MICROSOMAL MIXED-FUNCTION OXIDASES OF THE HOUSE FLY, MUSCA DOMESTICA (L): ENDOGENOUS FACTORS AFFECTING THEIR ISOLATION AND STABILITY

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ABSTRACT

MICROSOMAL MIXED-FUNCTION OXIDASES
OF THE HOUSE FLY, MUSCA DOMESTICA (L): ENDOGENOUS
FACTORS AFFECTING THEIR ISOLATION AND STABILITY

By

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The mixed-function oxidase NADPH-cytochrome c reductase has been shown to be tightly bound to the mammalian microsomal memgrane. House fly microsomal NADPH-cytochrome c reductase activity was extensively solubilized during the isolation of the microsomal fraction from mortar-and-pestle homogenates of whole house flies. In 23 isolations, only 20 ± 24.5% S.D. of the microsomal and soluble reductase activity was recovered in the pellet. Phenylmethylsulfonylfluoride, a protease inhibitor, and EDTA did not decrease the observed solubilization. The specific activity of the reductase was not significantly altered by these treatments and was 0.025 ± 0.008 μmoles S.D. of cytochrome c reduced/min/mg protein.

Microsomal fractions isolated from 30 abdominal homogenates prepared by glass-Teflon homogenization contained 80.3 ± 21.3% S.D. of the microsomal and soluble reductase activity recovered in the 105,000 xg pellet. The specific activity of the reductase for these isolations was 0.050 ± 0.025 moles S.D. of cytochrome c reduced/min/mg protein. The above differences obtained with the two methods of homogenization were significant

at the 0.01 and 0.05 level for the percentage of the reductase isolated in the microsomal fraction and its specific activity, respectively, when subjected to the Student's t-test. The aldrin epoxidase activities of the homogenates and microsomal fractions from glass-Teflon preparations were unstable. The homogenates lost 68% of their initial epoxidase activity in 30 minutes and the frozen microsomal fractions lost 50 to 60% of their activity within 24 hours.

Since the phenol oxidase complex has been implicated in the loss of aldrin epoxidase activity when whole southern armyworm homogenates were used, the effect of this system on rat liver microsomal mixed-function oxidases was examined. Phenol oxidase and its substrates are present in house fly homogenates and microsomal fractions, but have not been detected in rat liver microsomes. The incubation of rat liver phenobarbital-induced microsomes with tyrosinase and catechol at 4° C caused a significant decrease of cytochrome P-450 content, aldrin epoxidation, and NADPH-cytochrome c reductase activity. When rat liver microsomes were incubated with either cyanide-inhibited tyrosinase and catechol, tyrosinase alone, or catechol alone, the same enzymatic activity and cytochrome P-450 content as the untreated control was observed. Erythrocyte lysis, used as an indicator of the occurrence of free radical reactions, occurred when red blood cells were incubated with tyrosinase and catechol at 4° C. There was no lysis when they were incubated in the presence of tyrosinase alone or catechol alone. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of rat liver phenobarbital-induced microsomes incubated with tyrosinase and catechol indicated that cross-linkage of the proteins had occurred, since much of the sample failed to migrate into the gel. When a sample of microsomes incubated with cyanide-inhibited tyrosinase was electrophoresed,

it gave the same banding pattern as the untreated control.

The addition of catechol, a tyrosinase substrate, to the 10,000 xg supernatant of house fly homogenate gave a microsomal fraction without detectable cytochrome P-450 or P-420. Addition of cyanide to house fly homogenate significantly increased the cytochrome P-450 and aldrin epoxidase activity of the microsomal fraction. The aldrin epoxidation activity of the cyanide-treated microsomal preparation decreased 22% in 24 hours compared to 56% for the catechol-treated and untreated microsomal fractions.

The glass-Teflon homogenization method is superior to the mortar-and-pestle method for isolating house fly microsomes because of the extensive solubilization of the NADPH-cytochrome c reductase that occurred with mortar-and-pestle preparations. Microsomal aldrin epoxidation activity in house fly preparations obtained by glass-Teflon homogenization was unstable. The effect of tyrosinase and catechol on the rat liver microsomal mixed-function oxidases and of catechol and cyanide on house fly microsomal preparations indicate that the catechol oxidase complex may be an important factor involved in the instability of house fly microsomal mixed-function oxidases. The use of inhibitors and polymers to eliminate the effect of this complex should be a key factor in obtaining stable microsomal mixed-function oxidases from house flies.

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By

Daune L. Crankshaw

A DISSERTATION

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To my wife, Jacqueline

and our daughters,

Carolyn and Nadia

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Looking back over the time that I have spent at MSU, there are many people who have helped me during my doctoral program and deserve special thanks. Dr. Norman Leeling advised me during the first part of my research and also provided financial support for which I am grateful. My major advisor, Dr. Matthew Zabik, generously supported the latter part of my research. A special thanks goes to Dr. Stephen Aust, Biochemistry Department, for tolerating a "super fly" buzzing about his lab. His advice and encouragement helped me through the difficult and sometimes discouraging parts of my research. My association with the members of Dr. Aust's laboratory was a stimulating and helpful experience. The members of my advisory committee, Drs. A.W.A. Brown, Roger Hoopingarner and Robert Cook gave freely of their time and I appreciate their advice and help. Drs. Gordon Guyer and James Bath, the former and present chairmen, respectively, of the Department of Entomology, provided funds for assistantships and research for which I am grateful.

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INTRODUCTION

The role played by mixed-function oxidases in the detoxification of pesticides has become apparent only in the last decade. Insect detoxification systems are of special interest to man because they enable pest species to circumvent control measures and continue to compete with man for a limited food supply. The importance of microsomal mixed-function oxidases in pesticide detoxification is still being defined as more basic knowledge of this system accumulates.

Insecticide resistance is a complex phenomenon with factors, both enzymatic and non-enzymatic, contributing to the overall resistance of a species. Genetic analysis of susceptible and resistant insect strains has helped to characterize the factors involved. In vitro experiments employed to elucidate specific enzymatic detoxification mechanisms utilize subcellular fractions prepared from homogenates of insects. In addition, comparative studies using enzyme preparations from insect and mammalian sources can detect similarities and differences in the mechanisms utilized. Information of this nature aids in the design and development of pesticides that are less toxic to non-target organisms.

The importance of developing less toxic compounds lies not only in the fact that the use of pesticides has been growing nearly 10% a year, but in that some of the newer compounds being utilized are considerably more toxic to humans. With the recent banning of several persistent pesticides, such as DDT, there has been an increase in the use of the

less persistent organophosphorus compounds. These, although environmentally less persistent, are general biocides with some of the most widely used ones, such as parathion and azinphosmethyl, being quite toxic to man and other vertebrates.

The occurrence of resistance has been detected throughout the world and has appeared in most pest species of insects and acarines. Resistance has been demonstrated for each category of pesticides used to control these species. The recent development of insect hormone mimics as control agents was stimulated by the ubiquitous occurrence of insecticide resistance. It was hoped that the utilization of compounds which mimicked the actions of naturally occurring growth regulators would circumvent the problem of resistance. Such compounds would also be quite selective for the target organism. Research in this field has focused on the growth regulator juvenile hormone and its mimics. Evidence is beginning to accumulate that insecticide resistant species show cross-tolerance to juvenile hormone and its analogues. There are also reports of non-insecticide resistant strains developing tolerance to these compounds. The exact role that microsomal mixed-function oxidases play in the metabolism of juvenile hormone mimics is still unclear. The problem is complicated by the fact that esterases also have the capacity of deactivating these chemicals.

Due to the lack of a standardized method for the preparation of insect subcellular fractions to be used for in vitro studies, greater difficulties are encountered in trying to understand and interpret the importance of each detoxification mechanism. The great number of problems encountered in preparing enzymatically active fractions from insect homogenates, indicates that an optimum method of preparing these subcellular fractions is a prerequisite for the most accurate picture of the mechanism

involved. The optimum isolation procedure devised for each insect must take into consideration endogenous inhibitors and/or other unresolved factor(s) known to cause enzyme instability of *in vitro* preparations. The majority of these have yet to be completely identified and controlled in homogenates of insect tissue. The standard methods of analysis being used in related mammalian systems that are currently yielding the most significant information about microsomal enzymes demand stable preparations. Since insect homogenates lack this stability, the resolution of this problem is the major challenge facing researchers utilizing insect microsomal preparations.

LITERATURE REVIEW

The endoplasmic reticulum (ER) of certain tissues, such as liver of mammals and fat body of insects, contain enzymes that metabolize fatty acids, steroids and numerous xenobiotics (1). The homogenization of these tissues disrupts the ER, resulting in the formation of small membranous vesicles which can be isolated from other subcellular components, e.g. mitochondria, by differential centrifugation of the homogenate. The pellet of membranous vesicles obtained by high speed centrifugation of the microchondrial supernatant is called the microsomal fraction (2).

This fraction contains two electron transport systems. One, composed of NADH-cytochrome b_5 reductase and cytochrome b_5, is involved in fatty acid metabolism. The other is a mixed-function oxidase system which is involved in the detoxification of drugs and pesticides. This system is the one investigated in the research presented in this dissertation. Its composition, as currently understood, consists of NADPH-cytochrome c reductase and cytochrome P-450. This enzyme system requires atmospheric oxygen and reducing equivalents from NADPH. The electrons and oxygen are utilized in the hydroxylation of a wide variety of substrates. The mixed-function oxidases of mammals have been reviewed by Mason (1,3,4), Gillette (5,6), Orrenius et al. (7) and Siekevitz (8), and of insect systems by Terriere (9), Casida (10), Perry and Agosin (11), and Wilkinson and Brattsten (12). The role of cytochrome P-450 in insecticide resistance (13) and the induction of insect mixed-function oxidases by

pesticides has also been assessed in recent years (14).

Components of the Microsomal Mixed-Function Oxidase System:

The mixed-function oxidase components are diagrammatically represented in Fig. 1 (15). The flavoprotein, NADPH-cytochrome c reductase (often referred to as the reductase) accepts electrons from NADPH and reduces the cytochrome P-450. The exact mechanism of electron transfer is not completely understood. In studies with the purified enzyme, one electron at a time was transferred (16), however, the transfer mechanism for the second electron required per mole of substrate hydroxylated is still to be precisely identified, and the question mark in Fig. 1 indicates this area of uncertainty. The reductase is tightly bound to the microsomal membrane and is used as a marker enzyme for the microsomal fraction (17). In vitro assays for this enzyme employ electron acceptors such as cytochrome c and certain dyes.

Klingengberg (18) and Garfinkel (19) first described a unique cytochrome that appeared in difference spectra of the microsomal fraction of liver homogenates when it was flushed with carbon monoxide (CO) and reduced. Its unique spectrum shows only one, sharp maximum absorbance peak at 450 nm. Omura and Sato (20) have further characterized this cytochrome as a hemoprotein with several unusual properties; one property being the conversion of its typical P-450 form to a spectrally distinct form with its maxima at 420 nm by several reagents including deoxycholate (21,22). Conversion of cytochrome P-450 to P-420 was paralleled by the loss of enzymatic activity with the solubilized P-420 being extremely labile when reduced in the presence of oxygen. The biological function of this cytochrome was suggested by such observations as the inhibition of the steroid

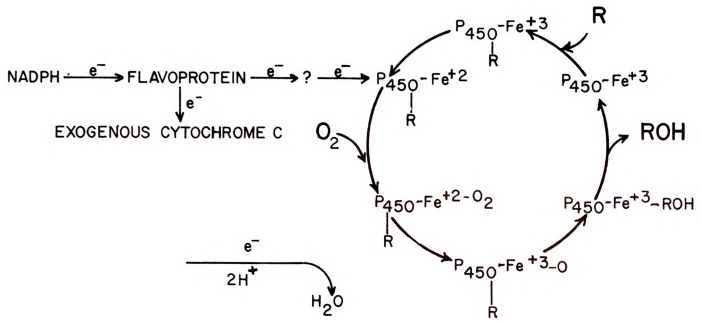

Figure 1. THE MIXED-FUNCTION OXIDASE ELECTRON TRANSPORT SYSTEM

C-21 hydroxylation activity of adrenal cortex microsomes by CO and the reversal of this inhibition by monochromatic light of 450 nm (23). Cytochrome P-450 has since been shown to be the oxygen-activating component of the mixed-function oxidase system (24).

Induction of the Microsomal Mixed-Function Oxidases:

The in vivo administration of certain drugs and pesticides such as phenobarbital and dieldrin elicit an increase in the rate of drug metabolism by the microsomal fraction. A corresponding increase occurs in the microsomal cytochrome P-450 content and NADPH-cytochrome c reductase activity (25,26).

This response to certain drugs and pesticides has aided investigators in elucidating how the system can metabolize a wide variety of substrates. Administration of phenobarbital (PB) or 3-methylcholanthrene (3-MC) to a wide variety of animals resulted in microsomes displaying different spectral forms, cytochrome P-450 and P-448 respectively (27), and different catalytic activities (28). Antisera obtained against partially purified cytochrome P-448 from the livers of 3-MC-treated animals were more specific for cytochrome P-448 from the liver microsomal fraction of 3-MC-pretreated animals than for the P-450 of PB-pretreated animals as determined by quantitative immunoprecipitation and Ouchterlony double diffusion analysis (29).

Welton (30) has compared the sodium dodecyl sulfate (SDS) polyacrylamide gel patterns of liver microsomes from control, PB- and 3-MC-treated rats. The gel protein and iodination profiles of the induced microsomes showed significant differences when compared to the control and to each other. The major differences were in the 43,000 to 53,000 molecular

weight band region of the gels. Gels of microsomes from PB-pretreated rat livers showed a dramatic increase in concentration and ^{125}I labelling of the 45,000 molecular weight band. Treatment with 3-MC induced a significant increase of the 53,000 band region in the gel. The major band in the gels of control microsomes was shown to occur in the region of 50,000 molecular weight (31).

An antibody obtained against cytochrome P-450 of PB-induced microsomes precipitated only the 45,000 molecular weight protein from the deoxycholate solubilized untreated, PB, and 3-MC microsomes, as demonstrated by polyacrylamide gel electrophoresis of the immunoprecipitate (32). Utilizing the same procedures, an antibody against purified NADPH-cytochrome c reductase from rat liver microsomes demonstrated that the reductase of control, 3-MC-, and PB-induced microsomes was antigenically and electrophoretically the same (33). This work explains how the mixed-function oxidases can metabolize a wide variety of substrates. Substrate specificity exists in the multiple forms of cytochrome P-450 which are reduced by the same reductase.

Fractionation of Tissue Homogenates:

Quantitative fractionation of the rat liver homogenate shows the above enzymes to be located almost exclusively within the microsomal fraction (34,35). Microsomal fractions are usually obtained by high speed centrifugation of the 10,000 xg supernatant and this procedure was employed in the present studies. The specific activity of rabbit NADPH-cytochrome c reductase, glucose-6-phosphatase, and aminopyrine demethylase in the microsomal fractions of rabbit lung and liver was found to be 2-4 times that in any other fraction. Microsomal glucose-6-phosphatase specific

activity in lungs was found to be about one-fiftieth of that in the liver. The specific activity of NADPH-cytochrome c reductase in the microsomal fraction from PB-treated rat liver was eight times the amount found in the homogenate. In the same study, marker enzymes for mitochondria, peroxisomes, and plasma membranes had specific activities lower than those found in the homogenate (36).

In one study of rat liver homogenate, the distribution of 25 enzymes in fractions obtained by differential centrifugation was examined. Eight of the enzymes studied, including glucose-6-phosphatase, NADPH-cytochrome c reductase, and aminopyrine demethylase, had 70% or more of their enzymatic activity recovered in the microsomal fraction and had similar distribution patterns. The microsomal specific activity of these enzymes was almost four times that found in the homogenate. Contamination of the microsomal fraction by mitochondria, lysosomes, and peroxisomes was 6% of the total microsomal protein. The microsomal fraction in this study was from the 41,000 xg supernatant of the mitochondria plus lysosome fraction (35).

Ultrastructural studies of the microsomal fractions from rat liver show that they consist primarily of endoplasmic reticulum and ribosomes. The microsomal fraction of rabbit lung contained tangles of filamentous material that trapped vesicle and free ribosomes. Liver microsomal sections rarely contained such structures (36).

Effect of Isolation Procedure:

The method and duration of homogenization, the gravitational forces used in centrifugation and the homogenization medium all affect the degree to which the microsomal fraction will be contaminated by other

cellular components. Hook et al. (36) compared four methods of homogenization and the effect of the length of homogenization for the four methods he employed in isolating microsomes from rabbit lung. As the duration of homogenization increased so did the contamination of the microsomal fraction by mitochondrial enzymes. The specific activity of microsomal enzymes also decreased with increased homogenization. By comparing the cytochrome P-450 concentration, mitochondrial contamination, and the release of aryl sulfatase from lysosomes, it was demonstrated that the glass-Teflon® method of homogenization produced a better microsomal preparation than did the glass-glass homogenizer, Waring Blendor® or VirTis® homogenizer.

