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I am submitting herewith a thesis written by Kave Noghrei Nikbakht entitled "Effects of Chemical and Environmental Stress on Cytochrome P-450 Activity and Phospholipid Composition of midgut microsomal fraction in southern armyworm Spodoptera eridania Cramer (Noctuidae). I recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Biochemistry.

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EFFECTS OF CHEMICAL AND ENVIRONMENTAL STRESS ON CYTOCHROME P-450 ACTIVITY AND PHOSPHOLIPID COMPOSITION OF MIDGUT MICROSOMAL FRACTION IN SOUTHERN ARMYWORM SPODOPTERA ERIDANIA CRAMER (NOCTUIDAE)

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ABSTRACT

The objective of this project was to study the effects of the temperature and two chemical inducers, namely pentamethylbenzene and phenobarbital on cytochrome P-450 activity and phospholipid composition of midgut microsomal fraction in southern armyworm Spodoptera eridania Cramer. We were also interested to know how the inductive properties of these chemicals might be modified in relation to the changes in the temperature. Finally, an analysis of fatty acyl composition of midgut microsomal phospholipids was performed at two temperatures (15 °C and 30 °C). Low temperature (15 °C) caused a significant induction of both microsomal phospholipids and cytochrome P-450 activity. The inductive effect of the low temperature in microsomal phospholipids was through decreasing PE/PC ratio as the rate of PC induction was much higher than PE induction. From quantitative analysis of microsomal phospholipids, it turned out that the major phospholipid is phosphatidylcholine followed by phosphatidylethanolamine. The other microsomal phospholipids included phosphatidylserine and phosphatidylinositol. Both chemical inducers caused a substantial induction of microsomal phospholipids and cytochrome P-450 activity at 15 °C but pentamethylbenzene turned out to be a stronger inducer than phenobarbital. At 30 °C, however, the induction caused by pentamethylbenzene was not as strong as its induction at 15 °C and that of phenobarbital totally disappeared. It was concluded that the shift in the temperature had somehow modified the inductive properties of these chemicals. We also found out that the induction of microsomal phospholipids by pentamethylbenzene

was through increasing the PE/PC ratio.

The induction of cytochrome P-450 activity which occurred along with the proliferation of the microsomal membranes was considered as a defensive adaptation mechanism developed by armyworms upon their exposure to the toxic foreign compounds. Fatty acyl analysis of microsomal phospholipids showed that at 15 °C, the concentration of myristic, palmitic, stearic and palmitoleic acid decreases, whereas concentrations of two unsaturated fatty acids, namely oleic and linoleic increases at that temperature.

Since membrane fluidity affects the properties of a number of membrane-bound enzymes as well as transport proteins, this tendency toward higher levels of unsaturation at the low temperature presumably had some adaptive values for armyworms in order to cope with the external stress imposed on them.

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CHAPTER I

INTRODUCTION

Among different mechanisms involved in the metabolism of lipophilic foreign compounds, biological oxidations undoubtedly play the major role. There is a wide spectrum of these compounds both synthetic and natural e.g. pesticides and other chemicals used in agriculture, a large number of drugs, industrial pollutants and naturally occurring plant allelochemicals. Although there is a great diversity in the chemical structures and physical properties of these compounds, the basic idea of their metabolism is the same, that is, to convert the lipophilic foreign chemical into a more polar species which the organism can easily rid itself of by means of its excretory system. A schematic view of foreign compound metabolism is presented below (Figure 1) (1). These compounds by the virtue of their lipophilicity have a great tendency to accumulate in the lipid-rich regions of the cell such as biological membranes which renders them potentially harmful at very low concentrations. For this reason the metabolism of these compounds which in most cases leads to their deactivation, is of vital importance to the organism.

In living organisms, biological oxidation of lipophilic foreign compounds is achieved by the microsomal mixed-function oxidases (MFOs) which catalyze the hydroxylation of a wide variety of chemicals in a cyclic sequence of reactions according to the following diagram (Figure 2).

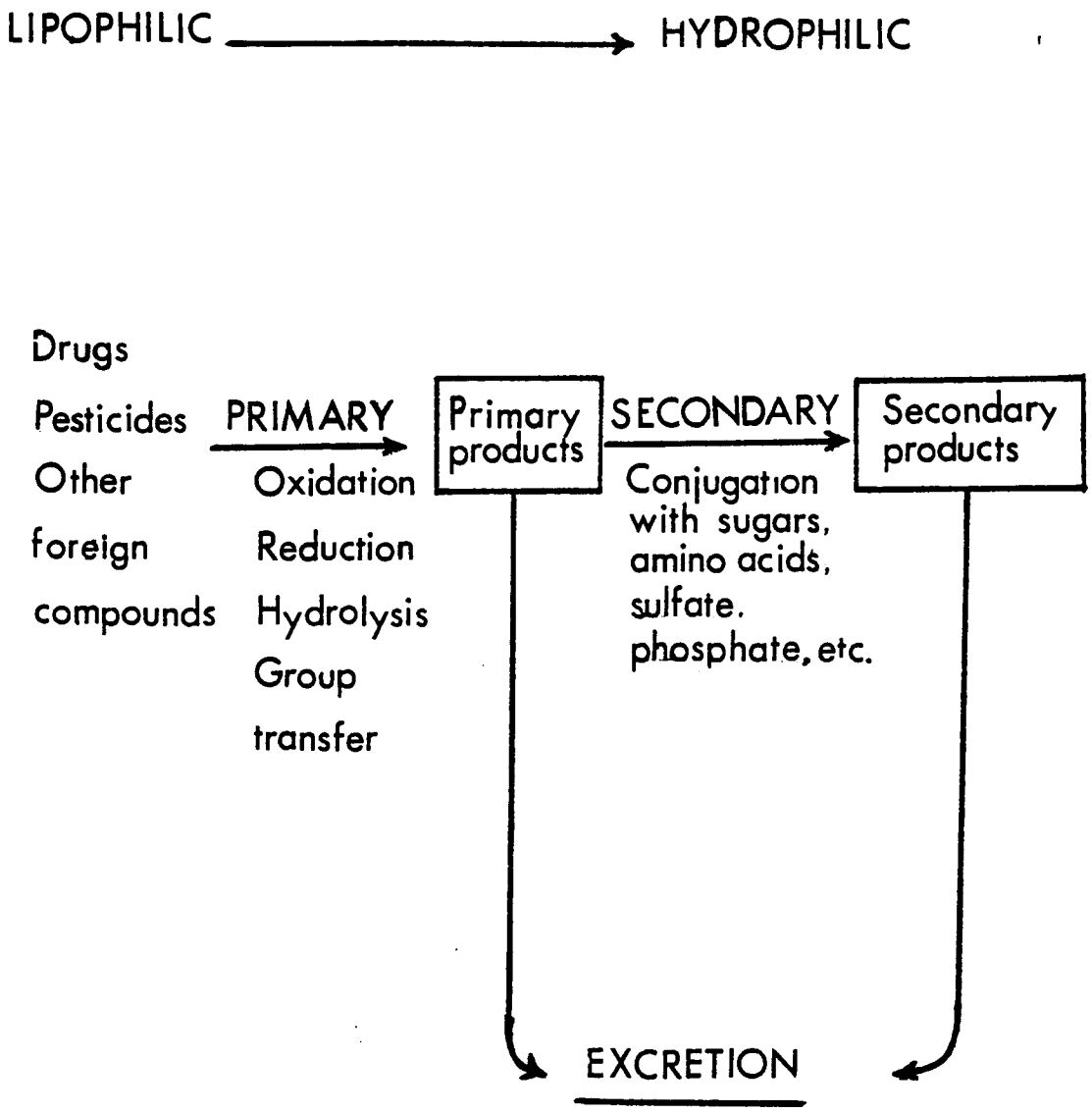

Figure 1: Schematic representation of the lipophilic foreign compound metabolism.

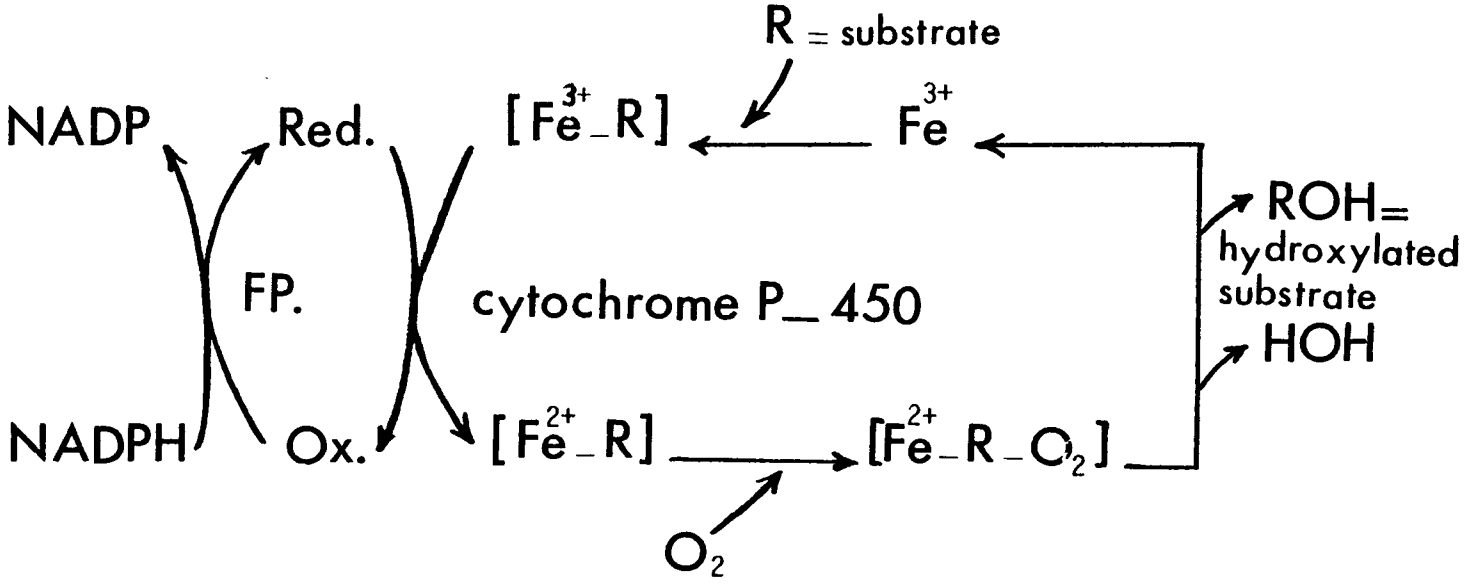

Figure 2: The cyclic sequence of reactions leading to biological oxidation of lipophilic foreign compounds by the microsomal mixed-function oxidases (MFOs).

The catalytic component of this system is cytochrome P-450, a heme protein which forms a complex with the substrate as well as with molecular oxygen during its catalytic activity. Cytochrome P-450 operates in conjunction with a flavoprotein enzyme, NADPH-P-450 reductase which transports reducing equivalents from NADPH to the heme iron of the cytochrome. Although the hydroxylation of these substrates is the first step in their metabolism (inactivation), it has been shown that in some cases (e.g. polycyclic aromatic hydrocarbons), the hydroxylation would stimulate the toxicity of a substrate which would otherwise be inactive (2).

