} or 10^{-5} M 20-hydroxyecdysone failed to synthesize any more vitellogenin than control cultures with no ecdysone which directly conflicted with the results of Fallon et al. (1974), Hagedorn et al. (1975), Hagedorn et al. (1979). In subsequent investigations by Lea (1982B) with Aedes aegypti and Redfern (1982) with Anopheles stephensi, injection of non-physiological, high doses of 20-hydroxyecdysone (0.5-10 µg per insect) into unfed females failed to elicit vitellogenesis, and in fact, could apparently cause some degeneration of primary follicles in Anopheles

stephensi . In view of the lack of agreement between various sources of data available in the literature concerning the possible role(s) of ecdysteroids in mosquito vitellogenesis and follicle growth, a physiological interpretation of the significance of our demonstration of an increase in ecdysteroid titers in Culex pipiens following a blood meal must await further progress in this area of research. A recent study utilizing radioimmunoassay techniques demonstrated peaks in ecdysteroid and juvenile hormone titers occurring in close succession about 40 hours after emergence in two autogenous mosquito species, Aedes detritus Haliday and Aedes caspius Pallas (Guilvard et al., 1984). The possible involvement of juvenile hormone in the vitellogenic phase of follicle growth in anautogenous mosquitoes must therefore be considered.

4.6. Determination of a Critical Period for Release of a Head Factor

The critical period in Culex pipiens proved to be very short, our estimate being between 4 and 8 minutes after beginning but before completion of the blood meal. These results are in substantial agreement with those of Clements (1956) who found that anautogenous females of Culex pipiens form berbericus decapitated or ligated as soon as 3 to 4 minutes after beginning the blood meal developed their primary follicles beyond the resting stage. He obtained similar results with the anautogenous species, Aedes aegypti and Anophele labranchiae atroparvus Thiel. But Gillett (1956), working with African strains of Aedes aegypti, failed to confirm his results and estimated the critical period to be several hours after the blood

meal. Larson and Bodenstein (1959) found that Culex pipiens decapitated from 1 to 30 minutes after the blood-meal did not develop their follicles past the resting stage. Some follicle growth was noted in mosquitoes decapitated 30 to 60 minuites after the blood meal and those mosquitoes decapitated more than 60 minutes past the blood meal developed mature follicles. However, it should be noted that their experiments involved only 33 insects distributed over 9 time points and the validity of their results are, therefore, called into question. Clements (1963), discussing the lack of agreement in results obtained in different laboratories, concludes that differences in experimental procedures are probably responsible. This serves as a reminder of the caution to be used in assessing reliability and/or drawing conclusions from the various data available on critical periods in mosquitoes.

Lea (1972) demonstrated that in anautogenous mosquitoes stretch receptors and direct neural connections to the corpora cardiaca were not responsible for release of egg development neurosecretory hormone (EDNH). This implied that the signal for release of EDNH is probably a hemolymph-borne factor which he confirmed with parabiosis experiments. In Aedes aegypti, the critical period has been found by a number of investigators to be measured in hours rather than minutes and it is easy to imagine accumulation of a hemolymph-borne factor to a level sufficient to initiate release of EDNH during such a time period. However, the shortness of the critical period in our strain of Culex pipiens implies that such a hemolymph-borne signal can accumulate to a level sufficient to initiate EDNH release before the

blood meal is even completed. This raised the interesting possibilites that the rate of accumulation of the signal is drastically slower in Aedes than in Culex, that the signals are different, or that their mode of action or the response of the target organ (corporus cardiacum) differ, or even that stretch receptors and/or neural connections could be involved.

A number of investigators have addressed the problem of determining the nature of the signal initiating EDNH release. Borovsky (1982), on the basis of ovariectomy experiments with Aedes aegypti, concluded that the ovaries were necessary for release of ENDH after a blood meal and that they released a corpus cardiacum stimulating factor (CCSF). Experiments involving injection of 20-hydroxyecdysone led him to rule this compound out as the CCSF and ovary transplantation experiments with Aedes taeniorhynchus Dyar indicated that CCSF was not species specific. On the basis of a bioassay procedure for synthetic activity in corpora cardiaca and ovariectomy and implantation experiments, Lea (1982) reached a conclusion similar to Borovsky's in that the ovaries appeared to be the source of the EDNH releasing factor. On the other hand, Spielman and Wong (1974) produced evidence that the distention of the midgut was crucial to the release of EDNH. Supplementation of midgut blood volumes (normally too small to produce subsequent follicle growth) with saline solutions resulted in growth of follicles, but injection of air to increase midgut volume did not result in follicle growth. This suggested a possible additional influence by the nutrients in the blood meal. Chang and Judson (1977) investigated various solutions of

amino acids, blood plasma fractions and proteolytically processed peptides as initiators of follicle growth. They concluded that a supernatant of a trypsin digested solution of blood was capable of causing EDNH release while amino acids were only marginally effective releasers of EDNH. This work, therefore, implicated presence of peptides in the hemolymph as the signal for release of EDNH.

This discusson indicates the lack of consensus existing in the literature on the nature of EDNH release in response to the blood meal. Our critical period results and the disagreements in the literature discussed above, furthermore, point out the pitfalls inherent in attempting to extrapolate results obtained in one genus of mosquitoes to other genera in the family. This is a point which rarely receives attention in the literature.

4.7. Conclusion

The groundwork for further investigation of cytochrome P-450 dependent monooxygenase activities in mosquitoes of different age and reproductive status has been laid. An improved procedure for obtaining microsomes which is faster than the classic differential centrifugation procedure and better suited to the small quantities of tissue available from Culex pipiens has been developed. An assay for microsomal aldrin epoxidation activity has been developed and information on the developmental expression of this activity and that of NADPH-cytochrome c reductase has been obtained. A follicle growth profile before and after the blood meal has been determined and correlated with the above enzymatic activities and ecdysteroid titers.

This information and the development of the procedures described in the body of this thesis provide a firm basis for design of additional experiments to continue investigation of possible interrelationships between ecdysteroid hormones and ontogenetic expression of cytochrome P-450 monooxygenases in the mosquito, Culex pipiens.

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