Microsomal fractions from glass-Teflon homogenization of whole house flies also were shown to have more enzymatic activity than those prepared by Waring Blendor or Sorvall Omnimixer® homogenization (37). Increasing the duration of homogenization of house fly abdomens gave preparations with decreasing enzymatic activity. Four passes with a glass-Teflon homogenizer was the maximum amount of homogenization attainable without a decrease in enzymatic activity. A tight-fitting pestle would also decrease the activity of the preparation (38).

The effect of centrifugation on the purity of a subcellular fraction is influenced by the tissue composition. The rate of NADPH oxidation and the ultrastructure of the microsomal fractions from house fly abdomens and the gut and fat body of the southern armyworm, Spodoptera eridania (Cramer), were examined following different centrifugation regimens. Electron micrographs of the microsomal fractions from the 15 minute 10,000 xg supernatant of house fly abdominal homogenates showed primarily ribonucleoprotein particles and membranous vesicles. Microsomal pellets

of S. eridania gut prepared with a preliminary 10,000 xg spin for 15 minutes contained numerous mitochondria and microorganisms. Increasing the preliminary spin to 15,000 xg removed the microorganisms and mitochondria. A preliminary spin of 20,000 xg removed large glycogen clusters. A more uniform preparation of small membranous vesicles were observed when the preliminary spin was increased to 30,000 xg. However, the specific activity of NADPH oxidase was not significantly reduced when the force of the preliminary spin was increased (39). The effect of the different centrifugation rates on microsomal drug metabolism, NADPH-cytochrome c reductase and cytochrome P-450 content was not examined. Wilkinson (personal communication, Cornell University) has not observed bacterial contamination of his microsomal preparation from the midgut of the armyworm. He suggested that the difference in ultrastructural observations might depend on the thoroughness with which the gut contents were removed by washing prior to homogenization.

Several workers (40 to 42) in assessing the distribution of drug and pesticide metabolizing activity in fractions of insect homogenates, have found the greater part of the activity in the microsomal fraction. The specific activity of the microsomal fraction relative to the total homogenate activity was reported to be two-fold greater in the house cricket, Acheta domesticus (L.) (40), 1.7-fold in armyworm midgut (41), and 2.8-fold in caeca of the Madagascar cockroach, Gromphadorina portentosa (Schaum (42).

Endogenous Inhibitors of Insect In Vitro Preparations:

Researchers isolating the microsomal fraction from insect tissues have found numerous endogenous inhibitors of mixed-function oxidases.

A potent inhibitor of these enzymes isolated from the gut contents of the armyworm was identified as a protease (43), that can solubilize the microsomal NADPH-cytochrome c reductase (44). Ninety to 100% solubilization of this enzyme occurred at low concentrations of the partially purified inhibitor. Since the amount of protein released during incubations with the inhibitor was only 5% of the total microsomal protein, this enzyme was very susceptible to proteolysis. Cytochrome P-450 was apparently not affected by the low concentrations of the partially purified inhibitor used in the study (44). The gut contents of the house cricket were also found to contain a protease that inhibited microsomal pesticide metabolism (45). Proteolytic activity has been detected in homogenates of other insect species including the house fly (43). Although every body segment of house flies contains an inhibitor(s) of mixed-function oxidases (46), the inhibitor from the head has been the one most extensively characterized.

Matthews and Hodgson (47) found the microsomal fraction from whole house flies to be inactive unless it had been extensively dialyzed. The freeze-dried dialysate obtained from the dialysis of the 15,000 xg supernatant of whole house fly homogenate inhibited the metabolism of p-nitrophenyl N,N-dimethylcarbamate by mouse liver microsomes. The inhibitor was heat stable, water soluble, and insoluble in most organic solvents. Tsukamoto and Casida (48) reported that fly abdomens gave much greater mixed-function oxidase activity than whole house flies, heads, or thoraces. The homogenates of heads and/or thoraces greatly reduced the activity of the abdominal preparation, and the inhibitor was again heat stable. However, this heat stable inhibitor was absent from the head and thoraces of house flies with the mutant yellow-eye (38). Schonbrod and

Terriere (49) found that single heads of wild-type house fly strains decreased microsomal naphthalene hydroxylation by 60 to 65%, whereas heads from white- or ocra-eyed mutant strains inhibited naphthalene metabolism by 10% or less. A subsequent report (50) by these workers described the isolation of the eye pigment, xanthommatin, from fly heads and its inhibition of mixed-function oxidases. The eye pigment acted as an electron sink by accepting electrons from NADPH-cytochrome c reductase. Concentrations as low as 5×10^{-7} M would inhibit aldrin epoxidation. Extrapolation of their published data suggests that the xanthommatin I_{50} of aldrin epoxidation would be 2×10^{-6} M. Wilson and Hodgson (51) demonstrated that purified NADPH-cytochrome c reductase from house flies would reduce xanthommatin, which incidentally stimulated the reduction of cytochrome c by this enzyme. The pigment prevented the reduction of cytochrome b_5, but did not interact with cytochrome P-450.

Other inhibitors present in house flies have not been characterized. Jordan and Smith (38) described two factors in house fly abdomen homogenates that decreased the mixed-function oxidase activity. One was called the linearity factor because it limited the time over which the reaction proceeded at a constant rate. The rate of biphenyl hydroxylation was not proportional to the amount of enzyme used when the homogenate concentration exceeded two abdomens/ml. Including bovine serum albumen (BSA) in the assay increased the time over which the reaction was linear. The second factor was called the decay factor, which reduced the hydroxylase activity of an improperly made preparation to negligible activity within 30 minutes after homogenization. Homogenates containing 25 abdomens/ml lost 80% of their hydroxylating activity in 30 minutes. Those containing 5 abdomens/ml decayed more slowly but still lost about

50% of their activity in 30 minutes. The rate of decay was essentially the same at 0° and 20° C. Substrates, BSA, and KCN prevented the loss of activity. BSA would restore the activity lost in a "decayed" preparation, and enhanced the enzymatic activity of the fresh homogenate. KCN increased the reaction rate but not the amount of time over which the reaction was linear.

Both of these factors were heat labile, proportional to the concentration of enzyme used, and were not removed by centrifugation at 10,000 xg. The release of these factors was also affected by the method of preparation. Prolonged homogenization, i.e., more than four strokes in a glass-Teflon homogenizer, and a tight-fitting Teflon pestle caused a decrease in the stability of the preparation.

Another factor that has been implicated in the instability of insect microsomal preparations is the polyphenol oxidase complex. Williamson and Schecter (52) found that the 12,000 xg filtered supernatant of lepidopterous larvae darkened rapidly on exposure to air. They attributed this to phenol oxidase activity. Ascorbic acid and phenylthiourea, both inhibitors of this enzyme, prevented the melanization of the supernatant. An increase in the concentration of the supernatant increased the degree of darkening and also significantly reduced its aldrin epoxidase activity. Increasing the supernatant concentration with phenylthiourea present gave an increase in the amount of dieldrin produced.

The microsomal fraction from the lepidopterous preparations darkened only over an extended period of time. Adding BSA to the microsomal incubation mixture nearly doubled the activity. Addition of KCN to the microsomal fraction caused a decrease in aldrin epoxidation activity which was the opposite of what occurred for the 12,000 xg supernatant.

Drug metabolism by rat liver microsomes is decreased by the addition of KCN (53). A KCN-binding protein has been isolated from rat liver microsomes (54,55). Addition of KCN to the microsomal fraction of house fly homogenates, however, increases the amount of pesticide metabolized (56, 57,58). Cyanide is known to inhibit a large number of enzymes (59) thus the stimulation of activity in house fly preparations by KCN may involve other factor(s) in addition to tyrosinase. This was suggested by the following observations of Krieger and Wilkinson (60); they bubbled air through the soluble fraction of southern armyworm homogenates to stimulate tyrosinase activity and found that the darkened soluble fraction when added to the incubation media sharply reduced aldrin epoxidation. Neither BSA nor KCN prevented the inhibition by the darkened supernatant. They concluded that it was the products of the tyrosinase activity that caused the inhibition.

Biochemistry of Catechol Oxidase:

The phenol oxidase complex (EC 1.10.3.1) (61 thru 65), also called polyphenol oxidase, catechol oxidase or tyrosinase, plays an important role in the process of cuticle hardening and tanning in arthropods (66). The purified enzyme appears to have two metabolic functions, namely, the o-hydroxylation of phenols and the oxidation of the phenols to the corresponding quinones (61,65). Tyrosine hydroxylation by this enzyme has an initial lag phase that can be eliminated by catalytic amounts of catechol. This has indicated to some workers that the enzyme has, in addition to the catalytic sites, a third modifier site (67). The function of tyrosinase in the process of cuticle formation (68,69) is shown in Figure 2. Since the enzyme incorporates one atom of oxygen into the

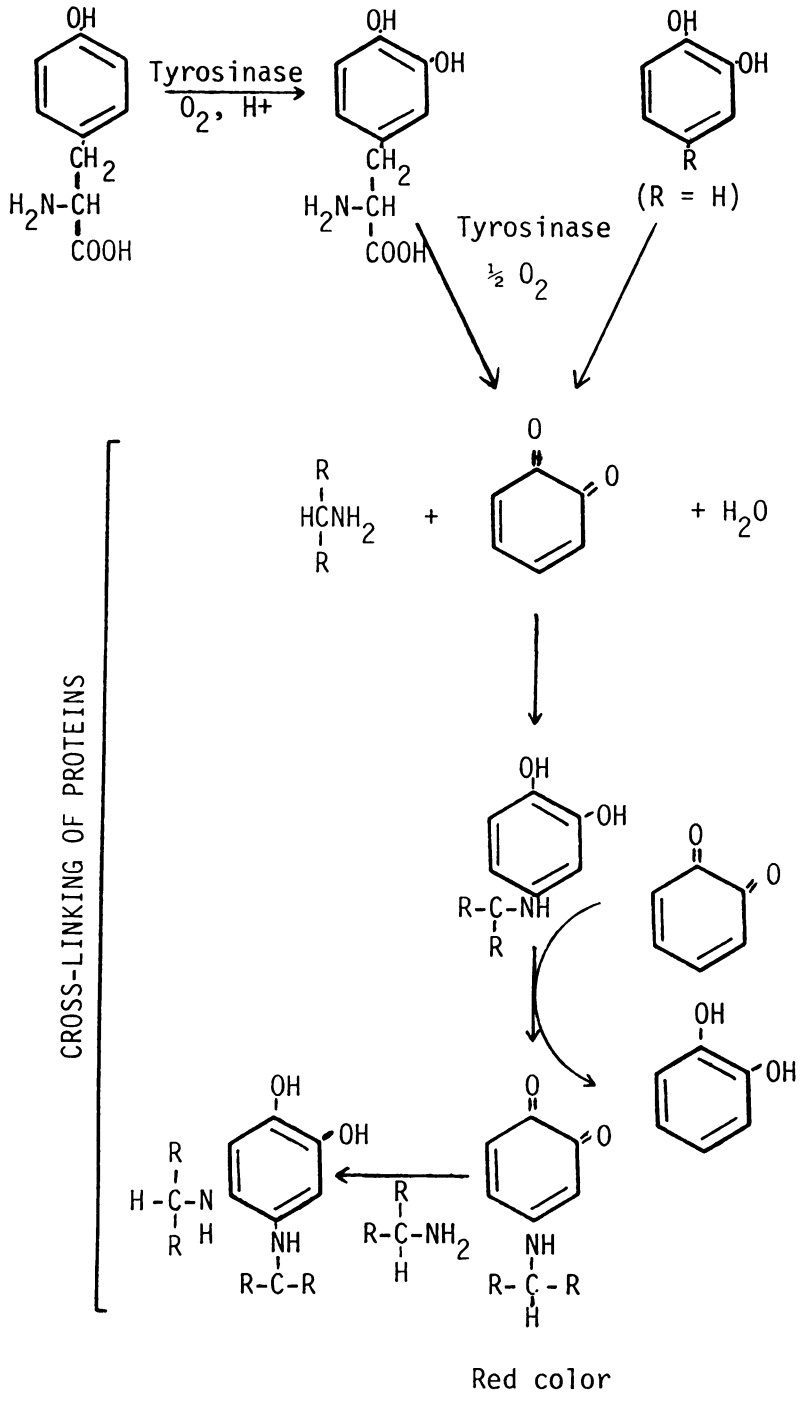

Figure 2. REACTIONS OF THE TYROSINASE COMPLEX

substrate and reduces the other atom to water, it is a mixed-function oxidase (3). The reducing equivalents may come from the oxidation of the phenols or such compounds as NADH, NADPH, cytochrome and FMN (61, 70). Tyrosinase is a copper-containing enzyme and is inhibited by KCN, phenylthiourea, glutathione, 2,3-dimercaptopropanol (BAL), and borate (63,71).

In insects, this enzyme has been found in the hemolymph and cuticle and is easily extracted from the cuticle by cold water (72). Tyrosinase activity in the soluble fraction of adult house fly homogenates was 6 times that detected in the microsomal fraction (58). The levels of the enzyme activity vary during the different stages of an insect's life cycle. In several insects there is a peak of tyrosinase activity just before pupation, followed by a dramatic decrease. In dipterous insects, the enzyme appears to be initially a proenzyme which is thought to be activated by a protease (73). Other workers postulate that the activation is an autocatalytic process (66).

Ohnishi (74) found the activation of tyrosinase to be inhibited by a NaCl concentration of 0.25 M or greater, and other salts had a similar affect. Since 0.25 to 1.0 M sucrose did not interfere with the activation he concluded that the inhibition observed was a function of ionic strength. Schweiger and Karlson (73) saw a similar inhibition by increased ionic strength of prophenoloxidase activation in samples from Calliphora larvae.

Mushroom tyrosinase has been observed to catalyze the oxidation of tyrosine residues in several proteins. Alcohol dehydrogenase lost 50%, and aldolase 82%, of their enzymatic activity when incubated with tyrosinase. Aldolase incubated in the presence of cyanide-inhibited tyrosinase

maintained control levels of activity. One of the other proteins whose tyrosine residues were oxidized by tyrosinase was BSA (75,76).

Phenols and Quinones in Biological Systems:

The quinones produced by tyrosinase are very reactive compounds (61,77,78). Webb (77) lists 63 enzymes that are inhibited by various quinones. The list included cholinesterase, cytochrome oxidase, deoxyribonuclease, dopa decarboxylase, NADH-tetrazolium oxidoreductase, proteinase and succinate dehydrogenase. The more common mechanisms by which quinones are known to inhibit enzymes are:

1) oxidation of enzyme groups
2) reaction with enzyme sulfhydryl groups
3) complexing with metal ions of enzymes
4) reactions with substrates or cofactors
5) production of hydrogen peroxide
6) nonspecific binding through aromatic rings
7) competition with quinonoid or polyphenolic substrates

It has been observed that the interaction of phenols with proteins caused marked changes in their structure and properties (79).

The reactivity of quinones lies in their ability to participate in oxidation-reduction reactions. Within the pH range of biological systems, the oxidation reduction potentials (E'_0) of most quinones fall between that of the cytochromes and the flavins, as follows:

	E_0' (pH + 7.0)
o-quinone	+ 0.378 v.
p-quinone	+ 0.284 v.
cytochrome b	+ 0.770 v.
cytochrome c	+ 0.250 v.
flavins	- 0.210 v.
pyridine nucleotides (NAD)	- 0.320 v.
thiols	- 0.350 v.

These values (77) indicate that quinones could interact with electron transport systems. Webb lists the following ways in which they could bring this about:

1) directly inhibit enzymes of the sequence
2) compete with or displace natural or endogenous quinones
3) serve as simple electron donors or acceptors
4) establish alternate or bypass pathways for electron flow
5) block specific site in the electron transfer sequence

The oxidation-reduction reactions of most quinones are thought to involve the free-radical (semiquinone) intermediate. Miller and Rapp (80) used electron spin resonance spectroscopy to observe the photochemical and enzymatic production of the semiquinone from catechols and the oxidation of ferrocytochrome c by the radical. The formation of a stable complex between the catechol semiquinone and the copper of superoxide dismutase has been observed by Rapp and co-workers (81). The development of electron spin resonance (ESR) signals was detected in cuticles of Calliphora erythrocephala as the white cuticle darkened during the process of melanization (82). The development of an ESR signal was also

observed in cultures of melanoma cells following the introduction of tyrosinase and catechol. Severe alteration in cellular morphology such as the deterioration of membranes was observed within 24 hours after the chemicals were introduced (83).

Several phenolic compounds have been isolated from insect homogenates. The compounds identified thus far have the catechol nucleus substituted with different groups at the 4 position of the ring (69).

The Effect of Free Radicals in Biological Systems:

The effects of free radical reactions occurring within biological systems has drawn increased attention recently. It has been proposed that these reactions are the mechanism through which several xenobiotics exert their toxic and carcinogenic effects (84). Lipid peroxidation is a free radical process that is thought to play a role in several diseases and in the aging process (85).