The MFO system is embedded in the smooth endoplasmic reticulum and the integrity of the membranous structure is essential for its catalytic activity; the MFO system loses its activity upon disruption of the smooth endoplasmic membrane. Despite the ubiquitous occurrence of the MFO system among the living organisms (it is found in plants, animals, fungi and aerobic microorganisms), its extensive study has been limited to only very few organisms including the small laboratory animals, a number of insect species, few species of fish and some microorganisms. Relative distribution of the MFO system is not equal in different parts of the body and basically depends on the strategic position of the organ. In mammals for instance, the highest concentration of the MFO system is found in the liver whereas other organs such as the kidney, the lungs and the intestine contain smaller proportions. In fish also, the liver is the major location of the MFO activity followed by the gills. Among insects, the greatest amount of MFO activity is found

in the midgut and fat body, but there is also activity in malpighian tubules, hindgut, etc.

There is a direct correlation between the MFO activity and different life stages of the organism, that is, the highest activity of the MFO is found when the organism is in its most active feeding state. A good example of such correlation appears in insects which are undergoing periodic transition to larger exoskeleton (or molting). It has been found that the MFO activity is lower during molting stages when the insect does not feed and increases during intermolt periods when it feeds vigorously. The insect does not have any MFO activity during dormant stages of its life cycle (insect eggs or insect pupae).

An important feature of the MFO system is its capability to adapt itself upon exposure of the organism to toxic environmental chemicals. Induction of the MFO activity in vertebrate liver which is accompanied by the proliferation of the smooth endoplasmic reticulum has been shown for a wide variety of compounds such as 3-methylcholanthrene, phenobarbital, pentamethylbenzene and low levels of plant allelochemical. Studies have also been carried out on insect MFO induction, particularly with houseflies in several laboratories (3,4). Induction of cytochrome P-450 (the catalytic component of the MFO system) along with the proliferation of the smooth endoplasmic reticulum is thought to serve as a defensive adaptation mechanism developed by organisms upon their exposure to the toxic chemicals.

Smooth endoplasmic reticulum is a membranous structure composed of both polar and non-polar lipids as well as proteins, including a number of enzymes. As said above, the integrity of the smooth endoplasmic reticulum is essential for the catalytic activity of the MFO system.

The lipid content of the endoplasmic reticulum not only plays an important role in keeping the microsomal membrane structure intact (5) but also influences the kinetic and thermodynamic properties of many constitutive enzymes (6-8). This conclusion has been derived from the fact that perturbation of membrane lipids, induced either by detergents or phospholipases, influences the properties of these enzymes. Also, temperature induced phase transitions within the lipid portion from the gel to the liquid-crystalline state have been shown to have similar effects (9-12). Due to the fact that as much as 80-90% of the total lipid content of the microsomal fraction obtained from mammalian liver tissue is contributed by phospholipids (13) the importance of phospholipids in catalytic activity of microsomal enzymes becomes obvious. In fact, for many years, scientists have shown that the MFO system for its activity absolutely depends on the phospholipids of the smooth endoplasmic reticulum. In 1969, Lu et al. in a reconstitution experiment showed that the hydroxylation of fatty acids not only required the presence of NADPH, molecular oxygen, cytochrome P-450, NADPH-cytochrome P-450 reductase, but also a heat-stable phospholipid fraction (14).

In view of these observations, the important question is the manner in which the phospholipid environment influences the

properties of the membrane-bound microsomal enzymes, including the MFO-system. Duppel and Ullrich in 1976 showed that the membrane transition from the gel to the liquid-crystalline state lowers the activation energies of the enzymatic reactions of membrane bound enzymes above the critical transition temperature and concluded that the phospholipid phase transition would affect the electron transfer from the flavo-protein reductase to cytochrome P-450 (15).

Generally speaking, the MFO substrates are classified into two categories, namely type 1 and type 2, according to the characteristics of their spectral perturbation when they combine with the oxidized cytochrome. The binding sites of these two groups of substrates is thought to be different. It is believed that the type 1 binding site is either in an undetermined hydrophobic region of cytochrome P-450 protein or in lipids of the microsomal membrane (8,16). The binding of the type 1 substrates to the hydrophobic regions is through hydrophobic interactions. The type 2 substrates bind directly to the cytochrome heme moiety (co-binding site of the hemoprotein). It should be kept in mind that most compounds known to be the MFO substrates have been classified as type 1 substrates.

A school of scientists believe that the phospholipids are somehow involved in binding of type 1 substrates. The reason for this suggestion stems from the convincing experiments of Chaplin and Mannering in 1970 (16). Using rat liver microsomal preparation these scientists selectively digested the phosphatidylcholine

and phosphatidylethanolamine with phospholipase c. Upon digestion, there was a total destruction of the type 1 binding site as well as a significant reduction in ability of the microsomal preparation to oxidize ethylmorphine and hexobarbital.

The proliferation of the smooth endoplasmic reticulum as a defensive adaptation mechanism against the toxic chemicals necessitates an increase both in lipid and protein contents of the membranous structure. The first account of the increased lipid content as a protective mechanism against the toxic chemicals has been given by Munson and Gottlieb in 1953 (17). They showed that those strains of the American cockroach, Periplaneta americana which are resistant to DDT have considerably higher total lipid content. Khan and Brown in 1966, showed that Dieldrin-resistant strains of the yellow fever mosquito, Aedes aegypti contained 20-50% more larval fat than their susceptible counterparts (18). They also concluded that the increase in lipid content involved both neutral lipids and phospholipids.

The reports showing the correlation between the lipid content and insects tolerance to the toxic chemicals are not always consistent, which indicates that more work has to be done on the subject. For instance, Micks and Singh in 1958 made a comparison of several multiresistant and chlordane resistant strains of houseflies with susceptible strains and observed no consistent

interstrain differences in lipid content (19). The same observation was made by Ascher and Neri in 1961 who carried out studies on a mosquito, Anopheles atroparvus (20). In 1959, Bridges and Cox reported that a highly dieldrin resistant strain of houseflies contained no more lipid than a susceptible strain (21). The most contradictory report has been given by Bradbury et al. in 1958. According to them, strains of housefiles and Anopheles gambiae Giles resistant to dieldrin and lindane contained equal or even smaller amounts of lipids than the susceptible counterparts (22).

Although there is a great diversity in the lipid patterns among different insect species, phospholipids are found to be qualitatively similar to those in mammals despite quantitative differences. Generally speaking, three phospholipid patterns have been reported among insects:

(1) a dipterous pattern, including flies, mosquitoes etc. in which phosphatidylethanolamine occurs in the greatest amount.

(2) a lepidopterous pattern which like mammals, contains phosphatidylcholine in the greatest amount. Examples from this group of insects are moths and butterfiles.

(3) A coleopterous pattern, including beetles in which phosphatidylethanolamine and phosphatidylcholine occur in equal amounts.

Although there is a great deal of information regarding the phospholipid composition in whole insects (23-27), studies on individual organs have not received adequate attention. In 1964, Crone, using housefly flight muscle sarcosomes, found that

the muscles are enriched with phosphatidylethanolamine while there was a smaller quantity of phosphatidylcholine compared to those of the intact flies (28). Allen and Newburgh have found that in Sarcophaga bullata, lysophosphatides occur in large quantities in fat body (29). According to Kulkarni et al. (30), the gut tissue of the tobacco hornworm contains most of the phospholipids of the larva and phosphatidylcholine occurs in the greatest amount. They also showed that the gut tissue contains most of the MFO activity. With respect to the subcellular fractions Khan and Hodgson, in 1967, reported that the microsomal fraction obtained from the housefly differs from other subcellular fractions in its higher content of phosphatidylcholine (31) which is similar to the phosphatidylcholine composition of the rat liver microsomes They also reported the presence of large quantities of lysophosphatidylethanolamine and ethanolamine sphingolipids in the microsomal fraction, whereas phosphatidylethanolamine was shown to occur in the greatest amount in the whole insect. The mitochondria have been reported to contain 5 percent cardiolipin whereas the microsomal fraction contained only 4 percent. These analyses indicate that there are variations in the phospholipid composition among different organs as well as different subcellular fractions.

The membrane fluidity which is influenced by the level of saturation of fatty acyl residues of phospholipids affect the activity of a variety of membrane-bound enzymes and transport proteins (8). For instance, it has been shown that β-Hydroxybutyric dehydrogenase not only requires phosphatidylcholine for its cata-

lytic activity, but also a high level of unsaturated fatty acyl chains (8). The dependence of the cytochrome P-450 activity upon the nature of the fatty acyl side chain has been demonstrated for purified cytochrome from beef adrenal cortex mitochondria (8); little or no activity was observed when fatty acyl chains were fully saturated, while activity increased when the phospholipid contained unsaturated chains. We have undertaken this project in order to provide unambiguous information regarding the combined effects of physical and chemical stresses on MFO activities as well as on the phospholipid composition of the midgut microsomal fraction of southern armyworm larva. We would like to know how the MFO activity is affected when the armyworm larvae are adapted to a low temperature environment (15°C) and to a high temperature (30°C) environment, and how the temperature influences the ability of the MFO system to be induced by known MFO inducers such as pentamethylbenzene and phenobarbital. A detailed qualitative and quantitative analysis of the phospholipid composition of the microsomal fraction under the experimental conditions described above has been designed in order to establish reliable information regarding the correlation between the MFO induction and changes in the endoplasmic membrane phospholipid composition. Since membrane fluidity depends on the saturation level of the fatty acyl residues of membrane phospholipids, an analysis of the fatty acyl composition at low and high temperatures was also done.

CHAPTER II

MATERIALS AND METHODS

Animals

The Southern armyworms, Spodoptera eridania Cramer originally obtained from the laboratory of Dr. C. F. Wilkinson, Cornell University were raised in a rearing room under suitable environmental conditions. In their early larval stages, the armyworms were fed the foliage of lima bean, Phaseolus lunatus which have smooth hairless leaves and in their latter stages of growth, foliage of red kidney bean, P. vulgaris was used (the larval growth period included 6 instars). The armyworms were collected during their 3rd and 4th instar and kept in the incubator for the remainder of their larval growth. The temperature acclimated animals were collected shortly after they molted into the 6th instar larval stage and were fed an artificial semi-defined diet (described below) either plain or loaded with the chemical inducer pentamethylbenzene (PMB) and phenobarbital as appropriate until their full growth. At high temperature (30 °C) the larvae become fully grown after 3 days, whereas at low temperature (15 °C) it takes as many as 7-9 days (unpublished data).

Incubation Condition

During incubation, the animals were kept in plastic boxes containing a layer of soil (Bacto autoclaved potting soil) to serve as an absorbent. Each batch contained from 20 to 80 armyworms and batches were kept at 15 °C (low) and 30 °C (high) according to the following scheme:

	Diet Containing PMB	Diet Containing Phenobarbital	Control Diet
temperature	15 °C	15 °C	15 °C
temperature	30 °C	30 °C	30 °C

The Diet

The semi-defined artificial diet contained 2.5% (w/v) bean leaf powder (freeze-dried leaves were ground to a fine powder in a Waring blender), 0.6% each of sucrose (Mallinckrodt, Inc.), alfacel (ICN pharmaceuticals), casein (vitamin free) (Nutritional Biochemicals Corp.), 0.1% each of wheat germ (regular) (Kretschmer, International Multifood), vitamin mixture for insects (Nutritional Biochemicals Corp.), salt mixture (Nutritional Biochemicals Corp.), cholesterol (U.S. Biochemicals Corp.), and linolenic acid (ICN Pharmaceuticals), 0.75% agar in water. When appropriate, a concentration of 0.2% (w/v) of pentamethylbenzene (PMB) and 0.25% of phenobarbital was included in the diet. Pentamethylbenzene and phenobarbital were purchased from Aldrich Chemical Co. and Sigma Chemical Co., respectively.

Preparation of the Midgut Microsomes

The fully grown armyworms were decapitated and the midguts were obtained after cutting the two ends of the whole guts, carefully cleaned, and kept in cold 0.2M phosphate buffer pH 7.8 with 1mM EDTA (approximately 0.5 ml of the buffer solution per gut). The midgut preparation was homogenized gently in a glass Ten Broeck homogenizer tube with a motor driven teflon pestle. The homogenate was centrifuged for 2 min. at 39,000 xg using a rotor type SS-34,

in a Sorvall centrifuge to remove nuclei, mitochondria and other debris and the supernatant was first filtered through glass wool and then centrifuged for 20 min. at 210,000 xg using a rotor type 60 Ti in a Beckman L5-75 preparative ultracentrifuge. The pellet (containing the microsomes) was suspended in a small volume of cold 0.25 M sucrose and kept in ice. The microsomal protein was measured by the method of Lowry et. al. (32), with bovine serum albumin as standard.

Measurement of the Cytochrome P-450 Concentration

The microsomal protein concentration was adjusted to 2 mg protein per ml using 0.3 M phosphate buffer, pH 7.8 containing 50% glycerol and then reduced by addition of a few milligrams of solid sodium dithionite. Using an Aminco-Chance DW-2 spectrophotometer a baseline was established for the reduced microsomal preparation between 400 and 490 nm. The sample cuvette was then saturated with CO by bubbling the gas for about 1 min. and the CO difference spectrum was obtained through the same wave length range (400-490 nm). The concentration of the cytochrome P-450 was estimated according to Omura and Sato (33) assuming a value of 91 cm^{-1} mM^{-1} for the molar extinction coefficient of the cytochrome.

Lipid Extraction of the Microsomal Fraction

The microsomal preparation was extracted in a separatory funnel with chloroform-methanol (2:1) (Fisher Scientific Co.) in the cold (34). The organic phase was then separated and washed with 0.15M cold KCL (20% v/v), (Fisher Scientific Co.). Total lipid was ob-

tained after evaporation of the organic phase (Buchi Rotavapor R) and measured gravimetrically and then kept under nitrogen environment in the refrigerator until use.

Fractionation of Total Lipid Through the Silicic Acid Column

Fractionation of total lipid was performed through a silicic acid column containing approximately 20 g of the activated (100 °C overnight) silicic acid (Silicar CC-4, Mallinckrodt) per 40 mg of the total lipid. The column elution was done by step wise application of the following solvent systems:

solvent systems:	chloroform	methanol
1st elution	150 ml	——
2nd elution	90 ml	10 ml
3rd elution	100 ml	20 ml
4th elution	——	100 ml

10 ml fractions were collected and each fraction was transferred into a small round bottomed flask (50 ml) and after evaporation of the organic phase, the lipid layer deposited at the bottom of the flask was dissolved in 0.5-1 ml of chloroform and transferred into a small vial. The chloroform was then evaporated under the nitrogen flush and the materials deposited at the bottom were kept under the nitrogen environment in the refrigerator until use.

Thin Layer Chromatography of Lipid Fractions and the Total Lipid

Thin layer chromatography of lipid fractions collected from the silicic acid column was carried out in one dimension using 250 μm TLC plates (K1 silica gel, Whatman). Each lipid fraction stored under the nitrogen was dissolved in 100 μl of chloroform and 15 μl

of every third fraction were applied to the TLC plate along with the standard lipids obtained from the laboratory of Dr. Blank, ORNL. The plate was run in the following solvent system (35): chloroform-methanol-acetic acid and water (25:15:4:2, v/v). Thin layer chromatography of the total lipid was performed in two dimensions using the following solvent systems (36); 1st dimension: chloroform-methanol-ammonia (65-25-5, v/v); 2nd dimension; chloroform-acetone-methanol-acetic acid-water (30-40-10-10-5, v/v).

Detection and Characterization of Lipids on TLC Plates

The following general and specific detection methods were used on TLC plates:

A. General detection methods

1. Sulfuric acid charring method: A solution of sulfuric acid, 30-40% aqueous, was prepared and the reagent was applied to the TLC plate by spraying the air-dried plate with an all-glass automizer. The plate was then placed in an oven (> 100 °C) for a few minutes. Lipids appeared as black spots on the TLC plate.

2. Iodine vapor method: The air-dried TLC plate was placed in a closed chamber saturated with crystal iodine vapor (Fisher Scientific Co.). The lipids were detected as the yellow spots appearing on the plate shortly after the exposure to the iodine vapor.

B. Phosphate specific detection method

Solution (a): 1.6 g of ammonium molybdate were dissolved in 12 ml of water. Solution (b): To 4 ml of concentrated HCL, 1 ml of mercury and 8 ml of solution (a) were added, shaken and then filtered. Spray reagent was prepared by adding to the remainder of

solution (a), 20 ml of concentrated sulfuric acid and all of solution (b) (37). The reagent was cooled and then diluted to 100 ml with water. The air-dried TLC plate was sprayed with reagent and the phospholipids appeared as blue spots on a white background within a few seconds. Ammonium molybdate and mercury were purchased from Fisher Scientific Co.

Quantitative Analysis of Phospholipids on TLC Plates

A. Phosphorous analysis of spots

The quantitation of phospholipid spots on TLC plates was performed according to the method described by Rouser, et. al. (38). Spots were first detected by placing the TLC plates in a chamber saturated with crystal iodine vapor. After appearance of yellow spots, each spot was circled and lettered for better identification and a blank area corresponding in size to the sample spots was marked off. All spots were scraped off the TLC plate and were aspirated into 30 ml Kjeldahl digestion flasks. The suction was accomplished by fitting a rubber stopper with two glass tubes into the Kjeldahl flask. One tube served as intake of the scraped material whereas the other was connected by a rubber tube to a water pump for suction. Each Kjeldahl flask contained 0.9 ml of 72% perchloric acid (Fisher Scientific Co.) in order to prevent the absorbent from passing out of the digestion flask during aspiration. The perchloric acid was subsequently used for digestion of the phospholipids. After completion of aspiration, the glass tubes were tapped to remove any dry powder into the lower bulb portion of the flask. An electrically heated Kjeldahl rack (Laboratory Construc-

tion Co.) was used to digest the flask contents and the escaping gas was removed by connecting a water-pump suction to the heated Kjeldahl rack. Digestion was continued for approximately 15 min. with gentle refluxing.

After completion of digestion, the walls of the flasks were rinsed with 5 ml of distilled water and the flask contents were vortexed for a few seconds. Subsequently, 1 ml of 2.5% ammonium molybdate solution was added and the flasks were swirled for mixing. Finally, to each flask, 1 ml of freshly prepared ascorbic acid (Fisher Scientific Co.) solution was added and the flask contents were vortexed for complete mixing. The flask contents were transferred to small centrifuge tubes and heated for approximately 5 min. at 52 °C in an oven, cooled and suspended absorbent removed by centrifugation for a few minutes. The supernatants (samples and blanks) were transferred to cuvettes and their optical density was determined at 820 nm using a spectronic 70 spectrophotometer (Bausch & Lomb) already calibrated to zero with water. The optical densities of the sample solutions were corrected after the optical density of the blank area corresponding in size to that of the sample was subtracted. A standard curve was prepared using $Na2HPO_4$ (heated overnight at > 100 °C) in 8 Kjeldahl flasks containing from 0 to 7 µg of the compound. Due to the sensitivity of the method throughout the entire experiment, all of the glassware was washed thoroughly in the acid bath (prepared from concentrated sulfuric acid and chromium trioxide purchased from Fisher Scientific) to avoid any phosphorous contamination resulting from

the detergent-washed glassware.

B. ^{32}P-radioisotope labeling method

The armyworms (fully grown on control and PMB-treated diets) were compartmentalized in an ice cube tray covered with a glass lid and starved for 2-3 minutes. After starvation period each worm was fed with a small piece of bean leaf loaded with approximately 1.0×10^{-4} mCi of ^{32}P (orthophosphate) (Amersham) followed by their normal diet. After approximately 4-5 hrs., armyworms were killed, their midgut isolated and the total lipids extracted as before. Two dimensional thin layer chromatography was performed on total lipids. After completion, the phospholipid spots (detected by crystal iodine vapor) as well as blank areas were scraped and transferred to the scintillation vials (containing 5 ml of PCS, Amersham) and radioactivity was determined in a Beckman LS 230 liquid scintillation counter.

Time-dependent Incorporation of ^{32}P Into Phospholipids

An experiment was performed in order to show the time-dependent incorporation of the radio labeled orthophosphate into phospholipids. Several batches of armyworms were compartmentalized and labeled with ^{32}P as described above. The groups of armyworms were killed at different time intervals (½, 1, 5, 10 and 20 hr.), their midguts were isolated and the total lipids were extracted and counted for radioactivity in a liquid scintillation counter.

Fatty Acid Analysis of Total Phospholipids

Total phospholipids were obtained after a column of silicic

acid loaded with the total lipids and already washed with chloroform (to remove neutral lipids and steroids) was eluted with methanol. To analyze the fatty acid constituents of the phospholipids, the fatty acyl residues were trans-esterified to the methyl group according to the following method:

1. Phospholipids (4-8 mg) dissolved in a small volume of chloroform were transferred into a conical tube and flushed with nitrogen until complete dryness.

2. 8 ml of 2% (v/v) concentrated sulfuric acid in methanol were added to the tube and after the tube's cap was screwed tightly and wrapped in parafilm, the tube was incubated at 40 °C overnight.

3. After completion of the incubation, 10 ml of the petroleum ether (Fisher Scientific Co.) and 5 ml water were added to the tube and shaken gently. Two phases appeared, the organic phase (top layer) was withdrawn and extraction was done two more times as before.

4. All organic phases were combined (approximately 30 ml total) and placed into a round bottomed flask.

5. The flask content was washed with 5 ml of water by gentle shaking to remove excess sulfuric acid; after appearance of two phases, the aqueous phase was withdrawn with a Pasteur pipette and checked for acidity using Hydrion paper (Fisher Scientific Co.). Washing of the organic phase was continued until no significant acidity in water was detected.

6. The organic solution was evaporated and the film deposited at the bottom of the flask was dissolved in a small volume

of chloroform and transferred into a small vial.

7. Chloroform was evaporated under the nitrogen flush until complete dryness and the materials deposited at the bottom were dissolved in 0.5-1 ml of hexane (Fisher Scientific Co.) and kept under nitrogen environment in the refrigerator until use.

Gas-Liquid Chromatography of Methyl Esters of Fatty Acids

The methyl esters of fatty acids were analyzed in a Fisher-Victoreen 4000 gas liquid chromatography equipped with a flame ionization detector and a column (1.8 m x 3.1 mm) packed with 10% SP-2300 cyanosilicone on 80/100 Supelcoport (Supelco). The experimental conditions were as follows: coarse attenuation = 10^4, fine attenuation = 16, chart speed = 1 inch/min., oven temp = 200 °C, detector temp ≃ 225 °C, injection temp ≃ 250 °C. The peaks were identified by comparison of their retention times with those of standard commercial methylated fatty acid samples purchased from Supelco.

CHAPTER III

RESULTS

Cytochrome P-450 Spectrum and Measurement of the Cytochrome Concentration

Figure 3 shows the CO difference spectrum of cytochrome P-450 after adjustment of the microsomal protein concentration to 2 mg protein per ml using 0.3M phosphate buffer, pH 7.8 containing 50% glycerol and reduction of the cytochrome by addition of a few milligrams of solid sodium dithionite. The concentration of the cytochrome was estimated according to Omura and Sato (33) assuming a value of 91 cm^{-1} mM^{-1} for the molar extinction coefficient of the cytochrome.