The process of lipid peroxidation can be described as follows (86):

Initiation: Rh----------R·

Propagation: R· + O_2----------ROO·

Termination: 2 ROO· ----------stable products + O_2

The reaction can be stimulated by catalytic amounts of transition metal ions such as iron which promotes the formation of radicals. It can be inhibited by antioxidants such as vitamin E and butylated hydroxytoluene (BHT) which trap the radicals thus preventing propagation. Lipid peroxidation has been demonstrated by oxygen consumption, NADPH oxidation and malondialdehyde formation (87). The NADPH-cytochrome c reductase of rat liver microsomes has been shown to be an enzymatic component of NADPH-dependent lipid peroxidation (88).

Malondialdehyde is a product of lipid peroxidation only when fatty acids having three or more methylene-interrupted olefin bonds are substrates. It can be detected in nanomolar quantities by the formation of a red colored chromagen in the presence of thiobarbituric acid (TBA) (89). Though lipid peroxidation has been demonstrated to occur in rat and goat liver microsomes, it has not been detected in microsomal fractions of insects. The addition of the house fly microsomal or soluble fraction to goat liver microsomes completely inhibited the formation of the TBA chromagen, and presumably, lipid peroxidation. House fly microsomes (heat killed), tyrosinase plus tyrosine and dihydroxyphenylalanine also inhibited the reaction. The addition of either tyrosine, tyrosinase or tyrosinase (heat killed) plus tyrosine did not prevent lipid peroxidation (57). Tyrosinase is known to be present in the house fly microsomal and soluble fraction (58,91). The endogenous substrates of tyrosinase have been shown to act as lipid antioxidants (90). The above data would indicate that the failure to detect lipid peroxidation in housefly microsomal preparations is because of the presence of this system.

Lipid peroxidation of microsomes causes the degradation of membrane structure and a decrease in enzymatic activity. A visible clarification of the normal turbidity of the microsomal suspension also occurs (92). Bidlack and Tappel found that in one hour lipid peroxidation caused a 50% loss of NADPH-cytochrome c reductase and a 90% decrease in glucose-6-phosphatase activity. Solubilization of protein from the microsomal membrane also occurred. Enzyme inhibition and protein solubilization was significantly reduced when an antioxidant was present during incubation (93). Cytochrome P-450 was also degraded during lipid peroxidation (92). The spectral shift from cytochrome P-450 to P-420 during the

peroxidation process was different from that seen during the incubation of microsomes with trypsin (94). The CO-difference spectra of trypsin-treated microsomes showed a parallel shift of the absorbance at 450 nm to 420 nm when the amount of protease was increased. An increase in lipid peroxidation resulted in a decrease of absorbance at 450 nm without a corresponding increase in the absorbance at 420 nm (92).

MATERIALS AND METHODS

The Fc strain of house flies used in this research was obtained from Dr. F. W. Plapp, Jr., Texas A & M University, College Station, Texas. The rat liver microsomes were supplied by Dr. S. D. Aust, Department of Biochemistry, Michigan State University.

NADP-isocitrate dehydrogenase (Sigma type IV), cytochrome c (Sigma type VI), bovine serum albumen, fraction V, $NADP^+$, NADPH, DL-sodium isocitrate, mushroom tyrosinase grade III, phenylmethyl-sulfonylfluoride, diisopropylfluorophosphate, dithiothreitol, EDTA, Tris base and Tris-HCl were all obtained from the Sigma Chemical Company, St. Louis, Missouri. Catechol was obtained from the Aldrich Chemical Company, Milwaukee, Wisconsin. Aldrin and dieldrin recrystallized, were obtained from Shell Chemical Company, New York, N.Y. Mushroom tyrosinase, B grade, was obtained from Calbiochem, La Jolla, California. All other reagents used were analytical grade. All aqueous solutions were prepared with distilled water that had been passed through charcoal and a mixed-bed resin column.

The House Fly Strain and its Culture:

The Fc strain of house flies has a high mixed-function oxidase activity when compared to susceptible strains and it is resistant to several pesticides such as DDT and dieldrin (95). The adults were reared under a 16:8 photoperiod at 80° C and 55% relative humidity. They were

fed a diet consisting of a 1:1 mixture of lowfat powdered milk and powdered sugar with tap water ad libitum. Every few months, the adults were exposed to 100 ppm of DDT in their food, in order to maintain the resistance level of the colony. The DDT was dissolved in acetone and mixed thoroughly with the mixture of ground sugar and powdered milk; the acetone was allowed to evaporate thoroughly before the mixture was fed to the flies.

Eggs were obtained by exposing the adults to NH_4CO_3 solution impregnated in a cloth, which was stretched over the wet CSMA medium employed to rear the larvae. The eggs were suspended in distilled water at 21° C and allowed to settle, and the water was decanted, the process being repeated 2 more times. Lots of approximately 300 eggs were then aspirated with a suction bulb into a calibrated glass tube for transferring to the larvae medium.

The CSMA larval medium (2400 ml) was mixed with malt syrup (25 ml), 5% yeast solution (75 ml), and lukewarm tap water (1200 ml). This mixture was then transferred into a No. 10 fruit can, to which the eggs were added, covered with paper toweling, and kept in a growth chamber at 80° F and 70% relative humidity. During the 2nd and 3rd days of larval development, a total of 250 mls of tap water was added to each can. In order to prevent the growth of mold which would interfere with complete larval development, it was necessary to stir the media on the 3rd day of larval development.

Under these controlled conditions, the larva pupated on the seventh day and were transferred into aluminum pans. Prior to their emergence on the 10th day of development, the pans were placed in cages. Occasionally, the adults were egged for maintenance of the colony. It was also observed

that after the 6th day following emergence the adults in the cage consisted almost entirely of females. Except for the study concerning the effect of age on enzymatic activity, the flies collected consisted primarily of 8 to 10 day old females.

Isolation of the Microsomal Fraction from House Fly Homogenates:

The isolation of the microsomal fraction from whole house flies or their abdomens from mortar-and-pestle (MP) homogenates followed the procedure outlined by Morello et al. (96). The flies were anesthetized in a walk-in freezer at -20° C, collected in a pre-chilled flask, and kept on ice. A buffer volume, 4 times the weight of the flies, was poured into a pre-chilled mortar and homogenized by gentle strokes of the pestle which was continued until a brei was obtained, 2 to 3 minutes later. The brei was then filtered through a double layer of cheesecloth into a cold flask and transferred into centrifuge tubes. The buffer used during the preliminary and aging studies was 0.15 M phosphate buffer that ranged from pH 7.4 to 7.8. The homogenate was then centrifuged at 10,000 xg for 20 minutes, followed by 105,000 xg for 90 minutes, in order to isolate the microsomal fraction. The microsomal pellet was then homogenized gently in a 1:1 ratio of 0.05 M Tris buffer and glycerol at pH 7.5 and stored under nitrogen at -20° C if not used immediately.

Distribution Studies of Marker Enzymes in MP Homogenates:

The MP method of homogenization was the same as used above, with the changes being made only in the buffer parameters and centrifugation regimen. Sucrose of 0.32 M concentration, isotonic to insect hemolymph, was employed to help preserve the integrity of mitochondria during the isolation. After filtering the first MP homogenate, it was then rehomogenized

by 2 strokes in a glass-Teflon homogenizer (GT) to insure a more complete disruption of the cells. The differential centrifugation of the homogenate was done at 800 and then 5,000 xg, each for 10 minutes, followed by 10,000 and then 20,000 xg, each for 20 minutes, and 105,000 xg for 90 minutes. The centrifugation of 20,000 xg and below were done in a Sorval RC2 refrigerated centrifuge using the GSA and SS-1 rotors. The centrifugation at 105,000 xg was performed in the Beckman model L ultracentrifuge with type 30 and Ti 50 rotors.

The pellets obtained from the 800 xg through the 20,000 xg centrifugations were resuspended in Medium A (see below). The microsomal fraction from the 105,000 xg centrifugation was resuspended in 0.05 M Tris plus glycerol, 1:1, at a pH of 7.5. The volumes for each fraction were recorded, and protein assays were made by the Lowry method (97). The distribution patterns of marker enzymes were calculated by the method of de Duve (98).

Sucrose Density Gradient Centrifugation:

The density gradient centrifugation method utilized a discontinuous gradient composed of 8 ml each of 10, 20, 41.5, and 50% sucrose. A 5 ml sample of the 800 xg supernatant in 0.32 M sucrose layered between the 10 and 20% layers of the gradient. The preparation was centrifuged for 2.5 hours at 24,000 rpm in the Beckman model L ultracentrifuge with a SW 27.1 rotor. The density gradient columns were monitored by the use of an ISCO density gradient fractionator coupled to a UA-2 ultraviolet analyzer set at 254 nm. Two-ml fractions were collected during the monitoring, kept at 4° C, and assayed for cytochrome oxidase and NADPH-cytochrome c reductase activity.

Isolation of the Microsomal Fraction from Abdominal GT Homogenates:

Adult house flies, 7 to 10 days after emergence, were anesthetized in a walk-in freezer, -20° C, and kept on ice. The whole flies were then transferred into a 2-ℓ Erlenmyer flask containing 6 mm glass beads that had been pre-chilled in a dry-ice acetone bath. During the following 20 minutes, the flask was turned several times to ensure the thorough freezing of the flies, after which it was shaken rapidly by hand for 30 seconds and replaced in the dry-ice-acetone bath. The flask was rotated in the bath for 2 minutes and then the shaking was repeated. Three cycles of shaking and freezing were required to separate the heads, abdomens, and thoraces from each other. The separated segments were replaced in the ice bath for 2 minutes and then emptied into a set of sieves held at -20° C. The beads were retained by the first sieve (US Standard Sieve Series, Tyler equivalent 10 mesh), the abdomens and thoraces by the second sieve (Tyler equivalent 6 mesh), and the heads were collected in a pan. The thoraces and abdomens were kept frozen and the abdomens separated from the thoraces by an aspiration procedure.

The abdomens were rinsed once in a buffer volume 4 times the sample weight (4° C) and homogenized in the same buffer volume using 2 strokes of the GT homogenizer with a loose-fitting pestle. The homogenate was filtered through 2 layers of cheesecloth into a chilled flask and centrifuged at 10,000 xg for 20 minutes. The 10,000 xg supernatant was centrifuged at 105,000 xg for 90 minutes. The resulting pellet was suspended by gently homogenizing it with a GT homogenizer in 0.2 M K_2HPO_4 and 50% glycerol at pH 8.0. If not used immediately, it was stored under N_2 at -20° C.

Enzyme Assays and Solution Preparation:

All solutions were prepared in deionized distilled water. The phosphate buffers were filtered through a 5 μ Millipore filter and then passed over a Dowex chelating column to remove impurities.

Assays of NADPH-Cytochrome c Reductase Activity:

The reduction of cytochrome c was measured by following the increase of absorbance at 550 nm with a Beckman DB spectrophotometer. The assays were made with 35 μM of cytochrome c and 0.16 mM of NADPH in 0.15 M phosphate buffer at pH 7.5 or 8.0. The rate of activity is expressed in μmoles of cytochrome c reduced/min/mg protein, using an extinction coefficient of 2.10×10^{-4} M^{-1} cm^{-1} (99).

Assay of Cytochrome Oxidase Activity:

The oxidized form of cytochrome c at a concentration of 0.175 mM in medium A was reduced with sodium dithionite crystals. Air was passed over a 10% NaOH solution and bubbled gently through the cytochrome c solution for 15 minutes. Medium A was composed of 15 mM KCl, 5 mM $MgCl_2$, 50 mM Tris, 2 mM EDTA, and 35 mM phosphate (100). The assays for the oxidation of the reduced cytochrome c were made with 17.5 μM of reduced cytochrome c and medium A at pH 7.5. The oxidation of reduced cytochrome c was measured by following the decrease in absorbance at 550 nm with a Beckman DB spectrophotometer.

Microsomal Pesticide Metabolism Assay:

The epoxidation of aldrin to dieldrin was used to assay the mixed-function oxidase activity of the microsomal preparations (101). The 5-ml reaction mixture contained microsomes or the homogenate, 0.2 M K_2HPO_4 at

pH 8.0, and a NADPH-generating system that consisted of 2 mM DL-isocitrate, 7 mM MgCl, 0.1 mM $NADP^+$, and 0.05 units of NADP-isocitrate dehydrogenase per ml. The mixture was pre-incubated in a water-bath shaker at 30° C for 5 minutes, and then 27.5 μM of aldrin was added in 0.1 ml ethanol. The dieldrin production was measured at various time intervals by pipetting 1-ml samples from the reaction mixture and mixing the sample immediately with 15 mls ethanol-acetone-water (2:1:1).

Extraction of Pesticides:

After the protein had precipitated, the sample was poured into a 60-ml separatory funnel and extracted 3 times with 10 mls redistilled n-hexane. The combined extracts were backwashed 3 times with approximately 30 mls deionized distilled water, and the hexane was then dried over anhydrous Na_2SO_4. A stream of N_2 was used to reduce the volume of the house fly extracts to 2 ml. The efficiency of this extraction procedure averaged 82% in its recovery of dieldrin. A 2-λ sample of the hexane extract was injected into the gas chromatograph for quantitation.

Gas Liquid Chromatographic Analysis:

A Packard 834 Gas Chromatograph equipped with a tritium-foil electron-capture detector was employed to determine the amount of dieldrin present in each sample. It was fitted with a 6 foot x 2 mm I.D. silanized glass column, packed with 3% XE-60 on 60/80 mesh Gas Chrom Q (Applied Science Laboratories). The instrument was operated at a column temperature of 200° C and a N_2 flow of 50 ml/min. The injection port temperature was 220° C and the detector temperature 210° C.

Standards of aldrin and dieldrin were injected at the beginning and end of each run, with intervening check being added during long runs.

The linearity of the detector was determined and only the samples injected within that range were taken for determination of the dieldrin concentrations. The retention times of aldrin and dieldrin under the conditions stated above were about 1.0 and 3.5 minutes respectively. The height and area of the peaks were measured and were then cut out and weighed; the figures obtained were similar and congruent with the computer determinations of the peak areas. The rate of dieldrin production was expressed as nmoles/min per mg protein.

Microsomal Cytochrome P-450 Analysis:

The cytochrome P-450 spectra were obtained by the method of Omura and Sato (20). The microsomal preparations, 1.5 to 4.0 mg protein per ml, were divided between two cuvettes. Carbon monoxide was bubbled gently through the sample cuvette for one minute. A few crystals of sodium dithionite were added to the sample and reference cuvettes and mixed gently until dissolved. The sample was scanned twice in a Beckman DB spectrometer. The scan was repeated a third time following the addition of several crystals of sodium dithionite making certain that the sample was fully reduced. The cytochrome content was calculated on the difference in absorbance at 490 and 450 nm and was expressed in nmoles/mg protein using an extinction coefficient of 91 mM^{-1} cm^{-1} (21).

Assay of Tyrosinase Activity:

The tyrosinase activity of microsomal preparations and mushroom tyrosinase were assayed using 5 mM catechol in a 0.1 M phosphate buffer at pH 7.0. The oxidation of catechol was measured by following the increase in absorbance at 470 nm (102) with a Beckman DB spectrophotometer.

Treatment of House Fly Homogenates with Catechol and Cyanide:

Equal quantities of house fly abdomens were homogenized with the GT homogenizer in 0.2 M K_2HPO_4 at pH 8.0. The buffer for the cyanide-inhibited preparation contained 10 mM KCN. Catechol was added to the homogenate without KCN after the 10,000 xg centrifugation to give a final concentration of 0.38 mM. The microsomal fractions were isolated from the 10,000 xg supernatants by centrifuging at 105,000 xg for 90 minutes, resuspending in 0.2 M K_2HPO_4 plus 50% glycerol at pH 8.0, and then assessing the aldrin epoxidase activity, NADPH-cytochrome c reductase activity, and cytochrome P-450. The microsomes not used in the above assays were stored under N_2 at -20° C and assayed the following day.

Incubation of Rat Liver Microsomes with Tyrosinase:

The incubations for determining the effects of tyrosinase on microsomal mixed-function oxidases were made with rat liver microsomes isolated from control and phenobarbital treated rats. The incubation mixture contained 5 to 6.5 mg microsomal protein per ml, 0.1 M K_2HPO_4, (pH 7.0), 2.8 to 5 mM catechol, and 0.31 to 2.5 µg tyrosinase per mg microsomal protein. The incubations were initiated with tyrosinase and kept on ice during the incubation, except for the studies made at 13° C. When tyrosinase was inhibited with KCN, they were incubated together at 21° C for 5 minutes before being added to the microsomal incubation mixture. Samples were removed at various times after the initiation of the reaction for the determination of cytochrome P-450 spectra, NADPH-cytochrome c reductase activity and aldrin epoxidation. The NADPH-cytochrome c reductase activity was usually expressed as the percent of the original reductase activity remaining at the time of sampling.

Erythrocyte Hemolysis:

The occurrence of free-radical intermediates have been detected by their ability to cause hemolysis of red blood cells (103). Sterile sheep erythrocytes were washed 3 times in isotonic saline and packed by centrifugation. One ml of the packed cells was diluted in 19 mls of isotonic saline. Two mls of this suspension were used to determine if hemolysis occurred during the oxidation of catechol by mushroom tyrosinase. After a 15 minute incubation of erythrocytes with tyrosinase alone, catechol alone, and tyrosinase plus catechol, on ice, the mixtures were centrifuged at 2000 rpm for 10 minutes. The supernatants were carefully aspirated and their absorbance at 541 nm was determined with a Beckman DB spectrophotometer. The results were expressed as the percent increase in the absorbance of the tyrosinase plus catechol supernatant over that of the treatments with catechol alone and tyrosinase alone.

Other Methods:

The determination of protein concentration was by the method of Lowry et al. (97) using dilutions of BSA solutions (1 mg/ml) for standardization. The sodium dodecyl sulfate polyacrylamide gel electrophoresis was done as described by Dr. Ann Welton (15) employing a modification of the method used by Fairbanks et al. (104). The polyacrylamide gels were stained with Coumassie blue.

RESULTS

The microsomal NADPH-cytochrome c reductase activity from mortar-and-pestle (MP) homogenates of whole flies was compared to that of abdominal MP preparations when first beginning this research. The microsomal reductase activity of the abdominal preparations in 2 isolations was slightly higher than that found for the whole house fly isolations (Table 1). Subsequent isolations of microsomal fractions from MP homogenates also showed wide variation in their enzymatic activity, not only for the adult but also for the larval homogenates. For example, Table 2, the NADPH-cytochrome c reductase activity for the 10 day old adult ranged from 0.5 to 50.0 nmoles of cytochrome c reduced/min/mg protein. This variation initiated a series of centrifugation studies designed to characterize the isolated microsomes.

Marker Enzyme Distribution Patterns of Mortar-and-Pestle Homogenates:

The marker enzyme distribution patterns of mitochondrial cytochrome oxidase and microsomal NADPH-cytochrome c reductase obtained after differential centrifugation of the MP whole house fly homogenates are shown in Figs. 3 and 4. Also shown for comparison are the patterns of mitochondrial and microsomal marker enzymes as found with rat liver homogenates (105). The distribution of the mitochondrial marker, cytochrome oxidase, differs significantly from that obtained with the rat liver preparations. Over 20% of the rat liver total nitrogen is located in the

Table 1

COMPARISON OF NADPH-CYTOCHROME C REDUCTASE ACTIVITY IN MICROSOMAL FRACTIONS FROM MORTAR-PESTLE HOMOGENATES OF ABDOMENS AND WHOLE FLIES

Homogenates were prepared from equal weights of abdomens and whole flies by the mortar-pestle technique. The 10,000 xg supernatant of each homogenate was centrifuged at 105,000 xg to obtain the microsomal fraction for the NADPH-cytochrome c reductase determinations. This was done with two groups of adult flies 7 and 10 days old.

Homogenization method	NADPH-cytochrome c reductase[a]	
	Isolation 1	Isolation 2
Whole flies	0.078	0.054
Abdomens	0.090	0.043

[a] μmoles of cytochrome c reduced/min/mg protein

Table 2

NADPH-CYTOCHROME C REDUCTASE ACTIVITY IN MICROSOMAL FRACTIONS FROM WHOLE HOUSE FLY MORTAR-PESTLE HOMOGENATES OF THE LARVAL AND ADULT STAGES

Whole larvae or adults were homogenized with a mortar and pestle and the microsomal NADPH-cytochrome c reductase activity was determined.

Isolation	NADPH-cytochrome c reductase[a]			
	Larva[b]		Adult[b]	
	4	5	5	10
1	0.002	0.001	0.006	0.0005
2	0.009	0.013	0.018	0.010
3	0.019	0.013	0.033	0.052
4	0.033		0.056	0.060
5	0.039			

[a] μmoles of cytochrome c reduced/min/mg protein

[b] Age of larval and adult stages in days

Figure 3. DISTRIBUTION OF CYTOCHROME OXIDASE IN SUBCELLULAR FRACTIONS OF RAT LIVER AND WHOLE HOUSE FLY HOMOGENATES.

Fractionation of whole house fly mortar-and-pestle homogenates in 0.32 M sucrose, pH 7.5, was done by differential centrifugation. The fractions isolated were the 0 to 800 xg nuclear (N), the 800 to 5,000 xg heavy mitochondrial (M_2), the 10,000 to 20,000 xg light mitochondrial (L), the 20,000 to 105,000 xg microsomal (P), and the 105,000 xg supernatant (S). The fractions are represented along the abscissa in the order in which they were isolated. The distribution patterns of rat liver marker enzymes are from studies done by de Duve and co-workers (105). The rat liver fractions, represented along the abscissa in the order they were isolated, are: nuclear (N), heavy mitochondrial (M), light mitochondrial (L), microsomal (P), and supernatant (S). The relative specific activity is the percent of the total enzyme activity in a fraction divided by the percent of the total nitrogen in the case of rat liver or for the house fly studies by percent of the total protein.

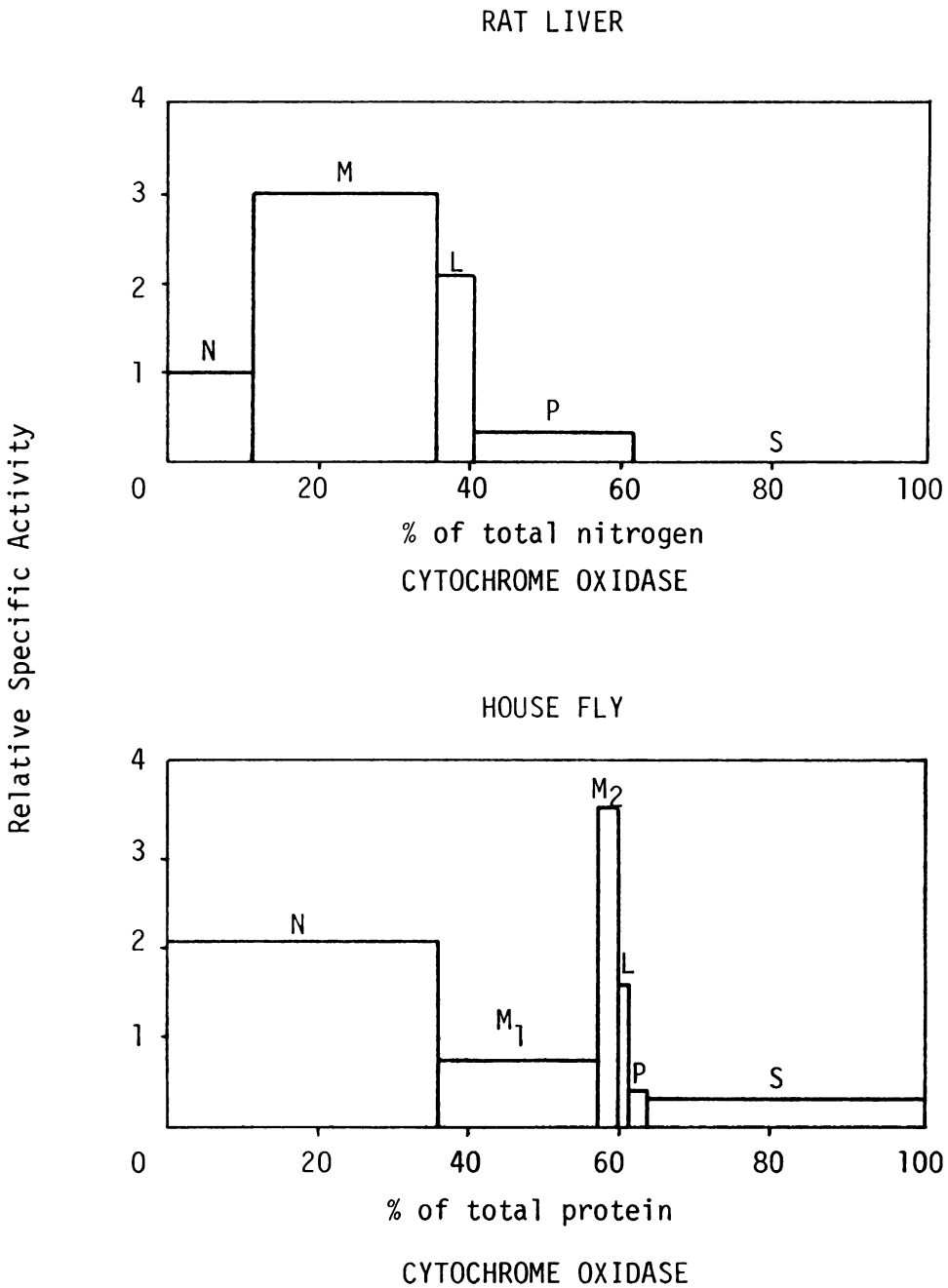
RAT LIVER
Relative Specific Activity
4
3
2
1
0
M
L
N
P
S
20
40
60
80
100
% of total nitrogen
CYTOCHROME OXIDASE
HOUSE FLY
4
3
2
1
0
M2
N
L
M1
P
S
20
40
60
80
100
% of total protein
CYTOCHROME OXIDASE

Figure 3

Figure 4. DISTRIBUTION OF MARKER ENZYMES IN SUBCELLULAR FRACTIONS OF RAT LIVER AND WHOLE HOUSE FLY HOMOGENATES

The distribution patterns of rat liver glucose-6-phosphatase and house fly NADPH-cytochrome c reductase are for the fractions of rat liver and house fly homogenates listed in Fig. 3.

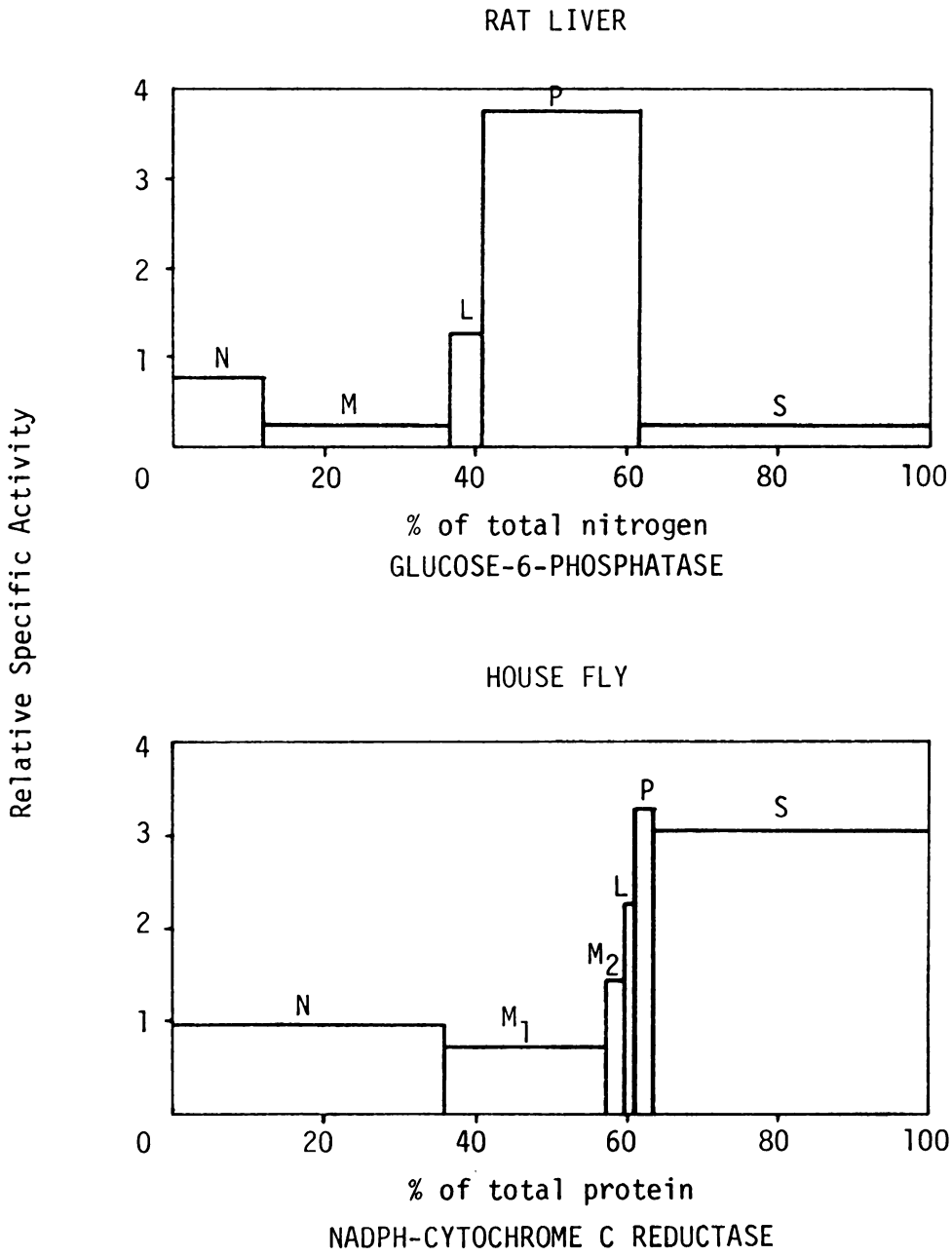
RAT LIVER
Relative Specific Activity
4
3
2
1
0
N
M
L
P
S
20
40
60
80
100
% of total nitrogen
GLUCOSE-6-PHOSPHATASE
HOUSE FLY
N
M1
M2
L
P
S
% of total protein
NADPH-CYTOCHROME C REDUCTASE

Figure 4

fraction containing the highest relative specific activity of the mitochondrial marker. The mitochondrial fractions of whole house fly homogenates with the highest relative specific activity has about 3% of the total protein. There also was activity in the soluble fraction which was not detected in the rat liver preparations.

The distribution of microsomal NADPH-cytochrome c reductase from the house fly homogenate also differed significantly from that of the rat liver microsomal marker enzyme, glucose-6-phosphatase (105). Both of these enzymes have been shown to have the same microsomal distribution pattern in subcellular fractions of rat liver (35). The relative specific activity of the house fly soluble fraction was nearly as high as the activity in the microsomal fraction. The amount of reductase activity recovered was only 63% of the total homogenate activity. Based on the amount recovered in the different fractions, 89% of the reductase activity was in the soluble fraction. Since this is the opposite of the results obtained with glass-Teflon (GT) homogenates (see below), it is evident that solubilization of the reductase occurred during the centrifugation of MP homogenates.

Density Gradient Centrifugation of Mortar-Pestle Homogenates:

Sucrose density gradient centrifugation of the 800 xg supernatant confirmed the solubilization of NADPH-cytochrome c reductase seen in the differential centrifugation studies. The distribution of reductase activity in fractions collected from discontinuous sucrose gradient and the 254 nm absorbance profile are shown in Fig. 5. Two major peaks of reductase activity were found in the gradient with 67% of the reductase activity in peak I and 31.6% in peak II (Table 3). The distribution of

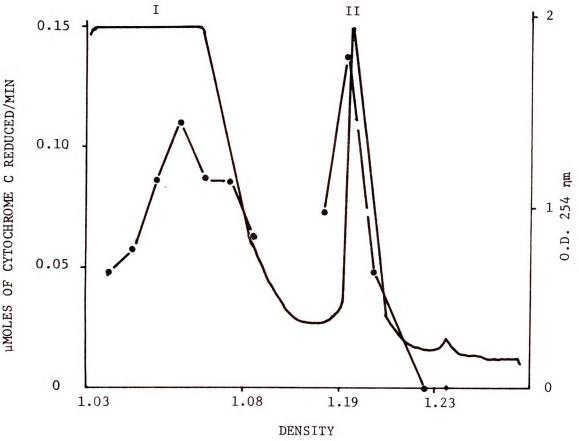

Figure 5. THE DISTRIBUTION OF NADPH-CYTOCHROME C REDUCTASE ACTIVITY AND PROTEIN PROFILE FOLLOWING SUCROSE DENSITY GRADIENT CENTRIFUGATION OF THE 800 XG SUPERNATANT OF MP HOMOGENATE

Whole house flies were homogenized with the mortar-and-pestle in 0.32 M sucrose and 10 mM EDTA, pH 7.5. The 800 xg supernatant (5 ml) was layered on a discontinuous gradient and centrifuged for 2.5 hours at 127,000 xg. Successive 2 ml fractions were assayed for NADPH-cytochrome c reductase. The dots on the broken line show the activity of each fraction. The solid line indicates the 254 nm absorbance profile of the gradient.

enzymatic activity in the fractions closely followed the protein distribution in the gradient. The fraction with highest activity occurred at the interface of the top sucrose layer and the slightly heavier layer of the 800 xg supernatant sample that formed the second layer in the gradient. Thus activity and protein migrated up the gradient as well as down. The second peak sedimented to a density of 1.19, which is lower than that of 1.14 observed for rat liver microsomes in a linear sucrose gradient (106). Peak II also had in the same fraction the highest cytochrome oxidase activity, which was negligible throughout the rest of the gradient.