Effect of the Temperature on Midgut Microsomal Total Lipid and Cytochrome P-450 Activity

Tables show the effect of the temperature on midgut microsomal total lipid and cytochrome P-450 activity. The armyworms were fed either plain diet or a diet containing pentamethylbenzene or phenobarbital. The microsomal total lipid was measured gravimetrically and the microsomal protein concentration was measured by the method of Lowry *et al.* (32). At lower temperature (15 °C), the armyworms contained a higher amount of microsomal total lipid as well as higher cytochrome P-450 activity. This temperature related induction not only was observed among the armyworms fed on control diet, but also among those treated with pentamethylbenzene or phenobarbital.

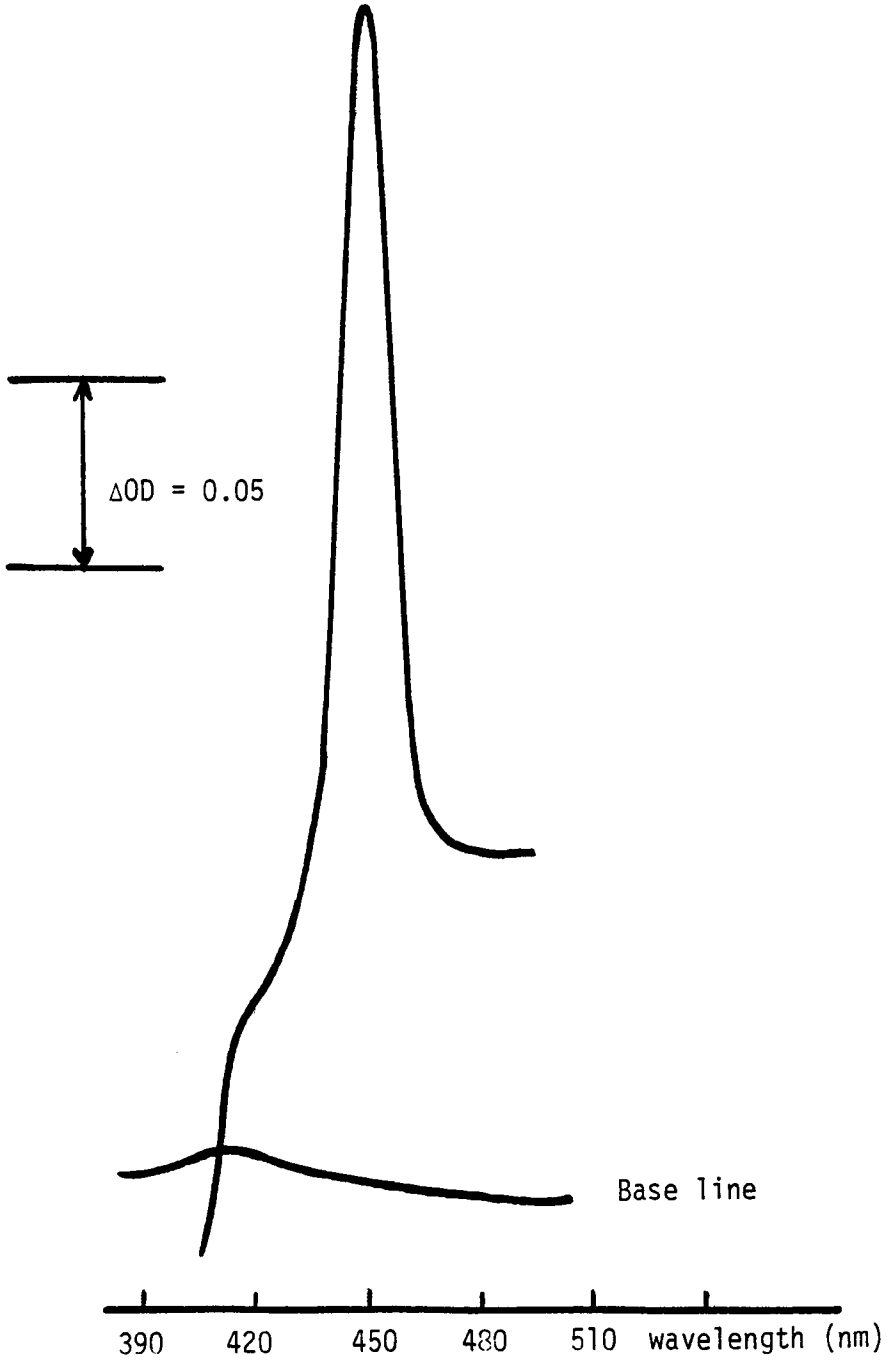

Figure 3: CO difference spectrum of cytochrome P-450 from midgut microsomal fraction. The spectrum was obtained using an Aminco-Chance DW-2 spectrophotometer.

Effect of Chemical Inducers on Midgut Microsomal Total Lipid and Cytochrome P-450 activity.

The data in Tables 1-3 have been simply rearranged in order to show the effect of chemical inducers pentamethylbenzene and phenobarbital on midgut microsomal total lipid and cytochrome P-450 activity. The new arrangement of the data appears on Tables 4 and 5. Whether the armyworms were raised at 15 °C or 30 °C a substantial induction of both microsomal total lipid and cytochrome P-450 activity was observed among the worms treated with pentamethylbenzene. Such induction, however, was less significant among the worms treated with phenobarbital. At 30 °C, phenobarbital had no inductive effect on cytochrome P-450 activity.

Fractionation of Microsomal Total Lipid Through the Silicic Acid Column and One Dimensional TLC of the Lipid Fractions

Figure 4 shows one dimensional thin layer chromatography of lipid fractions collected from the silicic acid column. A glass column containing approximately 20 g of the activated silicic acid was loaded with 30-40 mg of the microsomal total lipid and then eluted with different proportions of chloroform and methanol as described in methods and materials. In one dimensional TLC only two lipid spots appeard on the plate which were identified as phosphatidylcholine (PC) and phosphatidylethanolamine (PE) by comparison with standard lipid spots.

Two dimensional TLC of Microsomal Total Lipid and Detection of Phospholipids by Phosphate Specific Detection Reagent

The result of two dimensional TLC of microsomal total lipid is shown in Figure 5. 20-40 µg of microsomal total lipid were applied

Table 1: Effect of Temperature on Midgut Microsomal Total Lipid and Cytochrome P-450 Activity in Control Worms

Control Worms	30°C	15°C	% change & statistical significance*
microsomal total lipid (mg)/microsomal protein (mg)	0.59 ± 0.07	0.61 ± 0.10	4.2 (N)
microsomal total lipid (mg)/gram wet weight of worm	0.29 ± 0.06	0.38 ± 0.05	31.5 (S)
cytochrome P-450 (nmole)/microsomal protein (mg)	0.28 ± 0.08	0.42 ± 0.08	51.6 (S)
microsomal total lipid (mg)/cytochrome P-450 (nmole)	2.09 ± 0.10	1.44 ± 0.16	-31.1 (S)
cytochrome P-450 (nmole)/worm	0.06 ± 0.02	0.16 ± 0.03	161.4 (S)
microsomal protein (mg)/worm	0.22 ± 0.01	0.39 ± 0.02	73.3 (S)
microsomal total lipid (mg)/worm	0.13 ± 0.02	0.24 ± 0.04	80.4 (S)

The above data represent the mean values obtained from six experiments.
*Statistical significance of the differences between the mean values was verified after two-sample t testing at a = 0.05.

(S): The difference is statistically significant.
(N): The difference is not statistically significant.

Table 2: Effect of Temperature on Midgut Microsomal Total Lipid and Cytochrome P-450 Activity in PMB-treated Worms

PMB-Treated Worms	30 °C	15 °C	% change & statistical significance*
microsomal total lipid (mg)/microsomal protein (mg)	0.60 ± 0.16	0.70 ± 0.03	17.4 (N)
microsomal total lipid (mg)/gram wet weight of worm	0.56 ± 0.16	0.72 ± 0.07	26.4 (S)
cytochrome P-450 (nmole)/microsomal protein (mg)	0.65 ± 0.05	1.26 ± 0.16	93.8 (S)
microsomal total lipid (mg)/cytochrome P-450 (nmole)	0.92 ± 0.26	0.56 ± 0.08	-39.3 (S)
cytochrome P-450 (nmole)/worm	0.26 ± 0.04	0.87 ± 0.05	229.2 (S)
microsomal protein (mg)/worm	0.41 ± 0.06	0.70 ± 0.09	72.5 (S)
microsomal total lipid (mg)/worm	0.24 ± 0.07	0.50 ± 0.07	102.8 (S)

The above data represent the mean values obtained from six experiments.
*Statistical significance of the differences between the mean values was verified after two-sample t testing at a = 0.05.

(S): The difference is statistically significant.
(N): The difference is not statistically significant.

Table 3: Effect of Temperature on Midgut Microsomal Total Lipid and Cytochrome P-450 Activity in Phenobarbital-Treated Worms

Phenobarbital-treated Worms	30 °C	15 °C	% change & statistical significance*
microsomal total lipid (mg)/microsomal protein (mg)	0.56 ± 0.11	0.66 ± 0.14	19.0 (N)
microsomal total lipid (mg)/gram wet weight of worm	0.34 ± 0.07	0.51 ± 0.06	49.4 (S)
cytochrome P-450 (nmole)/microsomal protein (mg)	0.27 ± 0.04	0.77 ± 0.18	184.6 (S)
microsomal total lipid (mg)/cytochrome P-450 (nmole)	2.05 ± 0.63	0.85 ± 0.14	-58 (S)
cytochrome P-450 (nmole)/worm	0.06 ± 0.01	0.38 ± 0.05	472 (S)
microsomal protein (mg)/worm	0.25 ± 0.01	0.50 ± 0.05	99.6 (S)
microsomal total lipid (mg)/worm	0.14 ± 0.03	0.33 ± 0.04	137.5 (S)

The above data represent the mean values obtained from six experiments.
*Statistical significance of the differences between the mean values was verified after two-sample t testing at a = 0.05.

(S): The difference is statistically significant.
(N): The difference is not statistically significant.

Table 4: Effect of Chemical Inducers Pentamethylbenzene and Phenobarbital on Midgut Microsomal Total Lipid and Cytochrome P-450 Activity in Armyworms Raised at 15 °C.

Armyworms raised at 15 °C	Control Worms	PMB-treated Worms	% change & statistical significance*	phenobarbital-treated worms	% change & statistical significance*
microsomal total lipid (mg)/microsomal protein (mg)	0.61 ± 0.10	0.70 ± 0.03	14.7 (N)	0.66 ± 0.14	8.1 (N)
microsomal total lipid (mg)/gram wet weight of worm	0.38 ± 0.05	0.72 ± 0.07	89.4 (S)	0.511 ± 0.06	34.4 (S)
cytochrome P-450 (nmole)/microsomal protein (mg)	0.42 ± 0.08	1.26 ± 0.16	200 (S)	0.77 ± 0.18	83.3 (S)
microsomal total lipid (mg)/cytochrome P-450 (nmole)	1.44 ± 0.16	0.56 ± 0.08	- 61.1 (S)	0.85 ± 0.14	-40.9 (S)
cytochrome P-450 (nmole)/worm	0.16 ± 0.03	0.87 ± 0.05	443.7 (S)	0.38 ± 0.05	137.5 (S)
microsomal protein (mg)/worm	0.39 ± 0.02	0.70 ± 0.09	79.4 (S)	0.50 ± 0.05	28.2 (S)

Table 4 (Continued)

Armyworms raised at 15 °C	Control Worms	PMB-treated Worms	% change & statistical significance*	phenobarbital-treated worms	% change & statistical significance*
microsomal total lipid (mg)/worm	0.24 ± 0.04	0.50 ± 0.07	108.3 (S)	0.33 ± 0.04	37.5 (S)

The above data represents the mean values obtained from six experiments. *Statistical significance of the differences between the mean values was verified after two-sample t testing at a = 0.05.