Inhibition of Enzyme Solubilization in Mortar Homogenates:

Attempts to prevent the solubilization of the membrane-bound reductase in MP homogenates by using inhibitors of calcium-dependent phospholipases and proteases were unsuccessful. The addition of EDTA to chelate divalent metals and so inhibit phospholipases did not affect the percent of reductase retained in the microsomal fraction (Table 4) or its specific activity (Table 5). The same results were obtained when the protease inhibitor, phenylmethylsulfonylfluoride (PMSF), was used (Tables 4, 5). Table 6 shows the results of 3 isolations in which PMSF was added to the buffer to inhibit proteases, EDTA to inhibit phospholipases, and dithiothriotol (DTT) to protect against oxidation of enzyme sulfhydryl groups in the combinations listed. The first isolation in which PMSF was used gave 80% of the reductase in the microsomal pellet of the treated sample. This suggested that endogenous proteases solubilized the enzyme during the isolation (isolation A of Table 6). The results of two subsequent isolations failed to confirm this (isolations

Table 3

THE DISTRIBUTION OF NADPH-CYTOCHROME C REDUCTASE ACTIVITY FOLLOWING SUCROSE DENSITY GRADIENT CENTRIFUGATION OF THE 800 XG SUPERNATANT OF MP HOMOGENATE

The fractions collected from the gradient represented in Fig. 5 were combined into samples representing peak I and II. The NADPH-cytochrome c reductase activity of the samples were assayed and is expressed as a percentage of the total activity recovered from the gradient.

Total Activity	Peak I	Peak II	Pellet	Total
Total Activity[a]	10.66	5.04	0.24	15.94
Percent[b]	67.0	31.6	1.4	100

[a] Total activity = μmoles of cytochrome c reduced/min

[b] $\text{Percent} = \frac{\text{activity recovered in fraction}}{\text{total recovered activity}} \times 100$

Table 4

THE EFFECT OF ISOLATION CONDITIONS ON THE PERCENT OF NADPH-CYTOCHROME C REDUCTASE ACTIVITY FOUND IN THE MICROSOMAL FRACTION FROM HOUSE FLY HOMOGENATES

The isolations were performed as described in the Materials and Methods. The percent activity was calculated from the reductase activity in the microsomal pellet and supernatant retained in the pellet.

Method of Homogenization	Homogenization Medium	Number of Isolations	Percent Activity[a,b]
Mortar-pestle	0.32 M Sucrose	8	18.7±7.3 a
	0.32 M Sucrose + EDTA	4	8.0±1.3 a
	0.32 M Sucrose + PMSF	3	32.3±23.8 a
Glass-Teflon®	0.2 M K_2HPO_4	9	63.5±9.1 b
	0.32 M Sucrose	3	79.4±5.4 b
	0.57 M Tris	3	93.7±8.3 b
	0.2 M K_2HPO_4 + DFP	3	88.9±4.5 b

[a] $\% \text{ Activity} = \frac{\text{units in microsomal pellet}}{\text{units in pellet + units in supernatant}} \times 100 \pm \text{SE}$

units = μmoles cytochrome c reduced/min/mg protein x mg protein in fraction

[b] Pairs of means followed by the same letter were not significantly different at the 5% level using the Student's t-test

Table 5

THE EFFECT OF ISOLATION CONDITIONS ON THE SPECIFIC ACTIVITY OF NADPH-CYTOCHROME C REDUCTASE IN THE MICROSOMAL FRACTION FROM HOUSE FLY HOMOGENATES

The isolations were performed as described in the Materials and Methods.

Method of Homogenization	Homogenization Medium	Number of Isolations	Specific Activity[a,b]
Mortar-pestle	0.34 M Sucrose	9	0.023±0.008 a
	0.34 Sucrose + EDTA	5	0.027±0.007 a
	0.34 M Sucrose + PMSF	3	0.023±0.010 a
Glass-Teflon^{R}	0.2 M K_2HPO_4	10	0.061±0.033 b
	0.032 M Sucrose	3	0.040±0.019 b
	0.057 M Tris	3	0.033±0.005 b
	0.2 M K_2HPO_4	3	0.068±0.010 b

[a] Specific activity = μmoles cytochrome c reduced/min/mg protein ± SE

[b] Pairs of means followed by the same letter were not significantly different at the 5% level using Student's t-test

Table 6

INDIVIDUAL ISOLATION OF THE MICROSOMAL FRACTION FROM MP HOMOGENATES OF WHOLE HOUSE FLIES. THE EFFECT OF PMSF, DTT, AND EDTA ON MICROSOMAL NADPH-CYTOCHROME C REDUCTASE ACTIVITY

For each isolation, whole house flies were divided into equal groups for subsequent homogenization in one of the cold homogenization media listed below. After obtaining the microsomal fraction by differential centrifugation, the percent of reductase activity retained in the pellet and its specific activity were determined. The treatments were 0.32 M sucrose, pH 7.5 plus additive(s).

1. 0.32 M Sucrose, pH 7.5
2. 0.32 M Sucrose, 0.1 mM DTT
3. 0.32 M Sucrose, 0.2 mM PMSF
4. 0.32 M Sucrose, 0.2 mM PMSF, 10 mM EDTA
5. 0.32 M Sucrose, 0.4 mM DTT, 0.4 mM PMSF
6. 0.32 M Sucrose, 0.4 mM DTT, 0.4 mM PMSF, 10 mM EDTA

Table 6

Isolation	Treatment	% Activity[a]	Specific activity[b]
A	1	26	0.011
	2	56	0.013
	3	80	0.011
	4	79	0.019
B	1	13	0.023
	3	10	0.027
	5	16	0.025
	6	9	0.026
C	1	9.9	0.029
	3	7.5	0.030
	5	7.5	0.032
	6	8.5	0.037

[a] % activity = $\frac{\text{Enzyme units in microsomal pellet}}{\text{units in pellet + units in supernatant}} \times 100$

[b] Specific activity = μMoles of cytochrome c reduced/min/mg protein

B & C of Table 6). The percentage of reductase activity in the treated microsomal fractions of isolations B and C did not differ from that of the controls. The average of the specific activities in isolation A, however, was less than half of those obtained in B and C. Since the specific activities of the microsomal fractions in isolation A were the lowest of all the 25 MP isolations obtained it is probable that in this isolation some endogenous inhibitor had been released during the homogenization.

Glass-Teflon Homogenization and Solubilization:

In contrast to the solubilization of the reductase observed during the isolation of the microsomal fraction from MP whole fly homogenates, the GT method, which used only abdomens, gave preparations with most of the reductase activity in the microsomal pellet (Table 7). This method gave an average of 80.3 ± 21.3% of the reductase activity in the microsomal fraction as compared to 20.1 ± 24.5% for the MP method. When the differences were assessed for significance by Student's t-test they were significant at the 0.01 probability level. The specific activity of the reductase in the microsomal fractions obtained by the two methods was significantly different at the 0.05 probability level. The GT method gave an average specific activity twice that of the MP preparations (Table 8). The percent of NADPH-cytochrome c reductase activity recovered in the microsomal fraction with each method is shown in Fig. 6.

Microsomes isolated in the presence of EDTA or diisopropylfluorophosphate (DFP), a protease inhibitor, were not significantly different from the controls with respect to either percent of reductase activity recovered in the microsomal fraction of its specific activity (Tables 4, 5).

Table 7

THE EFFECT OF THE HOMOGENIZATION METHOD ON THE PERCENT OF THE MICROSOMAL AND SOLUBLE NADPH-CYTOCHROME C REDUCTASE ACTIVITY DETECTED IN THE MICROSOMAL FRACTION

The homogenizations and isolations were performed as described in the Materials and Methods.

Method of Homogenization	Number of Isolations	% Activity[a,b]	
Mortar-pestle	23	20.1±5.1	a
Glass-Teflon®	30	80.3±3.9	b

[a] % activity = $\frac{\text{Enzyme units in microsomal pellet}}{\text{units in pellet + units in supernatant}}$ X 100 ± SE

units = μmoles of cytochrome c reduced/min/mg x mg protein in fraction

[b] Pairs of means followed by the same letter were not significant at the 5% level using Student's t-test

Table 8

THE EFFECT OF THE HOMOGENIZATION METHOD USED TO PREPARE HOUSE FLY HOMOGENATES ON THE SPECIFIC ACTIVITY OF NADPH-CYTOCHROME C REDUCTASE IN THE MICROSOMAL FRACTION

The homogenizations and isolations were performed as described in the Materials and Methods.

Method of Homogenization	Number of Isolations	Specific Activity[a,b]	
Mortar-pestle	25	0.025±0.002	a
Glass-Teflon®	31	0.050±0.004	b

[a] Specific activity = μmoles of cytochrome c reduced/min/mg protein ± SE

[b] Pairs of means followed by the same letter were not significant at the 5% level using Student's t-test

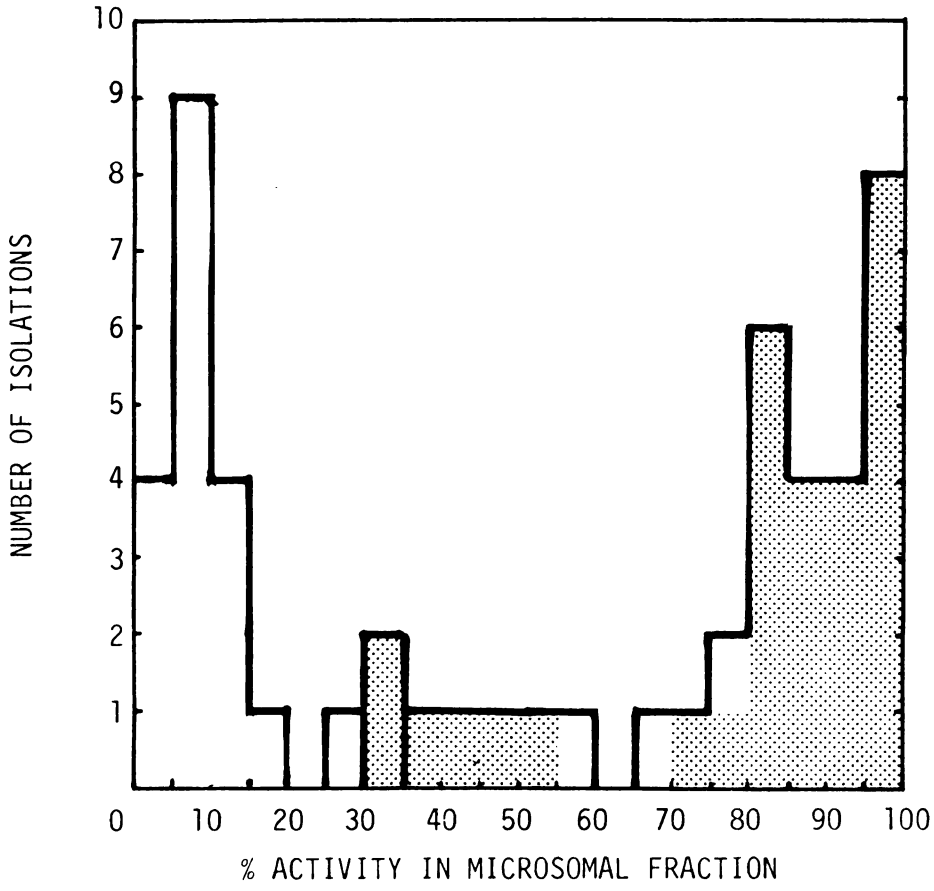

Figure 6. EFFECT OF HOMOGENIZATION METHOD ON THE PERCENT OF NADPH-CYTOCHROME C REDUCTASE ACTIVITY FOUND IN THE MICROSOMAL FRACTION OF HOUSE FLY HOMOGENATES

The percent of reductase activity in the microsomal fraction was calculated on the total amount of enzyme activity in the microsomal pellet and supernatant. The percent activity in each pellet was placed in the corresponding 5% interval. These results are from 23 mortar-pestle (▭) and 30 glass-teflon (▩) isolations.

Table 9

THE EFFECT OF THE PROTEASE INHIBITOR, DFP, ON THE NADPH-CYTOCHROME C REDUCTASE ACTIVITY IN THE MICROSOMAL FRACTION OF HOUSE FLIES USING THE GLASS-TEFLON® HOMOGENIZATION METHOD

In each isolation house fly abdomens were separated into two equal groups. One group was homogenized in 0.57 M Tris and the other in 0.57 M Tris with 1 mM DFP, pH 8.0. When the 105,000 xg centrifugation was completed, an aliquot of the supernatant was carefully pipetted from the centrifuge tube. This aliquot was used for the determination of the supernatant reductase activity. The pellets were carefully drained before resuspending in Tris-glycerol.

Isolation	Treatment[a]	% Activity[b]	Specific Activity[c]
1	Tris	100	0.033
	DFP	100	0.031
2	Tris	81	0.029
	DFP	85	0.028
3	Tris	100	0.038
	DFP	100	0.032

[a] Treatment = 0.57 M Tris ± 1 mM DFP

[b] % Activity = $\frac{\text{Enzyme units in microsomal pellet}}{\text{units in pellet + units in supernatant}} \times 100$

[c] Specific Activity = μmoles of cytochrome c reduced/min/mg protein

This is further illustrated by the results of three isolations made to determine the effect of DFP on the percent of the reductase retained in the pellet and its specific activity (Table 9). The conditions were the same for the control and the DFP-treated homogenate in each isolation. Two of the isolations had 100% of the reductase activity in the microsomal pellet, for both the treated and control, while the third isolation showed respectively, 85% and 80% of it in the pellet. This was typical of the results obtained with the GT method using two other buffers with or without DFP (Tables 4, 5). Evidently, the protease inhibitor did not significantly increase the amount of reductase retained in the microsomal fraction or its specific activity.

From the above results, it can be concluded that under the conditions utilized for each method, GT homogenization gave preparations with much more of the reductase activity in the microsomal fraction. Treatment with phospholipase or protease inhibitors did not give results that differed significantly from those obtained with the controls. This was true for each method. However, even though microsomal fractions from the GT preparations retained an average of 80% of the NADPH-cytochrome c reductase activity in the microsomal pellet and supernatant, the aldrin epoxidase activity of the homogenate and microsomal fraction was not stable. The homogenate lost 68% of its original aldrin epoxidase activity within 30 minutes after homogenization. The addition of DFP, a protease inhibitor, inhibited aldrin epoxidation by 62%, but the activity of the DFP-treated homogenate showed little change during the 60-minute sampling period (Fig. 7). The microsomal fraction, when stored as specified under Materials and Methods, lost 50% or more of the aldrin epoxidase activity within approximately 24 hours (Table 10).

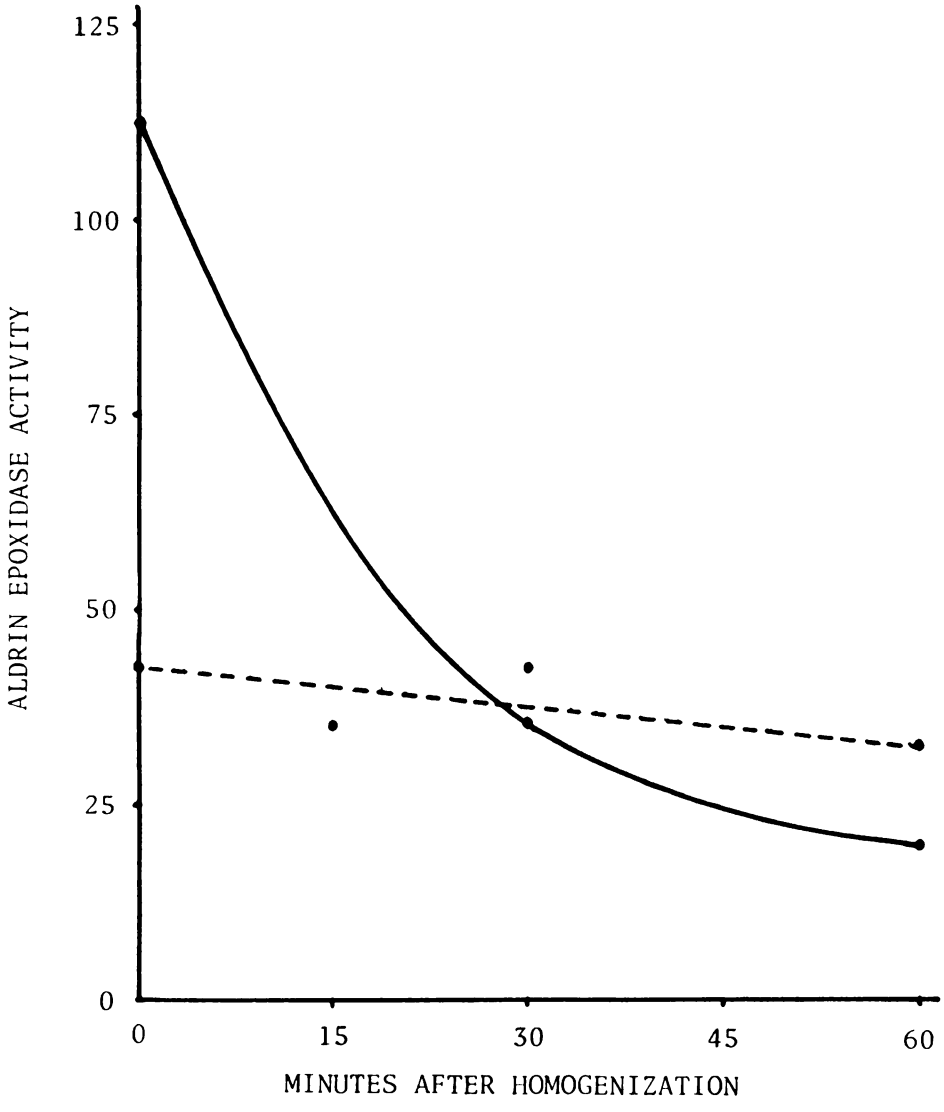

Figure 7. THE STABILITY OF ALDRIN EPOXIDASE ACTIVITY IN GLASS-TEFLON^{R} HOMOGENATES OF HOUSE FLY ABDOMENS

Equal quantities of the abdomens were homogenized in 0.2 M K_2HPO_4, pH 8.0 (•————•) and 0.2 M K_2HPO_4, pH 8.0 plus 1 mM DFP (•------•). Aliquots for aldrin epoxidation assays were taken after homogenization at the times indicated and preincubated at 21° C for 5 minutes in the assay mixture. Aldrin was added to initiate the reaction.