(S): The difference is statistically significant.
(N): The difference is not statistically significant.

Table 5: Effect of Chemical Inducers Pentamethylbenzene and Phenobarbital on Midgut Microsomal Total Lipid and Cytochrome P-450 Activity In Armyworms Raised at 30 °C.

Armyworms raised at 30 °C	Control Worms	PMB-treated Worms	% change & statistical significance*	phenobarbital-treated worms	% change & statistical significance*
microsomal total lipid (mg)/microsomal protein (mg)	0.59 ± 0.07	0.60 ± 0.16	1.6 (N)	0.56 ± 0.11	-5.0 (N)
microsomal total lipid (mg)/gram wet weight of worm	0.29 ± 0.06	0.56 ± 0.16	93.1 (S)	0.34 ± 0.07	17.2 (N)
cytochrome P-450 (nmole)/microsomal protein (mg)	0.28 ± 0.08	0.65 ± 0.05	132.1 (S)	0.27 ± 0.04	-3.5 (N)
microsomal total lipid (mg)/cytochrome P-450 (nmole)	2.09 ± 0.10	0.92 ± 0.26	-55.9 (S)	2.05 ± 0.63	-1.9 (N)
cytochrome P-450 (nmole)/worm	0.06 ± 0.02	0.26 ± 0.04	333.3 (S)	0.06 ± 0.01	0.0 (N)
microsomal protein (mg)/worm	0.22 ± 0.01	0.41 ± 0.06	86.3 (S)	0.25 ± 0.01	13.6 (N)

Table 5 (Continued)

Armyworms raised at 30 °C	Control Worms	PMB-treated Worms	% change & statistical significance*	phenobarbital-treated worms	% change & statistical significance*
microsomal total lipid (mg)/worm	0.13 ± 0.02	0.24 ± 0.07	84.6 (S)	0.14 ± 0.03	7.6 (N)

The above data represents the mean values obtained from six experiments. *Statistical significance of the differences between the mean values was verified after two-sample t testing at a = 0.05.

(S): The difference is statistically significant.
(N): The difference is not statistically significant.

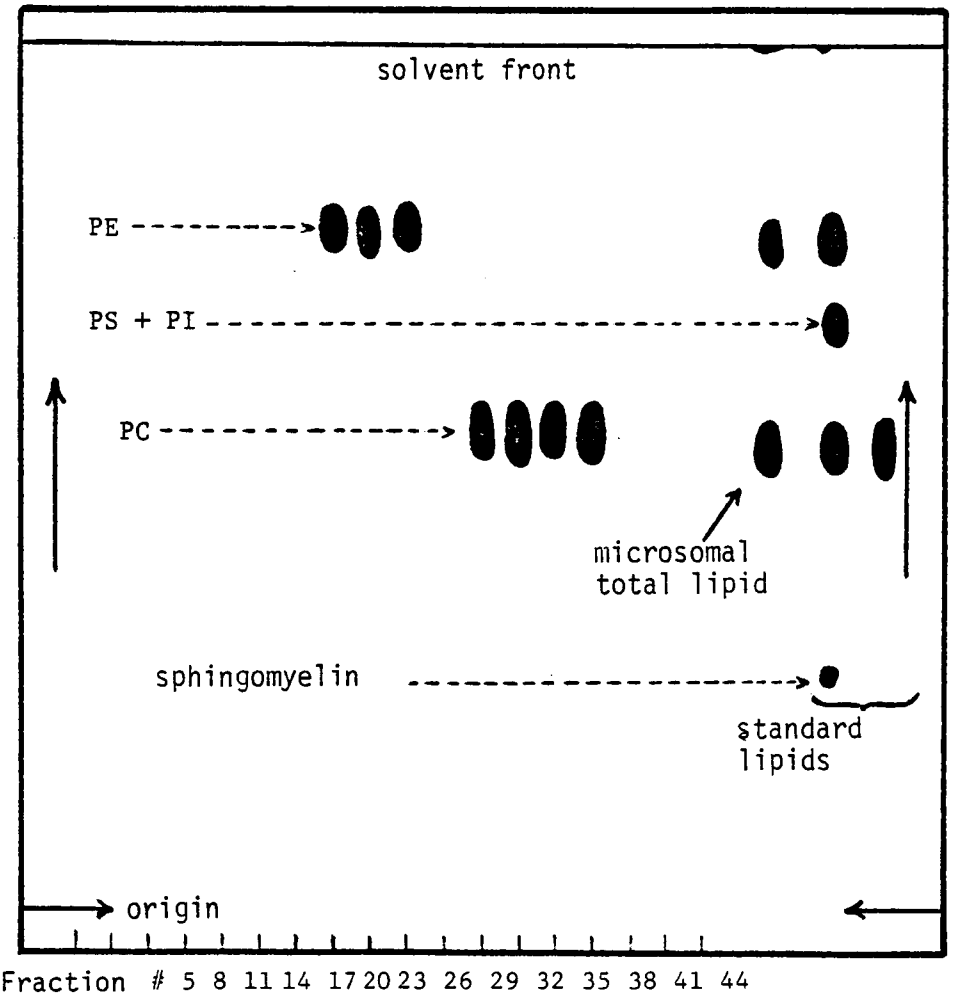

Figure 4: One dimensional TLC of lipid fractions collected from the silicic acid column and microsomal total lipid using 250 μm TLC plates (K1 silica gel, Whatman). The air-dried plates were developed in a closed chamber saturated with crystal iodine vapor and lipids appeared as yellow spots on the TLC plate .

Solvent system: chloroform-methanol-acetic acid-H_2O
50/30/8/4/ ml

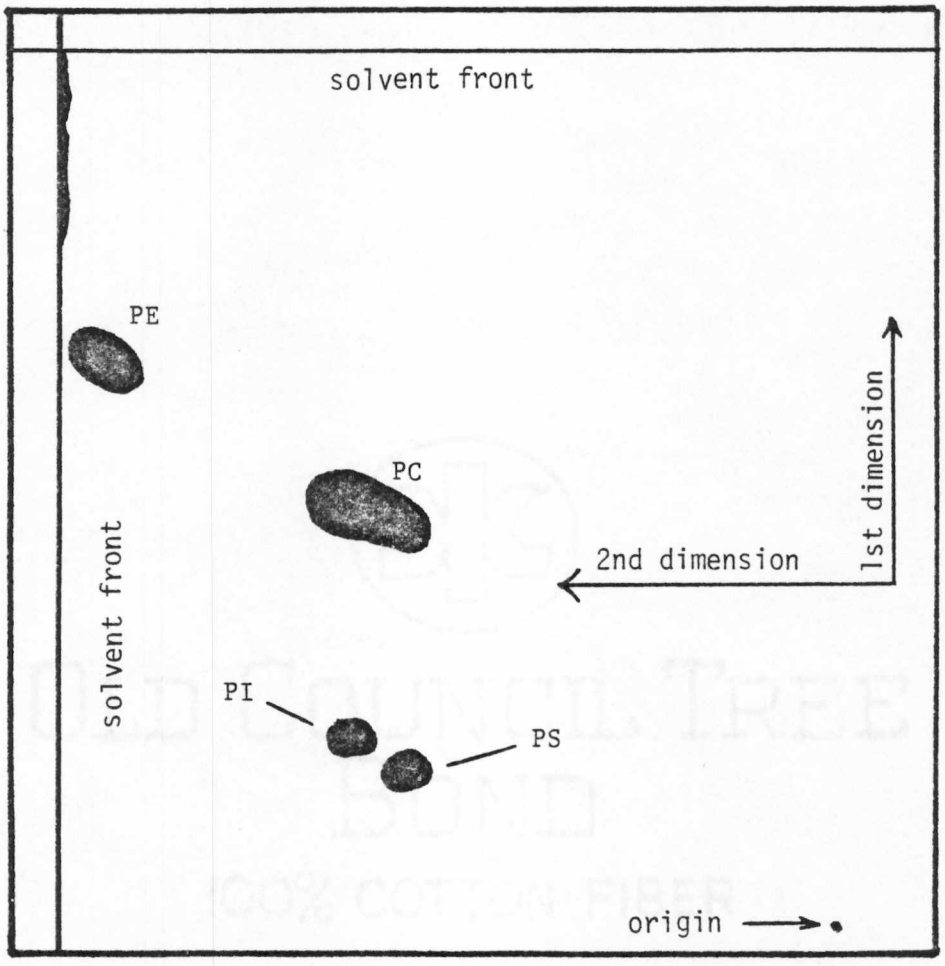

Figure 5: Two dimensional TLC of microsomal total lipid using 250 μm TLC plates (K1 silica gel, Whatman). The air-dried TLC plates were sprayed with phosphate specific detection reagent (37) and phospholipids appeared as blue spots shortly after spraying.

Solvent systems: 1st dimension - chloroform-methanol-ammonia-(65/25/5 ml)

2nd dimension - chloroform-acetone-methanol-acetic acid-water (30/40/10/10/5 ml)

to the TLC plate and phospholipids appeared as blue spots shortly after spraying the air-dried TLC plate with phosphate specific detection reagent (37). Four spots appeared on the TLC plate which were identified as phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol. Phosphatidylcholine and phosphatidylethanolamine appeared to be the major microsomal phospholipids.

Time-dependent Incorporation of ^{32}P Into Microsomal Phospholipids

Time dependence of ^{32}P incorporation into microsomal phospholipids is shown in Figure 6. In this experiment carried out at the room temperature, armyworms divided into several batches were separated and labeled with ^{32}P as described before. The armyworms were killed at different time intervals and after isolation of their midguts, the microsomal total lipids were extracted and counted for radioactivity in a liquid scintillation counter. The rate of ^{32}P incorporation appeared to be very high up to 5 hours after which the curve started leveling off.

Quantitation of Phosphatidylcholine and Phosphatidylethanolamine by Radioisotope Labeling Method and Effect of Pentamethylbenzene on PE/PC Ratio

Quantitation of PE and PC, the major phospholipids of the midgut microsomal fraction, was carried out after microsomal total lipid containing labeled phospholipids was run in two dimensions on the TLC plate. After completion of thin layer chromatography and detection of phospholipids by crystal iodine vapor, the phospholipid spots as well as blank areas were scraped off the plate and counted