Table 10

MIXED-FUNCTION OXIDASE ACTIVITY IN MICROSOMAL FRACTIONS ISOLATED FROM HOUSE FLY HOMOGENATES TREATED WITH EITHER CATECHOL OR CYANIDE

House fly abdomens were homogenized by the glass-Teflon method in either 0.2 M K_2HPO_4, pH 8.0 or in 0.2 M K_2HPO_4 with 10 mM KCN, pH 8.0. The preparation treated with catechol was homogenized in 0.2 M K_2HPO_4, pH 8.0, with catechol being added to the 10,000 xg supernatant.

Treatment	NADPH-cytochrome c reductase[a]	Cytochrome P-450[b]	Aldrin epoxidation[c]	
CONTROL	0.095	0.197	0.046[e]	0.020[f]
Catechol	0.081	ND[d]	0.067[e]	0.029[f]
KCN	0.124	0.397	0.237[e]	0.185[f]

[a] μmoles of cytochrome c reduced/min/mg protein

[b] nmoles of cutochrome P-450/mg protein

[c] nmoles of dieldrin produced min/mg protein

[d] Not detected

[e] Assays done as soon as the microsomal fraction was isolated

[f] Assays done the day following the isolation

Effect of Endogenous Tyrosinase on House Fly Microsomal Enzymes:

One isolation was made to see if the stimulation and inhibition of tyrosinase activity in house fly homogenates would affect the activity on stability of microsomal enzymes from the house fly. Catechol was added to the 10,000 xg supernatant of a glass-Teflon abdominal homogenate, since it is known to stimulate the hydroxylation of tyrosinase, and cyanide, an inhibitor of tyrosinase, was added to another (Table 10). Addition of catechol reduced two of the parameters assayed when compared to the control. NADPH-cytochrome c reductase activity was reduced by 15%, and the preparation had no detectable cytochrome P-450 content. Aldrin epoxidation was slightly higher than in the control. The NADPH-cytochrome c reductase activity in the cyanide-treated preparation was 30% more than in the control and 35% more than that observed in the catechol-treated preparation. The aldrin epoxidation of the cyanide-treated preparation was 5 times that detected in the control and 3.5 times that of the catechol-treated isolation.

Of significant interest was the fact that the control and catechol-treated isolations lost 56% of their aldrin epoxidation activity by the next day. For the cyanide treatment the loss of activity in one day was 22%. Assays made 3 weeks later gave the same amount of aldrin epoxidation as observed the day after the isolation. These results suggest that the phenol oxidase complex did decrease the activity of microsomal enzymes.

Effect of Mushroom Tyrosinase on Rat Liver Microsomal Enzymes:

Using catechol as the substrate tyrosinase activity was detected in house fly microsomes but not in the rat liver microsomal preparation. The effect of mushroom tyrosinase plus catechol on rat liver microsomes was examined to see what effect exogenous tyrosinase would have on their enzymatic activities.

Looking first at NADPH-cytochrome c reductase, increasing concentrations of tyrosinase, in the presence of catechol, gave a corresponding decrease in reductase activity (Fig. 8). Activity decreased with time following an initial lag period (Fig. 9), with the lower concentration of tyrosinase used in the incubations. The rate at which activity was lost also increased with a rise in temperature (Fig. 10). Whereas at 4° C 10% of the activity was lost in 20 minutes, at 13° C almost 50% of the original reductase activity was lost in 10 minutes.

The effect of tyrosinase on different microsomal parameters following a 22 hour incubation on ice is shown in Table 11. The reduction of cytochrome c by NADPH-cytochrome c reductase was not affected by catechol alone, but it was stimulated 12% by tyrosinase, when compared to the control. Potassium cyanide reduced the reductase activity by 17%. In the presence of tyrosinase and catechol, the activity was reduced from 0.204 down to 0.026 μmoles of cytochrome c reduced per minute per mg protein. The reductase activity was 26% greater than the control for microsomes treated with cyanide-inhibited tyrosinase plus catechol. The carbon monoxide difference spectra were essentially the same for all treatments except for the tyrosinase plus catechol (Fig. 11). This treatment reduced the cytochrome P-450 to 36% of the control value (Table 11). The difference spectrum of the tyrosinase

Figure 8. THE EFFECT OF TYROSINASE CONCENTRATION ON NADPH-CYTOCHROME C REDUCTASE ACTIVITY OF RAT LIVER MICROSOMES

Liver microsomes (6.0 mg/ml) from PB-pretreated rats were suspended in 0.1 M K_2HPO_4 with 2.5 mM catechol and incubated on ice for 15 minutes. At the end of the incubation period, the reductase activity was assayed. The following concentrations of tyrosinase were used:

1. 0 µg tyrosinase/mg microsomal protein
2. 0.31 µg tyrosinase/mg microsomal protein
3. 0.62 µg tyrosinase/mg microsomal protein
4. 1.25 µg tyrosinase/mg microsomal protein
5. 2.50 µg tyrosinase/mg microsomal protein

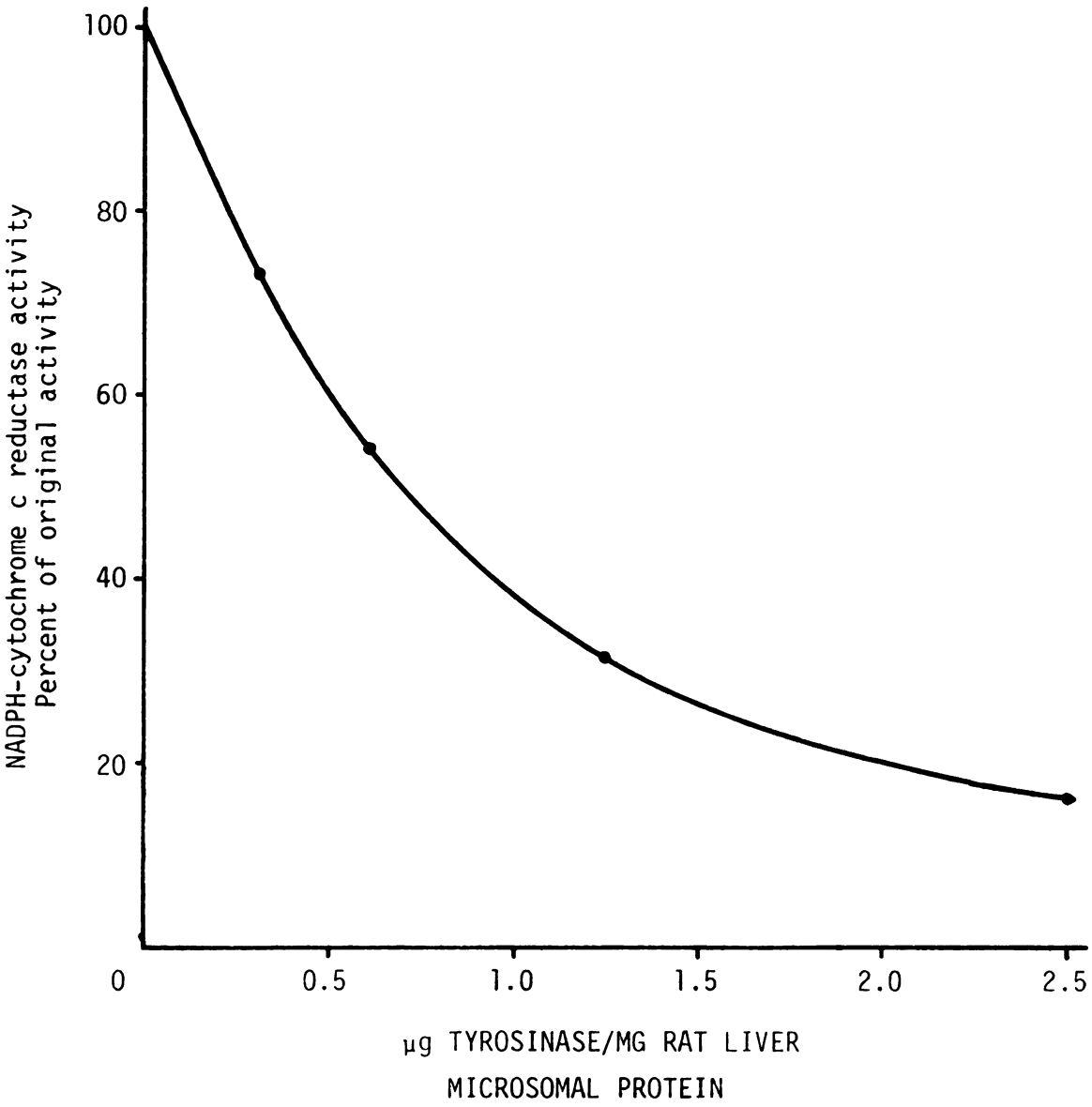
NADPH-cytochrome c reductase activity
Percent of original activity
100
80
60
40
20
0
0.5
1.0
1.5
2.0
2.5
µg TYROSINASE/MG RAT LIVER
MICROSOMAL PROTEIN

Figure 8

Figure 9. THE EFFECT OF TYROSINASE PLUS CATECHOL ON RAT LIVER MICROSOMAL NADPH-CYTOCHROME C REDUCTASE ACTIVITY: EFFECT OF TYROSINASE CONCENTRATION

Two levels of tyrosinase, 0.31 and 0.62 μg/mg microsomal protein, were added to liver microsomes (6.0 mg/ml) from PB-pretreated rats in 0.1 M K_2HPO_4 with catechol (5mM), pH 7.0. The rate of inhibition was followed by assaying the reductase activity at five or ten minute intervals.

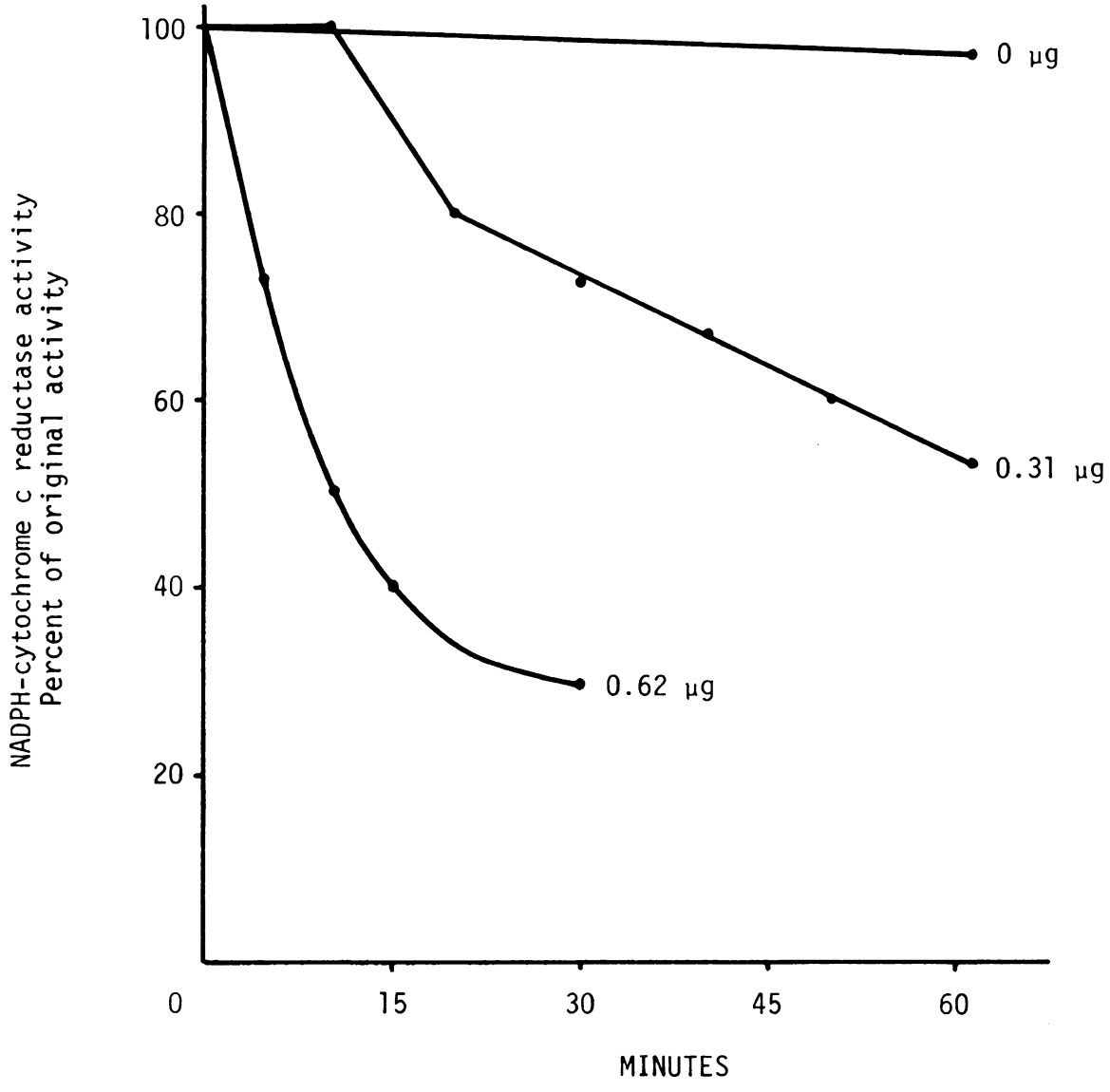
NADPH-cytochrome c reductase activity
Percent of original activity
100
80
60
40
20
0
15
30
45
60
MINUTES
0 µg
0.31 µg
0.62 µg

Figure 9

Figure 10. THE EFFECT OF TYROSINASE PLUS CATECHOL ON RAT LIVER MICROSOMAL NADPH-CYTOCHROME C REDUCTASE ACTIVITY: RATE OF INHIBITION

Liver microsomes (6 mg/ml) from PB-treated rats were suspended in 0.1 M K_2HPO_4 with 5 mM catechol, pH 7.0. They were preincubated for 10 minutes at 4° C and 13° C before adding tyrosinase (0.31 µg/mg microsomal protein). The activity of the reductase was assayed at 5 or 10 minute intervals. Reductase activity did not decrease when microsomes were incubated alone, with catechol or with tyrosinase.

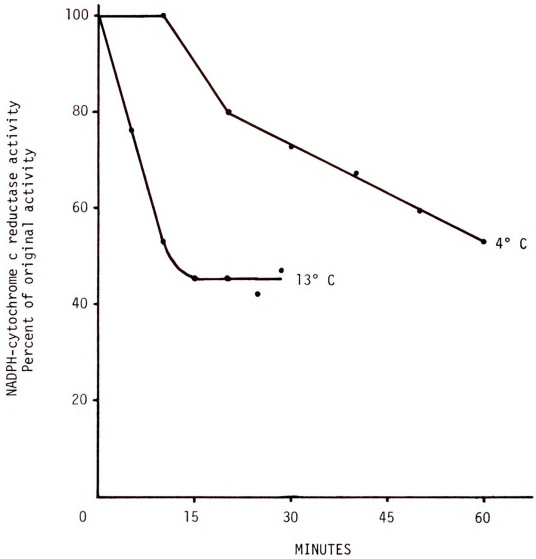
NADPH-cytochrome c reductase activity
Percent of original activity
100
80
60
40
20
0
15
30
45
60
MINUTES
4° C
13° C

Figure 10

Table 11. EFFECT OF TYROSINASE ON CYTOCHROME P-450 AND ENZYMATIC FUNCTIONS OF LIVER MICROSOMES FROM PHENOBARBITAL-PRETREATED RATS

Liver microsomes from PB-pretreated rats were suspended in 0.1 M K_2HPO_4, pH 7.0 and incubated for 22 hours on ice.

Cytochrome P-450, NADPH-cytochrome c reductase activity and aldrin epoxidation were assayed at different times during the incubation period. The results shown here are those obtained at 22 hours. Assays were done as specified in the Material and Methods section.

1. Microsomes (6.5 mg/ml) in 0.1 M K_2HPO_4, pH 7.0 containing 0.001% BHT.
2. As in one plus throsinase (1 μg/mg microsomal protein).
3. As in one plus catechol (2.8 mM).
4. As in one plus potassium cyanide (0.8 mM).
5. As in one plus tyrosinase (1 μg/mg microsomal protein) and catechol (2.8 mM).
6. As in one plus cyanide (0.8 mM) inhibited tyrosinase (1 μg/mg microsomal protein) and catechol (2.8 mM).