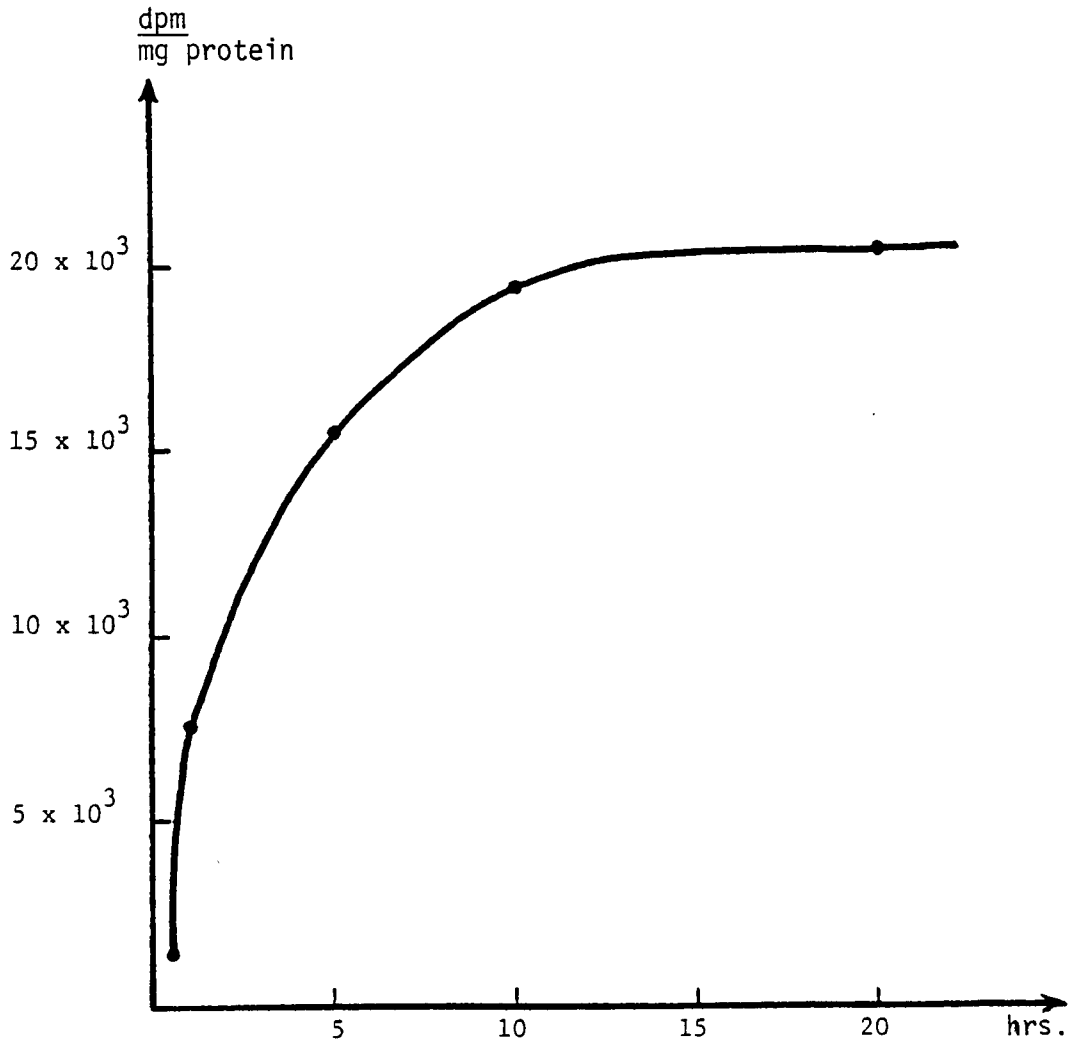

Figure 6: Time-dependent incorporation of the radio labeled orthophosphate, (^{32}P), into microsomal phospholipids. The experiments were carried out at the room temperature (25 °C) using control worms.

for radioactivity as described above. Table 6 shows the results of PE and PC quantitation by radioisotope labeling method and effect of PMB on PE/PC ratio. The amount of phosphatidylcholine appeared to be greater than phosphatidylethanolamine among the worms fed control diet. The PE/PC ratio however, increased when the armyworms were treated with the chemical inducer pentamethylbenzene.

Effect of Temperature on Midgut Microsomal Total Phospholipids and PE/PC Ratio

The data on Tables 7-9 were obtained after two dimensional thin layer chromatography of midgut microsomal total lipid and phosphorous analysis of phosphatidylcholine and phosphatidylethanolamine spots according to the method described by Rouser et. al. (38). At lower temperature (15 °C), in the presence or absence of chemical inducers, there was an increase in the amount of total microsomal phospholipids. The PE/PC ratio, however, showed smaller values at 15 °C in all experiments compared to those at 30 °C.

Effect of Chemical Inducers on Midgut Microsomal Total Phospholipids and PE/PC Ratio

The data in Tables 7-9 were rearranged in order to show the effect of chemical inducers pentamethylbenzene and phenobarbital on midgut microsomal total phospholipids and PE/PC ratio. Studies were carried out at two different temperatures (15 °C and 30 °C) and the results appear in Tables 10 and 11. When armyworms were raised at 15 °C, both chemicals caused an increase in the amount of midgut microsomal total phospholipids. Pentamethylbenzene however, showed a stronger inductive effect than phenobarbital.

Table 6: Quantitation of Phosphatidylcholine and Phosphatidylethanolamine by Radioisotope Labelling Method and Effect of Pentamethylbenzene on PE/PC Ratio

Control Worms	Total microsomal phospholipids applied to the TLC plate (dpm)	PE after correction for blank spot (dpm)	PC after correction for blank spot (dpm)	% PE of total microsomal phospholipids	% PC of total microsomal phospholipids	$\frac{PE}{PC}$
experiment 1	1123	231	437	20.5	38.9	0.52
experiment 2	1815	299	599	16.4	33	0.49
			Mean	18.45 ± 2.05	35.95 ± 2.95	0.5 ± 0.015
PMB-treated worms	Total microsomal phospholipids applied to the TLC plate (dpm)	PE after correction for blank spot (dpm)	PC after correction for blank spot (dpm)	% PE of total microsomal phospholipids	% PC of total microsomal phospholipids	$\frac{PE}{PC}$
experiment 1	992	245	301	24.7	30.3	0.815
experiment 2	1723	501	453	29	26.3	1.1
			Mean	26.85 ± 2.15	28.3 ± 2	0.95 ±0.14

Table 7: Effect of Temperature on Midgut Microsomal Total Phospholipids and PE/PC Ratio in Control Worms

Control Worms	30 °C	15 °C	% change & statistical significance*
microsomal total phosphorous (nmole) / microsomal protein (mg)	339 ± 24.50	362 ± 35.81	6.7 (N)
microsomal total phosphorous (nmole) / microsomal total lipid (mg)	607.70 ± 35.50	541.40 ± 38.83	-10.9 (N)
microsomal total phosphorous (nmole) / worm	74.70 ± 8.41	142.40 ± 15.11	90.6 (S)
microsomal total phosphorous (nmole) / gram wet weight of worm	162 ± 34.00	224 ± 20.92	38.2 (S)
% microsomal total phosphorous of microsomal total lipid	68.51 ± 0.51	75 ± 1.58	9.4 (N)
PE (nmole)/microsomal protein (mg)	16.10 ± 2.66	21 ± 2.85	30.4 (S)
PC (nmole)/microsomal protein (mg)	32 ± 6.22	61.15 ± 7.39	91 (S)
PE (nmole)/microsomal total lipid (mg)	28.66 ± 3.51	32 ± 2.95	11.6 (N)
PC (nmole)/microsomal total lipid (mg)	56.52 ± 7.38	94.40 ± 19.20	67 (S)
PE (nmole)/gram wet weight of worm	7.20 ± 2.01	12.96 ± 1.22	80 (S)
PC (nmole)/gram wet weight of worm	15.31 ± 4.55	38.11 ± 7.48	148.9 (S)
PE (nmole)/worm	3.55 ± 0.61	8.26 ± 1.05	132.6 (S)

Table 7 (Continued)

Control Worms	30 °C	15 °C	% change & statistical significance*
PC (nmole)/worm	7.04 ± 1.54	23.99 ± 2.81	240.7 (S)
% PE of microsomal total phospholipids	4.40 ± 0.62	5.60 ± 0.48	27.2
% PC of microsomal total phospholipids	9 ± 1.10	16.72 ± 3.10	85.7
PE/PC	0.50	0.34	-32

The above data which represent the mean values from four experiments were obtained after two dimensional TLC of midgut microsomal total lipid and phosphorous analysis of PE and PC spots according to the method of Rouser et al. *Statistical significance of the differences between the mean values was verified after two-sample t testing at a = 0.05.

(S): The difference is statistically significant.
(N): The difference is not statistically significant.

Table 8: Effect of Temperature on Midgut Microsomal Total Phospholipids and PE/PC Ratio in PMB-treated Worms

PMB-treated worms	30 °C	15 °C	% change & statistical significance*
microsomal total phosphorous (nmole) / microsomal protein (mg)	311 ± 13.31	328 ± 13.95	5.4 (N)
microsomal total phosphorous (nmole) / microsomal total lipid (mg)	628 ± 34	465.21 ± 30.69	-25.9 (S)
microsomal total phosphorous (nmole) / worm	121.8 ± 17.20	244.52 ± 17	100.7 (S)
microsomal total phosphorous (nmole) / gram wet weight of worm	291.81 ± 55	348.20 ± 27	19.3 (N)
% microsomal total phosphorous of microsomal total lipid	70 ± 1	78 ± 8	11.4 (N)
PE (nmole)/microsomal protein (mg)	19.41 ± 1.21	24.61 ± 1.31	26.7 (S)
PC (nmole)/microsomal protein (mg)	23.60 ± 1.28	62.32 ± 9	164 (S)
PE (nmole)/microsomal total lipid (mg)	39.41 ± 3.42	34.89 ± 3.24	-11.64 (N)
PC (nmole)/microsomal total lipid (mg)	47 ± 12	88.81 ± 17.20	88.9 (S)
PE (nmole)/gram wet weight of worm	18.42 ± 3.97	26 ± 0.78	41.1 (S)
PC (nmole)/gram wet weight of worm	22 ± 3.53	65 ± 0.21	195.4 (S)
PE (nmole)/worm	7.94 ± 1.34	18.31 ± 1.12	130.6 (S)

Table 8 (Continued)

PMB-treated Worms	30 °C	15 °C	% change & statistical significance*
PC (nmole)/worm	9.20 ± 1.02	46 ± 2.61	400 (S)
% PE of microsomal total phospholipids	6.10 ± 0.34	7.50 ± 0.57	22.9
% PC of microsomal total phospholipids	7.51 ± 0.25	18.72 ± 2.50	149.2
PE/PC	0.83	0.40	-51.8

The above data which represent the mean values from four experiments were obtained after two dimensional TLC of midgut microsomal total lipid and phosphorous analysis of PE and PC spots according to the method of Rouser et al. *Statistical significance of the differences between the mean values was verified after two-sample t testing at a = 0.05.

(S): The difference is statistically significant.
(N): The difference is not statistically significant.

Table 9: Effect of Temperature on Midgut Microsomal Total Phospholipids and PE/PC Ratio in Phenobarbital-treated Worms

Phenobarbital-treated worms	30 °C	15 °C	% change & statistical significance*
microsomal total phosphorous (nmole) / microsomal protein (mg)	283 ± 12	364 ± 27.78	28.6 (S)
microsomal total phosphorous (nmole) / microsomal total lipid (mg)	489 ± 33.30	551.12 ± 30.20	10.6 (N)
microsomal total phosphorous (nmole) / worm	68.91 ± 5.11	186.51 ± 17.41	170.6 (S)
microsomal total phosphorous (nmole) / gram wet weight of worm	166.82 ± 10	263.10 ± 21.34	57.7 (S)
% microsomal total phosphorous of microsomal total lipid	80 ± 3	84 ± 2.55	5 (N)
PE (nmole)/microsomal protein (mg)	30.70 ± 5.82	30.72 ± 2	0.06 (N)
PC (nmole)/microsomal protein (mg)	30.3 ± 1.24	89 ± 9.97	193.7 (S)
PE (nmole)/microsomal total lipid (mg)	53.11 ± 4.28	46.49 ± 6.42	-12.4 (N)
PC (nmole)/microsomal total lipid (mg)	53.95 ± 10	133.50 ± 20	147.4 (S)
PE (nmole)/gram wet weight of worm	15 ± 3.11	23.82 ± 3	58.8 (S)
PC (nmole)/gram wet weight of worm	18 ± 1.70	68 ± 6.81	277.7 (S)
PE (nmole)/worm	7.51 ± 1.62	15.85 ± 1.37	111 (S)

Table 9 (Continued)

Phenobarbital-treated Worms	30 °C	15 °C	% change & statistical significance*
PC (nmole)/worm	7.43 ± 0.65	45.40 ± 3.69	511 (S)
% PE of microsomal total phospholipids	10.20 ± 1.50	8 ± 1.29	− 21.5
% PC of microsomal total phospholipids	10.71 ± 0.95	23 ± 3	114.7
PE/PC	≈ 1.0	0.35	− 65

The above data which represent the mean values from four experiments were obtained after two dimensional TLC of midgut microsomal total lipid and phosphorous analysis of PE and PC spots according to the method of Rouser et al. *Statistical significance of the differences between the mean values was verified after two-sample t testing at a = 0.05

(S): The difference is statistically significant.
(N): The difference is not statistically significant.

Table 10: Effect of Chemical Inducers Pentamethylbenzene and Phenobarbital on Midgut Microsomal Total Phospholipids and PE/PC Ratio in Armyworms Raised at 30 °C

Armyworms raised at 30 °C	Control Worms	PMB-Treated Worms	% change & statistical significance*	Phenobarbital-treated worms	% change & statistical significance*
microsomal total phosphorous (nmole) / microsomal protein (mg)	339 ± 24.50	311 ± 13.31	-8.2 (N)	283 ± 12	-16.5 (N)
microsomal total phosphorous (nmole) / microsomal total lipid (mg)	607.70 ± 35.50	628 ± 34	3.3 (N)	498 ± 33.30	-18 (N)
microsomal total phosphorous (nmole) / worm	74.70 ± 8.41	121.8 ± 17.2	63 (S)	68.91 ± 5.11	- 7.7 (N)
microsomal total phosphorous (nmole) / gram wet weight of worm	162 ± 34.00	291.81 ± 55	80.1 (S)	166.82 ± 10	2.9 (N)
% microsomal total phosphorous of microsomal total lipid	68.51 ± 0.51	70 ± 1	2.1 (N)	80 ± 3	16.7 (N)
PE (nmole)/microsomal protein (mg)	16.10 ± 2.66	19.41 ± 1.21	20.5 (N)	30.70 ± 5.82	90.6 (S)
PC (nmole)/microsomal protein (mg)	32 ± 6.22	23.60 ± 1.28	-26.2 (S)	30.3 ± 1.24	- 5.3 (N)
PE (nmole)/microsomal total lipid (mg)	28.66 ± 3.51	39.41 ± 3.42	37.5 (S)	53.11 ± 4.28	85.3 (S)
PC (nmole)/microsomal total lipid (mg)	56.52 ± 7.38	47 ± 12	-16.8 (N)	53.95 ± 10	-4.5 (N)
PE (nmole)/gram wet weight of worm	7.20 ± 2.01	18.42 ± 3.97	155.8 (S)	15 ± 3.11	108.3 (S)

Table 10 (Continued)

Armyworms raised at 30 °C	Control Worms	PMB-treated Worms	% change & statistical significance*	Phenobarbital-treated worms	% change & statistical significance*
PC (nmole)/gram wet weight of worm	15.31 ± 4.55	22 ± 3.53	43.6 (S)	18 ± 1.70	17.5 (N)
PE (nmole)/worm	3.55 ± 0.61	7.94 + 1.34	123.6 (S)	7.51 ± 1.62	111.5 (S)
PC (nmole)/worm	7.04 ± 1.54	9.20 ± 1.02	30.6 (S)	7.43 ± 0.65	5.5 (N)
% PE of microsomal total phosopholipids	4.40 ± 0.62	6.10 ± 0.34	38.6	10.20 ± 1.50	131.8
% PC of microsomal total phospholipids	9 ± 1.10	7.51 ± 0.25	-16.5	10.71 ± 0.95	19
PE/PC	0.50	0.83	66	≈1.0	100

The above data which represent the mean values from four experiments were obtained after two dimensional TLC of midgut microsomal total lipid and phosphorous analysis of PE and PC spots according to the method of Rouser et al. *Statistical significance of the differences between the mean values was verified after two-sample t testing at a = 0.05.

(S): The difference is statistically significant.
(N): The difference is not statistically significant.

Table II: Effect of Chemical Inducers Pentamethylbenzene and Phenobarbital on Midgut Microsomal Total Phospholipids and PE/PC Ratio in Armyworms Raised at 15 °C

Armyworms raised at 15 °C	Control Worms	PMB-treated worms	% change & statistical significance	Phenobarbital-treated worms	% change & statistical significance*
microsomal total phosphorous (nmole) / microsomal protein (mg)	362 ± 35.81	328 ± 13.95	-9.3 (S)	364 ± 27.78	0.5 (N)
microsomal total phosphorous (nmole) / microsomal total lipid (mg)	541.4 ± 38.83	465.21 ± 30.69	-14 (N)	551.12 ± 30.20	1.7 (N)
microsomal total phosphorous (nmole) / worm	142.40 ± 15.11	244.52 ± 17	71.7 (S)	186.51 ± 17.41	30.9 (S)
microsomal total phosphorous (nmole) / gram wet weight of worm	224 ± 20.92	348.20 ± 27	55.4 (S)	263.10 ± 21.34	17.4 (N)
% microsomal total phosphorous of microsomal total lipid	75 ± 1.58	78 ± 8	4 (N)	84 ± 2.55	12 (N)
PE (nmole)/microsomal protein (mg)	21 ± 2.85	24.61 ± 1.31	17.1 (N)	30.72 ± 2	46.2 (S)
PC (nmole)/microsomal protein (mg)	61.15 ± 7.39	61.32 ± 9	1.9 (N)	89 ± 9.97	45.5 (S)
PE (nmole)/microsomal total lipid (mg)	32 ± 2.95	34.89 ± 3.24	9 (N)	46.49 ± 6.42	45.2 (S)
PC (nmole)/microsomal total lipid (mg)	94.40 ± 19.20	88.81 ± 17.20	-5.9 (N)	133.50 ± 20	41.4 (S)
PE (nmole)/gram wet weight of worm	12.96 ± 1.22	26 ± 0.78	100.6 (S)	23.82 ± 3	83.7 (S)

Table 11 (Continued)

Armyworms raised at 15 °C	Control Worms	PMB-treated worms	% change & statistical significance*	Phenobarbital-treated worms	% change & statistical significance*
PC (nmole)/gram wet weight of worm	38.11 ± 7.48	65 ± 0.21	70.5 (S)	68 ± 6.81	78.4 (S)
PE (nmole)/worm	8.26 ± 1.05	18.31 ± 1.12	121.6 (S)	15.85 ± 1.37	91.8 (S)
PC (nmole)/worm	23.99 ± 2.81	46 ± 2.61	100 (S)	45.40 ± 3.69	89.2 (S)
% PE of microsomal total phospholipids	5.60 ± 0.48	7.50 ± 0.57	33.9	8 ± 1.29	42.8
% PC of microsomal total phospholipids	16.72 ± 3.10	18.72 ± 2.50	11.9	23 ± 3	37.5
PE/PC	0.34	0.40	17.6	0.35	2.9

The above data which represent the mean values from four experiments were obtained after two dimensional TLC of midgut microsomal total lipid and phosphorous analysis of PE and PC spots according to the method of Rouser et al. *Statistical significance of the differences between the mean values was verified after two-sample t testing at a = 0.05.

(S): The difference is statistically significant.
(N): The difference is not statistically significant.

The PE/PC ratio increased in the presence of pentamethylbenzene but did not change when the worms were treated with phenobarbital. At 30 °C, the inductive effect of PMB, although not as strong as at 15 °C, still was significant. Phenobarbital, on the other hand, did not cause any substantial induction at 30 °C. At this temperature, PE/PC ratio increased in the presence of both chemical inducers.

Effect of Temperature on Fatty Acyl Composition of Midgut Microsomal Total Phospholipids

The effect of temperature on fatty acyl composition of midgut microsomal total phospholipids is shown in Table 12. Generally speaking, there was a tendency toward higher levels of unsaturated fatty acids when armyworms were raised at lower temperature (15 °C). An exceptional case however, was observed for palmitoleic acid which represented a higher concentration at high temperature (30 °C).

Table 12: Effect of Temperature on Fatty Acyl Composition of Midgut Microsomal Total Phospholipids

Fatty acid	Retention times (min) of methyl esters of fatty acids			% relative number of moles		% Change
	Standard[a]	Sample (15 °C)[b]	Sample (30 °C)[b]	15 °C[b]	30 °C[b]	
C14:0 Myristic	1.100 ± 0.06	1.095 ± 0.01	1.080 ± 0.04	2.25 ± 0.01	2.80 ± 0.01	-19.64
C16:0 Palmitic	1.820 ± 0.05	1.790 ± 0.10	1.760 ± 0.04	10.47 ± 0.21	13.31 ± 0.08	-21.33
C16:1 Palmitoleic	2.244 ± 0.10	2.240 ± 0.07	2.230 ± 0.03	4.27 ± 0.03	5.08 ± 0.01	-15.94
C18:0 Stearic	----	3.040 ± 0.08	2.980 ± 0.06	36.24 ± 3	41.50 ± 4.10	-12.67
C18:1 Oleic	3.472 ± 0.10	3.520 ± 0.04	3.400 ± 0.03	18.74 ± 0.24	14.93 ± 0.20	25.50
C18:2 Linoleic	3.860 ± 0.13	4.160 ± 0.04	4.080 ± 0.05	27.78 ± 2.10	22.57 ± 2	23

a: Mean values from 3 runs
b: Mean values from 2 experiments

CHAPTER IV

DISCUSSION

The fully grown larvae at high temperature (30 °C), although representing higher feeding activity throughout three days incubation period, achieved smaller size compared to those raised at low temperature (15 °C) for longer periods of time (7-9 days). At the end of the incubation period, the larvae raised at 30 °C usually weighed from 0.35 to 0.45 g, whereas those raised at 15 °C achieved a weight of 0.5 - 0.6 g. It seemed therefore, that the larger size of armyworms at 15 °C had been caused by longer incubation periods and was not related to the feeding habits at two different experimental conditions. Such differences in the larval size were clearly reflected in the amounts of the midgut microsomal total lipid as well as microsomal total protein as shown in Tables 1-3. It is seen that when the armyworms were raised at 15 °C, the amount of midgut microsomal protein per worm increased significantly under all three experimental conditions (control, PMB and phenobarbital). Such increase was as low as 72.5% for PMB-treated worms and as high as 99.6% for phenobarbital-treated worms. In control worms, a substantial increase of 73.3% was observed. This temperature-caused induction was even higher for midgut microsomal total lipid and values of 80.4, 102.8, and 137.5% increase were obtained in control, PMB and phenobarbital treated worms, respectively. Due to the fact that the proliferation of the smooth endoplasmic reticulum necessitates an increase both in lipid and protein contents of the membranous

structure, the ratio microsomal total lipid (mg)/microsomal protein (mg) did not vary significantly at 15 °C and 30 °C as shown in Tables 1-3. The cytochrome P-450 activity was also significantly induced at low temperature not only in control worms, but also in PMB and phenobarbital-treated worms. This induction expressed as nmoles of cytochrome P-450 per mg microsomal protein was 51.6% for control, 93.8% for PMB-treated and 184.6% for phenobarbital-treated worms. Due to the fact that both microsomal protein and cytochrome P-450 activity were induced at low temperature the presented values of 51.6, 93.8 and 184.6, although seem to be significant, may still not well represent the strong inductive effect of the low temperature. Another approach was then to present the data in terms of cytochrome P-450 (nmole)/worm instead of cytochrome P-450 (nmole)/mg microsomal protein. In this way, values of 161.4, 229.2 and 472% increase were obtained for control, PMB and phenobarbital-treated worms. The differential induction (induction not related to the growth) of microsomal total protein, microsomal total lipid and cytochrome P-450 activity expressed as the amounts of these three parameters per gram wet weight of worms appears in Tables 1-3 and 13. All three parameters showed significant differential inductions at the low temperature.