Table 11

Treatment	NADPH-cytochrome c reductase[a]	Cytochrome P-450[b]	Aldrin Epoxidation[c]
1. untreated control	0.204	2.2	2.1
2. tyrosinase	0.229	2.3	2.2
3. catechol	0.204	2.2	2.1
4. KCN	0.190	2.1	1.7
5. tyrosinase + catechol	0.026	0.8	0
6. tyrosinase + catechol + KCN	0.257	2.1	1.7

[a] μmoles of cytochrome c reduced/min/mg protein

[b] nmoles of cytochrome/mg protein

[c] nmoles of dieldrin produced/min/mg protein

plus catechol-treated microsomes had a small shoulder at 420 nm in 30 minutes, but with time it did not increase in proportion to the decrease of absorbance observed at 450 nm, as it had been reported to do in the presence of proteolytic enzymes (94).

Aldrin epoxidation was the same for control, tyrosinase-treated, and catechol-treated microsomes (Table 11). The cyanide and cyanide-tyrosinase plus catechol treatments reduced the epoxidation from 2.1 down to 1.7 nmoles of dieldrin produced per minute per mg protein, a 20% decrease. Tyrosinase plus catechol completely inhibited aldrin epoxidation.

Another effect of tyrosinase on membranes was observed in an experiment using erythrocyte lysis as an indicator of the occurrence of free radical reactions. The 1200 xg supernatant of washed red blood cells had a 3.5-fold more hemoglobin than red blood cells alone or red blood cells plus tyrosinase or catechol, following centrifugation after a 15-minute incubation with tyrosinase and catechol at 0 to 5° C. SDS polyacrylamide gel electrophoresis of rat liver PB induced microsomes incubated with tyrosinase indicated that cross linkage of the proteins had occurred, since much of the sample failed to migrate into the gel (Fig. 12). A sample of microsomes incubated with cyanide-inhibited tyrosinase when electrophoresed gave the same banding pattern as untreated rat liver PB microsomes.

Figure 11. EFFECT OF MUSHROOM TYROSINASE ON CYTOCHROME P-450 OF LIVER MICROSOMES FROM PHENOBARBITAL-PRETREATED RATS

Liver microsomes from PB-pretreated rats were suspended in 0.0 M K_2HPO_4, pH 7.0 and incubated for 22 hours on ice. Microsomal samples, 1.3 mg protein, were flushed with carbon monoxide, reduced with sodium dithionite, and scanned at different times during the incubation. The treatments are the same as 1 and 5 listed in Table 11. The other treatments did not differ significantly from the spectrum of 1 during the incubation period.

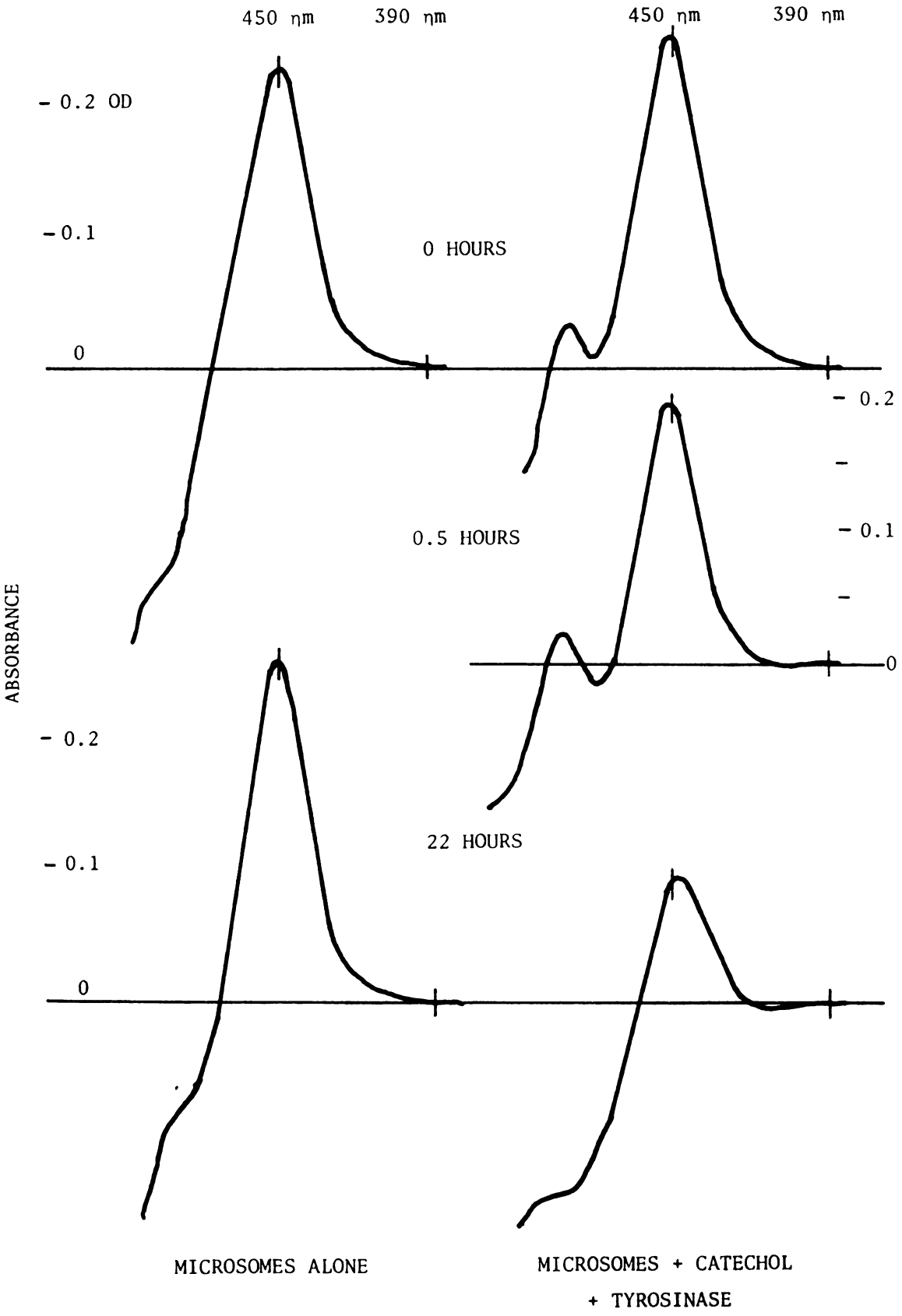
450 nm
390 nm
450 nm
390 nm
- 0.2 OD
- 0.1
0
0 HOURS
- 0.2
- 0.1
0
0.5 HOURS
ABSORBANCE
- 0.2
- 0.1
0
22 HOURS
MICROSOMES ALONE
MICROSOMES + CATECHOL
+ TYROSINASE

Figure 11

Figure 12. THE EFFECT OF TYROSINASE AND CATECHOL TREATMENT ON THE SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS PROTEIN PROFILE OF THE LIVER MICROSOMAL FRACTION FROM PB-PRETREATED RATS

Rat liver PB-induced microsomes (6.0 mg/ml) were incubated with tyrosinase (0.31 µg/mg microsomal protein) and/or catechol (2.5 mM) for 15 minutes at 4° C. The samples were prepared for SDS-polyacrylamide gel electrophoresis as described by Welton (30). The tyrosinase- and catechol-treated microsomes were the same as the untreated sample.

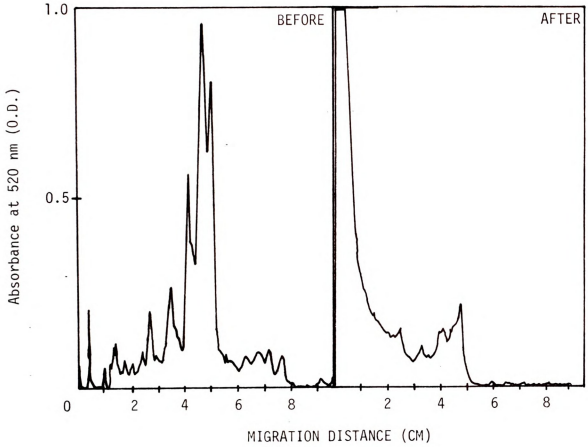
1.0
0.5
0
BEFORE
AFTER
Absorbance at 520 nm (O.D.)
2
4
6
8
2
4
6
8
MIGRATION DISTANCE (CM)

Figure 12

DISCUSSION

The methods of quantitative cell fractionation introduced by Albert Claude (107) represented a fundamental change in the preparative fractionation of tissue homogenates. It shifted the emphasis from the purification of a given visible intracellular entity to a quantitative analysis of the enzymatic activities present in the original homogenate. This approach focused on the actual objects of analysis, the enzymes or other biochemical components, and examined the manner in which these entities were distributed between all the fractions separated from the homogenate. The focus on enzymatic activities and their quantitative recoveries provided a powerful tool for the study of any such cellular component not irretrievably lost in the initial homogenization of the cells.

Even though the fractionation procedures allow an almost infinite number of systems and enzymes to be studied, it is not easily adapted to a diffuse variety of tissues. The majority of this work has been focused on mammalian tissues, such as the liver. The wealth of information obtained by the fractionation of liver homogenates has enticed researchers to apply cell fractionation to a wide variety of other tissues and systems. It is apparent from the literature that the analysis of insect preparations lacks the thoroughness of the mammalian studies. Consequently, assumptions have been made concerning the stability and homogeneity of insect preparations which cannot

be supported by this worker's research on house fly *in vitro* preparations.

When this research was first begun, Morello and co-workers (96) published one of the first articles to deal extensively with microsomal cytochrome P-450 of house flies and in this they advocated homogenizing house flies by the mortar-and-pestle (MP) technique when isolating the microsomal fraction. This method, according to these workers, gave the highest microsomal naphthalene hydroxylation activity per nmole of cytochrome P-450 hitherto reported, and less contamination of the microsomal fraction. The MP method gave less than half of the microsomal protein per 10 g of flies as compared to microsomal fractions prepared from Sorvall^{R} -homogenized whole flies. This result was interpreted by these workers as indicating the attainment of "clean" microsomes, free from the sarcosomal contamination which would interfere with the analysis of cytochrome P-450. This technique allowed the use of whole flies, without the necessity of separating the abdomens.

The NADPH-cytochrome c reductase activity of the microsomal fractions from homogenates prepared by glass-Teflon (GT) homogenization of abdomens was compared by this investigator with that of the MP whole house fly preparations. In two isolations, the specific activity of microsomal NADPH-cytochrome c reductase average was slightly lower for the MP microsomal fractions at 0.060 μmoles of cytochrome c reduced/min/mg protein, than that of the GT preparations at 0.072 μmoles (Table 1). This difference did not seem great enough to justify the three to four hours required to separate and collect the large numbers of abdomens required for each isolation.

Having made this assumption, a number of isolations were made with house flies at various developmental stages using the MP method to see

if the reductase activity followed the same fluctuations within these various stages as was shown for naphthalene hydroxylation. Naphthalene hydroxylation by house fly microsomal fractions showed a peak of activity just prior to larval pupation. The level of activity was very low during the pupal stage and increased again after ecdysis, with the highest level of enzymatic activity occurring 10 to 12 days later (108). It had been thought that the last larval instar might provide a good source of enzyme activity, thereby eliminating the time-consuming process of rearing the flies to their adult stage. However, the microsomal NADPH-cytochrome c reductase specific activity from 94 isolations ranged as much as 10-fold for a specific age (Table 1). Since these preparations were made from whole insects, it was then thought that a cleaner microsomal fraction would give more consistent results.

At this time, differential and density gradient centrifugation studies were conducted in this laboratory to see if a combination of these methods might reduce the observed variability. They revealed that most of the reductase was being solubilized during the isolation procedure. When the use of phospholipase and protease inhibitors failed to prevent solubilization, it was realized that a new isolation procedure would have to be established in order to isolate the house fly microsomal fractions. This procedure utilized the GT homogenization of abdomens only (46), and buffers of 0.57 to 0.60 ionic strength (109). The results obtained by this method contradicted those obtained with the MP method (Fig. 6, Table 7).

In establishing a reliable isolation procedure, the heterogeneity of the mixture subjected to further fractionation must be given careful consideration. A homogeneous tissue possesses a cell population which

contains a variety of intracellular particulate entities that form a number of distinct populations upon homogenization. Heterogeneous tissues normally composed of many cell types multiply the complexities of cellular fractionation. Whole or abdominal house fly homogenates contain greater numbers of complex elements not present in liver homogenates, yet to date the isolation procedure for house fly microsomes has remained essentially the same as that used in the isolation of liver microsomal fractions. Although there are vast differences in composition between the insect and liver homogenates this does not mean a priori that the procedure will differ, but careful quantitative fractionation studies are needed to determine whether or not the established procedure used to isolate liver microsomes is the correct one for insects.

The term microsome is a biochemical one designating the subcellular fraction of membranes obtained by high speed centrifugation of the mitochondrial supernatant. It does not refer to a specific organelle as do the terms mitochondria or lysosome. Rather, the microsomal fraction contains membranous elements such as endoplasmic reticulum and ribosomes, originating from a heterogeneous population of cells with the quantity of each component determined by the isolation procedure. Therefore, the optimal conditions for isolating the microsomal fraction from each type of tissue and/or insect must be thoroughly established, since each may contain elements that uniquely affect the isolation of this fraction.

The analytical procedure that defines the most optimal isolation conditions involves the quantitative analysis of marker enzymes and their distribution patterns in the fractions obtained by differential centrifugation of the homogenate. The procedure resulting from such studies incorporates individual methods that give the best overall

results for enzymatic stability, purity and yield. The distribution study of marker enzymes in fractions from rabbit lung and liver illustrates how such results can aid in establishing a procedure that gives the fraction possessing the greatest specific activity of microsomal enzymes and the least amount of contamination by other enzymes or cellular components (36). In isolating the microsomal fractions by differential centrifugation from house cricket Malpighian tubules, it was found that most of the microsomal activity was obtained at less than 12,000 xg. A two-step density gradient procedure was found to provide a means of isolating a fraction containing most of the pesticide metabolizing activity. However, this isolated fraction was unstable and rapidly lost its enzymatic activity (40).

Though several excellent studies have been made on the enzymatic distribution patterns using marker enzymes and taking into account morphological characteristics of subcellular fractions from rat liver homogenates (107), quantitative studies of this type have yet to be done for insect preparations. In the few studies that have analyzed the enzyme distribution in subcellular fractions of insects, pesticide metabolism alone has been used as the sole indicator of the distribution of microsomal enzymes. These studies assume that the mixed-function oxidase complex was a complete and functional unit whenever present in a fraction. Pesticide metabolism, which serves as the primary indicator of the distribution of insect microsomal enzymes, fails to detect the location of membranous fragments containing an incomplete complement of the mixed-function oxidase system. Therefore, the use of a complex system drastically reduces the resolving power that could be attained by using a marker enzyme, consisting of one functional component. Even

when several single enzyme species are employed as markers to assess the composition of each fraction, great caution must be exercised in evaluating their distribution patterns, since the resolving power of the techniques used is rarely commensurate with the complexity of the material being analyzed.

The resolving power of the separation procedures presently available greatly depends on differences in such physical properties as particle size, shape, density, and the resulting sedimentation coefficient. In some instances, factors such as electrical charge or solvent affinity can even influence the isolation of a component. These isolation procedures are severely limited, however, by the necessity of preserving the structural and biochemical integrity of the components during the utilization of these methods. They are further complicated by the fact that the populations of these particles are not composed of identical individual members as is the case in isolating a single species from a chemical mixture.

Several artifacts may occur during the isolation of subcellular fractions and can arise from the disruption of cellular organelles, the adsorption of soluble material onto the organelles and cellular particles, and enzyme inhibition or activation. The use of complex enzymatic systems to determine the distribution pattern of an enzyme can also distort the results. Quantitation of the fractionation procedure keeps a complete record of the original level of enzymatic activities, their distribution patterns and their recoveries enabling the investigator to detect any of the problems mentioned above. When inhibition or activation does occur, the recombination of fractions can assist in determining if the inhibitor or activator is associated

with an organelle or is soluble.

The presence of endogenous factors causing loss of enzymatic activity can also be detected by this procedure. The occurrence of adsorption, inhibition, solubilization, and/or decay of enzymatic activity can alter the apparent distribution pattern of an enzyme so that the major proportion of its activity is mistakenly associated with the wrong fraction. Fig. 4 shows how solubilization can alter the distribution pattern of an enzyme. The recovery of 89% of the house fly NADPH-cytochrome c reductase activity in the soluble fraction of MP homogenates suggested that this enzyme in house flies is a soluble one. The rat liver reductase, however, is tightly bound to the microsomal membrane with 70% or more of the total homogenate activity recovered in the microsomal fraction, similar to the distribution pattern of glucose-6-phosphatase (35). The distribution pattern of the house fly reductase was assumed to be an artifact of the MP homogenization method; the results from GT isolations confirmed this assumption (Fig. 6, Table 7).