In the measurement of cytochrome concentration which was done primarily by measuring the absorbance difference between 490 nm and the peak at λ max, we assumed the same value for the molar extinction coefficient of the cytochrome (91 cm^{-1} mM^{-1}) although this value for the molar extinction coefficient of cytochrome P-450

Table 13: Effect of Temperature on Midgut Microsomal Total proteins and Cytochrome P-450 in Control, PMB-Treated and Phenobarbital-Treated Worms

Control Worms	30 °C	15 °C	% change & statistical significance*
cytochrome P-450 (nmole)/gram wet weight of worm	0.14 ± 0.04	0.26 ± 0.05	85.7 (S)
microsomal protein (mg)/gram wet weight of worm	0.49 ± 0.07	0.62 ± 0.08	26.5 (S)
PMB-treated worms	30 °C	15 °C	———
cytochrome P-450 (nmole)/gram wet weight of worm	0.60 ± 0.12	1.28 ± 0.11	113.3 (S)
microsomal protein (mg)/gram wet weight of worm	0.94 ± 0.17	1.02 ± 0.09	8.5 (N)
phenobarbital-treated worms	30 °C	15 °C	———
cytochrome P-450 (nmole)/gram wet weight of worm	0.16 ± 0.03	0.6 ± 0.11	275 (S)
microsomal protein (mg)/gram wet weight of worm	0.60 ± 0.04	0.77 ± 0.13	28.3 (S)

The above data represent the mean values obtained from six experiments.
*Statistical significance of the differences between the mean values was verified after two-sample t testing at a = 0.05.

(S): The difference is statistically significant.
(N): The difference is not statistically significant.

was obtained with cytochrome isolated from rabbit liver. It has become a common practice to employ this extinction coefficient for all forms of the cytochrome not readily available in pure form. Careful measurements of the cytochrome from the armyworm midgut showed a λ max of 449.8 $\pm$ 0.4 nm with no significant differences discernable between tissues from the different treatments (Brattsten, personal communication).

The induction of midgut microsomal total lipid and cytochrome P-450 activity caused by the low temperature did not occur at the same rate as a significant difference existed between the values of microsomal total lipid (mg)/cytochrome P-450 (nmole) at two temperatures in control as well as in PMB and phenobarbital-treated worms. The possible mechanisms of the temperature-caused induction of cytochrome P-450 will be discussed later.

The presence of chemical inducers pentamethylbenzene and phenobarbital in the diet, although had strong inductive effects on midgut microsomal protein, microsomal total lipid and cytochrome P-450 activity, did not cause any change in the larval size either at 15 °C or at 30 °C as compared to the size of control worms. The data presented in Table 4 indicates that the induction of midgut protein, microsomal total lipid and cytochrome P-450 activity at 15 °C by chemical inducers did not take place at the same rate and was somehow weaker for phenobarbital. In the presence of pentamethylbenzene, the amounts of microsomal protein and microsomal total lipid per worm increased by 79.4 and 108.3%, respectively; whereas phenobarbital caused only 37.5% increase for microsomal

total lipid. With respect to the induction of cytochrome P-450 activity, the induction caused by pentamethylbenzene was almost three times stronger than that caused by phenobarbital (the amount of cytochrome P-450 per worm showed an increase of 443.7% in PMB-treated worms whereas phenobarbital did not cause more than 137.5% change). At 30 °C, the inductive effect of phenobarbital became even less conspicuous and was not statistically significant as shown in Table 5. Since phenobarbital is generally known as a potent chemical inducer of cytochrome P-450 among mammals, the weak inductive effect of this compound in armyworms might suggest that the mechanism by which the MFO system is induced by phenobarbital is different in mammals and insects. At high temperature (30 °C) the induction of cytochrome P-450 activity by pentamethylbenzene although is quite significant (333.3% increase), yet shows a drop compared to its induction by the same chemical inducer at 15 °C (443.7%). It seems therefore, that the increase in temperature somehow affects the ability of the microsomal oxidase system to become induced.

The phospholipid analysis of midgut microsomal fraction by radioisotope labeling method indicates that phosphatidylcholine is the predominant phospholipid in this subcellular fraction and phosphatidylethanolamine is present in relatively smaller quantity (Table 6). Phosphatidylcholine comprised 35.95 percent of the total microsomal phospholipids whereas phosphatidylethanolamine contributed only 18.45%. The data presented here are comparable to those reported by Glaumann and Dallner (13) for phospholipid

composition of microsomal fraction in rat liver (47.5 and 19.0% for PC and PE, respectively), but contrast with the data given by Khan and Hodgson (31) who studied the phospholipid composition of the microsomal fraction in housefly. According to these workers, phosphatidylethanolamine was the predominant phospholipid in the microsomal fraction comprising 37.8% of the total phospholipid whereas the amount contributed by phosphatidylcholine was only 27.7%. Knowing the fact that the armyworm larvae will transform into a moth (lepidoptera) and the housefly is a member of diptera, it seems that the phospholipid composition of the microsomal fraction follows the pattern of the phospholipids in the whole insect, that is, largest amount of PC in lepidoptera and largest amount of PE in diptera (Page 9). When the armyworm larvae were exposed to pentamethylbenzene at the room temperature, a drastic change in PE/PC ratio occurred and an increase of 90% was observed. In the presence of PMB, both phosphatidylcholine and phosphatidylethanolamine contributed almost equally to the microsomal total phospholipids (26.83% PE and 28.3% PC). It was concluded that pentamethylbenzene affected the turnover rate of either one or both microsomal phospholipids. The data obtained from the quantitative analysis of microsomal phospholipids by radioisotope labeling method have limited usefulness for two reasons. First, since the experiments were performed at the room temperature, it is not safe to compare these data with those obtained from the experiments carried out at different temperatures (15 °C and 30 °C) by phosphorous analysis method. The second drawback arises from the fact that when the

armyworms were killed 4 or 5 hours after labeling with ^{32}P, the rate of phospholipid biosynthesis was higher than the rate of its breakdown (as shown in Figure 6). Therefore, the quantitative analysis of the phospholipids labeling during this period does not represent the amounts of phospholipids achieved at the steady state where the rate of synthesis is equal to the rate of breakdown. As it is shown in Figure 6, the steady state was achieved approximately 13 hours after labeling.

The total phosphorous analysis of the microsomal fraction at 15 °C indicated that phospholipids comprised approximately 75% of the microsomal total lipid (Table 7). Although this value is somehow smaller than the value obtained for the microsomal fraction of mammalian liver tissue (80-90%) (13), yet, quantitatively is quite significant and shows the importance of phospholipids in the structural organization of the microsomal membranes. The shift in temperature from 30 °C to 15 °C caused a significant induction of microsomal total phospholipids as expressed in terms of nmole microsomal total phosphorous per worm. This induction amounted to 90.6% for control, 100.7% for PMB and 170.6% for phenobarbital-treated worms (Tables 7-9). Despite the substantial induction of the microsomal total phospholipids the percentage of phospholipids in microsomal total lipid remained the same at the two temperatures under all three experimental conditions and changes of 9.4, 11.4 and 5% were shown to be statistically not significant. This observation demonstrated that the induction of microsomal lipids include both polar and non-polar species. As it was expected, the low temperature caused a significant induction of both phosphatidylethanolamine

and phosphatidylcholine as the amount of these phospholipids were expressed in terms of nmole PE or PC per worm. The interesting observation made at this point was that the induction of PC occurred at a higher level than than of PE and this gave rise to smaller values of PE/PC ratio at low temperature as seen in Tables 7-9. It was therefore concluded that low temperature exerts its inductive effect on PE/PC ratio in a different manner than does pentamethylbenzene; that is, one decreases the ratio whereas the other increases. The chemical inducer pentamethylbenzene caused an induction of 63% of microsomal total phospholipids per worm at 30 °C (Table 10) and an induction of 71.7% at 15 °C (Table 11). The 30.9% induction caused by phenobarbital at 15 °C, is statistically significant, although totally abolished at 30 °C. It was concluded that the shift in temperature had somehow modified the properties of these chemicals in inducing midgut microsomal phospholipids, a phenomenon observed previously with respect to the induction of cytochrome P-450 activity by such inducers. Here again, despite the induction of the microsomal total phospholipids, the percentage of the phospholipids in the microsomal total lipid did not vary significantly, indicating a parallel induction of both polar and non-polar lipid species. Pentamethylbenzene induced phosphatidyl-ethanolamine as much as 123.6% at 30 °C whereas phosphatidylcholine was induced only by 30.6%. Therefore, induction of PE at a higher rate compared to that of PC caused a higher value for PE/PC, 0.83, for PMB-treated worms (PE/PC = 0.5 for control worms).

The induction of cytochrome P-450 activity by chemical inducers

pentamethylbenzene and phenobarbital which took place along with the proliferation of the smooth endoplasmic reticulum (i.e. higher amounts of microsomal total lipid and microsomal protein), was considered as a defensive adaptation mechanism developed by armyworms in response to the presence of the toxic chemical.

The fatty acid analysis of the microsomal total phospholipids showed a decrease in concentrations of four fatty acids, myristic, palmitic, stearic and palmitoleic when the temperature was shifted from 30 °C to 15 °C. At low temperature, however, the concentrations of two unsaturated fatty acids, namely, oleic and linoleic showed substantial increases. The standard methyl esters of fatty acids purchased from Supelco, did not contain methyl palmitoleate and identification of the sample peaks was done not only by comparing their retention times with those of standards, but also through the supplementary chromatograms provided by the supplier company.

A strict interrelationship between the temperature of the medium and fatty acid composition as well as the physical state of the lipids has been repeatedly demonstrated in investigations of cells from different origins (39-43). A possible mechanism which explains the higher content of unsaturated fatty acids in the membrane at reduced temperature is that low temperature activates the enzymes that desaturate fatty acids and introduce these acids into phospholipids. Such observation has been made previously by Okuyama et. al. using E. coli cell cultures (44). Since the kinetic and thermodynamic properties of some of the membrane bound enzymes are influenced by the level of saturation of fatty acyl residues of phospholipids (8), this tendency toward higher levels of unsatura-

tion at low temperature is considered as having adaptive values for the armyworm.

To explain how the low temperature induces higher amounts of cytochrome P-450 protein in the endoplasmic reticulum, one should notice that the appearance of active cytochrome P-450 protein in the membranes is preceded by a multistep sequence, beginning with the transcription of its specific mRNA, formation of the macromolecule (translation) and its final incorporation into the endoplasmic reticulum membranes. The low temperature might cause induction at any of these three steps and to know precisely which particular step undergoes the induction requires further investigation. Rate limiting changes may also result from low ambient temperature in the sequence of events leading to heme synthesis and its attachment to the protein portion of the cytochrome. Other possible mechanisms include changes in the half-life or the synthesis rate of the flavoprotein reductase, as well as in the membrane phospholipid metabolism. Recently Evdokimova et. al. have studied the effect of low (20 °C) and high (37 °C) temperatures on alkaline phosphatase synthesis (a cytoplasmic membrane associated enzyme) in bacterial cells (45). They showed that the low temperature induced the enzyme synthesis by exerting its inductive effect at the level of transcription. These investigators have ascribed the higher content of the mRNA to either higher rate of its synthesis or its more stable character at this temperature on account of the greater activity of nucleases at high temperature (37 °C). The stability of the mRNA as well as the rate of its synthesis are

important factors in determining the level of protein synthesis.

Since the binding of chemical inducers pentamethylbenzene and phenobarbital (type I substrates) to the MFO system is through hydrophobic interactions, we suggest that the modified inductive properties of these chemicals caused by the low temperature might be related to the changes occurred at the hydrophobic regions of the microsomal membranes at that temperature.

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