Removal of an organ that contains the desired enzyme(s) prior to homogenization is a major purification step in the isolation procedure. In the case of insects, their small size usually prevents this simple step. The studies conducted with the southern armyworm and the house cricket illustrate how the utilization of a single organ, as opposed to the whole insect, increased the enzyme activity of the preparation. The whole larval armyworm and cricket homogenates had no detectable epoxidation activity. Homogenates of the fat body, Malpighian tubules and washed gut all epoxidized aldrin, with the highest activity being found in the microsomal fractions of the armyworm midgut and cricket Malpighian tubules.

Careful evaluation of the isolation procedure is necessary because

the procedure can have the effect of combining subcellular components as well as fractionating them. Subfractionation of the rat liver mitochondrial fraction has revealed two distinct types of minor components, lysosomes and microbodies. The result of this subfractionation was that only 7 of the 18 enzymes previously thought to be mitochondrial were actually associated with that fraction (105). The association of mitochondrial with microsomal activity in peak II of Fig. 3 may have been a result of the densities employed in the discontinuous sucrose gradient.

Homogenization of the tissue followed by centrifugation produces an unnatural environment for the cellular components, as well as the mechanical stress of centrifugation. These conditions often result in the leakage of soluble entities from their organelles followed by their readsorption onto the surface of other cellular particulates. Whenever tissues are homogenized, all the barriers within a cell that separates the cellular constituents are destroyed, placing the enzyme(s) in what may be a distinctly foreign or hostile environment. This is certainly true when whole insects or their body parts are homogenized, as indicated by the numerous reports concerning the endogenous inhibitors of insect microsomal preparations and their instability. Microsomal fractions from whole armyworm and cricket homogenates failed to metabolize pesticides; this was considered to be due to the solubilization of the NADPH-cytochrome c reductase by an endogenous protease (43,44). In homogenates of the house fly abdomens containing 1 g of abdomens per 4 ml of buffer, the mixed-function oxidase activity decreased by 80% in 30 minutes at 0° C (38). The exact factors causing this decay have yet to be isolated and identified. Results from the MP isolations reported here demonstrated the solubilization of NADPH-cytochrome c reductase

by a factor(s) which also have yet to be identified.

The distribution of NADPH and oxygen-requiring DDT metabolism was examined in what is apparently the only study published showing the distribution of enzyme activity in subcellular fractions from MP homogenates (110). Whole German cockroaches, Blattella germanica (L.), were homogenized with a mortar-and-pestle, and the subcellular fractions were obtained by differential centrifugation. Forty-five percent of the enzymatic activity was found in the microsomal fraction and the remaining 34% of the enzymatic activity in the nuclear and mitochondrial fractions. Since the activity recovered in the microsomal fraction was only 11% more than that recovered in the other two fractions, the procedure failed to fractionate the desired enzymatic activity into an enriched fraction. In assaying for a complex enzyme system, the procedure also failed to indicate the complete distribution of microsomal membranes.

The distribution of the marker enzymes, cytochrome oxidase (Fig. 3) and NADPH-cytochrome c reductase (Fig. 4) in the subcellular fractions of whole house fly MP homogenates also failed to give an enriched fraction of either enzyme that contained a significant proportion of the total protein. These results from the cockroach and house fly MP homogenates differed significantly from the results obtained with rat liver homogenates. The distribution of the rat liver microsomal enzymes, NADPH-cytochrome c reductase, glucose-6-phosphatase and aminopyrine demethylase, showed 70% of the total homogenate activity in the microsomal fraction (35). This difference might best be explained as the result of using whole insects, which would reduce the relative proportion of protein contained in the enriched fraction. However, the obvious solubilization of NADPH-cytochrome c reductase in MP homogenates in contrast to the GT

preparations, would indicate that the difference is a result of the method used. The failure to isolate a given enriched fraction containing the desired enzyme may also be related to aggregation caused by endogenous tyrosinase and phenols, which have been shown to cross-link proteins. The association of the microsomal with the mitochondrial enzymatic activity in the density gradient (Fig. 5) may have resulted from aggregation.

Another difference between rat liver and insect preparations is apparent when one considers the amount of initial enzymatic activity recovered in the fractions. Whereas in the cockroach and house fly isolations only 79 and 64%, respectively, of the total enzymatic activity of the homogenate was recovered, the recoveries for the three rat liver enzymes mentioned above averaged 100%.

The failure to recover enzyme activity in the insect fractions may be due to several reasons such as incomplete breakage of cells or loss of enzymatic activity through inactivation by endogenous factors released during homogenization. The decay of enzyme activity detected in house fly abdominal homogenates (38) (Fig. 7) would account for at least part of the failure to recover enzymatic activity. The solubilization of NADPH-cytochrome c reductase is also known to destroy microsomal drug metabolism (44). If solubilization occurred in the cockroach MP preparations, as it did in the house fly MP isolations, it could have resulted in the failure to recover 21% of the total homogenate DDT-metabolizing activity in the fractions.

Even though eye pigments had been reported previously as endogenous inhibitors of mixed-function oxidase in house fly microsomal preparations (38,49), the high rate of naphthalene hydroxylation by

MP preparations indicated that the pigment was not released during homogenization (96). However, subsequent work found that xanthommatin would inhibit house fly microsomal aldrin epoxidase at concentrations as low as 5×10^{-7} M (50). In this research, MP homogenization of whole flies would occasionally show the red smear of a crushed head on the side of the mortar. Even though the homogenization was done gently, there would be isolations when the low speed centrifugation yielded a pellet containing a zone of red pigment, thus suggesting that ommatidia had been broken during homogenization. These observations indicate that another of the difficulties encountered between one MP preparation and the next was the maintenance of a consistent degree of homogenization. The solubilization of NADPH-cytochrome c reductase, the erratic distribution patterns of marker enzymes from MP preparations, and the low recovery of enzymatic activity would all indicate that the MP homogenization technique of whole flies is not a suitable method for isolation of the house fly microsomal fraction. These findings also indicate that this isolation procedure had been adapted for insect studies without the experimental information needed to properly evaluate its suitability.

The next major problem to be considered was the dramatic loss of mixed-function oxidase activity within a relatively short period of time for the homogenate and microsomal fraction of house flies. Because of this rapid decay of microsomal activity during the isolation procedure (38), it was not feasible to study the distribution patterns of marker enzymes for the determination of an optimum isolation procedure. The microsomal fraction isolated from GT homogenates was unstable when stored (Table 10) under the conditions listed in the

Materials and Methods. Storage of microsomal fractions from house flies in dilute solutions containing BSA prevented the loss of enzymatic activity (111). This does not, however, indicate what the factor(s) was that produced the observed instability. BSA is known to be an alternate protease substrate, and also protects enzymes from inactivation by lipids (112) or phenols (113).

Therefore, an important question is: What could be present in house fly microsomes but absent from rat liver microsomes that would cause instability? It has been reported that potassium cyanide added to house fly microsomal incubations stimulated pesticide metabolism by 15 to 25% (56,57,58). Similar concentrations of KCN inhibit rat liver microsomal metabolism by 15% (Table 9). Tyrosinase, which is inhibited by KCN, has not been detected in goat liver microsomes (57) nor was it detected in rat liver microsomes by this researcher. The microsomal supernatant of house fly abdominal homogenates, which had 6 times the tyrosinase activity detected in the pellet, inhibited pesticide metabolism when added to house fly microsomal incubations (58). As mentioned earlier, the stimulation of this enzyme's activity decreased aldrin epoxidation in preparations from lepidopterous larvae (52,60). Jordan and Smith (38) also observed that KCN prevented the decay of mixed-function oxidase activity in house fly homogenates. Even though KCN is known to inhibit a large number of enzymes (59), an isolation was made to see if the addition of catechol, which stimulates tyrosinase hydroxylation activity, and KCN, which inhibits tyrosinase, would respectively decrease and increase microsomal enzyme activity when compared to the control. As seen in Table 9, the results fulfilled the expectation. These results suggested that catechol

oxidase and its substrates adversely affected microsomal enzymes. Since rat liver microsomes did not contain any detectable tyrosinase activity, they provided a system for the evaluation of the effects of exogenous tyrosinase on microsomal mixed-function oxidases.

The effect of tyrosinase and catechol on the three microsomal constituents examined were quite dramatic. During the incubations of rat liver microsomes with tyrosinase and catechol, the turbid microsomal suspension underwent a visible clarification. A decrease in turbidity of the microsomal suspension has been observed during lipid peroxidation (92). The loss of NADPH-cytochrome c reductase activity occurs during lipid peroxidation (93) as well as during the tyrosinase and catechol treatments. When microsomes were incubated with a protease, the reductase activity was observed to increase significantly before showing a decrease. Also during proteolysis, the cytochrome P-450 peak shifted to 420 nm as the 450 nm peak decreased (94). After the tyrosinase plus catechol incubation was started, cytochrome P-420 began to appear within a few minutes, but failed to increase as the absorbance at 450 nm decreased. In a study of cytochrome P-450 degradation during lipid peroxidation (92), the spectral changes observed with time resembled those observed when the rat liver microsomes were incubated with tyrosinase and catechol (Fig. 8).

As reviewed earlier, lipid peroxidation has been shown to be a free-radical process. It is presently unclear as to whether or not the semiquinone free radical forms of the phenols are the substrates for tyrosinase. It has been suggested that semiquinones are involved in the oxidation-reduction interactions of phenols with quinones

(Fig. 2), but this has yet to be rigorously established. The observed lysis of erythrocytes in the presence of tyrosinase and catechol would indicate that free radicals are involved in the degradation of microsomal enzymes when tyrosinase and the phenol, catechol, are present. Experiments using red blood cells to detect free radicals, tyrosinase, and other phenols with more stable semiquinones could more thoroughly examine the role that free radicals might play in tyrosinase function and in the oxidation-reduction of phenols and quinones.

Research done on the inhibition of plant enzymes during their isolation in subcellular fractions by phenols and quinones (114,115) have indicated some ways that this problem can be eliminated. The utilization of polymers such as polyvinylpyrrolidone to trap quinones in the plant homogenates gives preparations with much higher enzymatic activities than those prepared without the polymer (116). Other agents, such as BSA, which react with quinones also provide preparations with higher enzymatic activities than those without (115). The inclusion of reagents that trap quinones during the isolation of phenyl oxidase from tea leaves, resulted in the enzyme being found only in the soluble fraction (117). It would appear that the presence of tyrosinase in other fractions was an artifact resulting from the binding of tyrosinase to membranes that sedimented in the different fractions. Examination of this point in future experiments with house fly preparations might yield the same results. If this should be the case, then the utilization of reagents to protect enzymatic activities from inactivation by tyrosinase and phenols would yield a microsomal fraction free from these contaminants. Such procedures might provide a way to prepare a stable microsomal fraction from house flies. Studies along these lines would be important not

only to researchers interested in mixed-function oxidases, but to all those doing in vitro analysis with insect material.

Obtaining a stable preparation would also permit workers to analyze the isolation procedure quantitatively, providing important information basic to the rational use of subcellular fractions from insects. There are obviously many problems facing the investigator when attempting such an analysis on homogenates of most insects, but such work is impossible when the preparations are unstable.

The importance of stability cannot be over emphasized. In preparations that are not stable, the degree of instability is often variable depending on the quantity of the unknown factor(s) causing instability released during each isolation. Under such unstable conditions, studies to determine the optimum isolation procedure cannot be undertaken. When the differential studies of whole house fly MP homogenates were being made, each isolation varied in the distribution of cytochrome oxidase and NADPH-cytochrome c reductase.

Welton (15) examined the effect of proteolysis on rat liver phenobarbital-induced microsomes in the presence and absence of BHT, an inhibitor of lipid peroxidation. When microsomes were incubated at 25° C for 60 minutes in the presence of trypsin and BHT, approximately 25% of the microsomal protein was solubilized into 105,000 xg supernatant of the post-treatment centrifugation. SDS polyacrylamide gels of trypsin-treated ^{125}I-labelled microsomes, centrifuged down after treatment, showed a significant alteration of the protein and ^{125}I profiles. The ^{125}I labelling had a more dramatic decrease than the protein profile. The destruction of the microsomes increased when proteolysis was carried out in the absence of BHT with the ^{125}I remaining bound to the proteins falling

essentially to zero. In another experiment, when the peroxidase labelling of rat liver microsomes with ^{125}I was performed with and without BHT present, the results showed that the presence of BHT increased by 100% the amount of label incorporated. The BHT also completely inhibited lipid peroxidation as measured by the malondialdehyde assay. This data clearly demonstrates the importance of maintaining the integrity of the microsomal membrane and its enzymatic functions during experimental procedures. Lipid peroxidation alone decreased iodination by half, and when allowed to occur during proteolysis completely disrupted the integrity of the microsomal membrane, as shown by the complete inhibition of iodination and the severe alteration of the gel protein profile.

At this point, it is uncertain how many degradative and/or inhibitory factors are present in house fly preparations. The combined effect of proteolysis and lipid peroxidation on rat liver microsomes should suggest to researchers working with insect preparations that the solution of one problem may only reveal another. It is obvious that much remains to be done to characterize the factors affecting the integrity and stability of insect microsomal fractions.

Armyworm preparations appear to be stable and can be stored without the loss of aldrin epoxidation activity or degradation of cytochrome P-450 (118). In this case, the preparations are made from the larval midgut which reduces the heterogeneity of the homogenate. The abrasive action of chitin which may also degrade enzymes and which is unavoidable in glass-Teflon homogenates of whole or parts of house flies, is also eliminated. A quantitative analysis of this system using marker enzymes for the various organelles identified in mammalian systems would provide information which is clearly absent from insect biochemical

literature.

The work presented here concerning the effect of tyrosinase plus catechol on microsomal mixed-function oxidases is only an introduction into the effect of endogenous tyrosinase, phenols and quinones on the stability and activity of the enzymes in insect homogenates. If these compounds are among the primary causes of the notorious instability and low enzymatic activity of most in vitro insect preparations, numerous avenues are open for a much fuller utilization of insecticide-resistant strains. Careful experimentation that utilizes the biological differences of house fly strains should help to further elucidate the molecular mechanisms of the mixed-function oxidase complex and its regulation. Such biological and biochemical information would contribute greatly to our understanding of this important system and, hopefully, contribute to a more rational design of pesticides.

SUMMARY

During the isolation of the microsomal fraction by differential centrifugation from MP homogenates of whole flies, the NADPH-cytochrome c reductase was extensively solubilized. In 23 isolations, only 20 ± 24.5% S.D. of the 105,000 xg pellet and supernatant reductase activity was in the pellet. Inhibitors of calcium dependent phospholipases, EDTA, and proteases, phenylmethylsulfonylfluoride, did not decrease the solubilization. In 30 isolations obtained from glass-Teflon homogenates of house fly abdomens, 80.3 ± 21.3% S.D. of the reductase activity was recovered in the microsomal fraction. The difference in percentage of the reductase isolated in the microsomal fraction by the two homogenization methods was significant at the 0.01 level when tested for significance by the Student's t-test. The specific activity for the MP preparations was 0.025 ± 0.008 S.D. of cytochrome c reduced/min/mg protein and for the GT preparations it was 0.050 ± 0.025 μmoles S.D. of cytochrome c reduced/min/mg protein. These differences were significant at 0.05 level when compared by the Student's t-test. Even though microsomal fractions isolated from GT homogenates contained most of the reductase activity, the aldrin epoxidase activity of the homogenates and microsomal fractions from GT isolations was unstable. The homogenate lost 69% of its epoxidase activity in 30 minutes and when stored at -20° C the microsomal fraction lost 50 to 60% of its activith within 24 hours.

The catechol oxidase complex has been reported to cause loss of insect mixed-function oxidase activity. When tyrosinase was inhibited by cyanide in house fly homogenates, the levels of reductase activity, aldrin epoxidation, and cytochrome P-450 content was higher than in untreated preparations. The reductase activity of a catechol-treated preparation was lower than the untreated control. Aldrin epoxidation activity was the same as the control, but neither cytochrome P-450 nor P-420 was detected in the catechol-treated sample. When liver microsomes from phenobarbital-induced rats were treated with tyrosinase and catechol for 22 hours at 4 C, the reductase activity decreased to 13% of control value, aldrin epoxidation was completely inhibited, and cytochrome P-450 was reduced to 36% of the concentration detected in untreated controls. Tyrosinase alone and catechol alone and cyanide-inhibited tyrosinase and catechol did not degrade microsomal mixed-function oxidases. The lysis of erythrocytes when incubated with tyrosinase and catechol suggest that free radicals are involved in the loss of microsomal enzymatic activity. Incubation of red blood cells with catechol alone or tyrosinase alone did not cause lysis. SDS-polyacrylamide gel electrophoresis of microsomes treated with tyrosinase and catechol showed that cross-linking of proteins had occurred during the degradation process. Most of the sample stayed at the origin and failed to migrate into the gel.

The effect of tyrosinase and catechol on rat liver microsomal mixed-function oxidases indicates that endogenous tyrosinase complex present in house fly homogenates causes the loss of enzymatic activity. The use of inhibitors and polymers should eliminate the effect of tyrosinase, phenols and quinones thus allowing the isolation of a stable microsomal

fractions from house flies. This will have to be examined carefully as the resolution of this problem may only reveal that other endogenous factors, such as proteases, also cause loss of house fly microsomal mixed-function oxidases.

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