THE <u>IN</u> <u>VITRO</u> HYDROXYLATION
OF NAPHTHALENE-1-C^{14} BY HOUSEFLY MICROSOMES

by

ROBERT ORTIZ ARIAS

A THESIS

submitted to

OREGON STATE UNIVERSITY

in partial fulfillment of
the requirements for the
degree of

DOCTOR OF PHILOSOPHY

June 1962

APPROVED:

███████████████████████████

Professor of Insect Toxicology and Biochemistry

In Charge of Major

███████████████

Chairman of Department of Entomology

████████████████████

Chairman of School Graduate Committee

████████████████

Dean of Graduate School

Date thesis is presented   August 14, 1961

Typed by Eloise Allison

ACKNOWLEDGMENT

The author wishes to express his appreciation to Dr. Paul O. Ritcher, Chairman, Department of Entomology, Oregon State University, for the research fellowship which made this investigation possible. The author is especially indebted to Dr. Leon C. Terriere, Professor of Insect Toxicology and Biochemistry, Oregon State University, under whose advice the work was completed, and for his patience and constructive criticisms in the preparation of this dissertation.

The author also wishes to express his sincere appreciation to Dr. Leman F. Remmert, Professor of Biochemistry, Oregon State University, who made available equipment for the research and for his invaluable advice during the investigation.

Acknowledgments are gratefully made to the following persons for assistance and encouragement during the experimental work and in the preparation of this thesis: Robert D. Schonbrod, Research Chemist; Richard B. Boose, Research Chemist; Allen O'Berg, Graduate Student; and Mrs. Myrna Smith, Laboratory Technician, all of the Department of Entomology, Oregon State University. The author is also very grateful to Dr. Kenneth R. Hobbs, Professor of Entomology, California State Polytechnic College, for his unselfish concern and sincere encouragement throughout

the entire period of study.

The author wishes to express his gratitude to his wife, Rachel T. Arias, for her kindness, understanding and encouragement throughout the preparation of this investigation.

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Each of us....at the outset of his or her individ-
ual life story is microscopic and one sole cell. By
that cell's multiplication and by its descendants'
coherence, each of us attains....final form and size.
The doings of this cell-assembly are....those of a being
which is....a unity. Yet each of its constituent cells
is a life centered in itself....managing itself, feeding
and breathing for itself, separately born and destined
separately to die. Further, it is a life help by and in
its turn helping the whole assembly, which later is the
corporate individual.

                                        Sir Charles Sherrington

THE IN VITRO HYDROXYLATION
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INTRODUCTION

Studies on the metabolism of foreign organic com-
pounds in mammals have been carried on for nearly one hun-
dred years and considerable information has accumulated on
these degradative processes. Until the development of
insecticide resistance such studies with insects have been
very limited. Resistance brought on by the widespread use
of DDT and other synthetic insecticides called attention
to the lack of information on the biochemistry of these
organisms and thus stimulated a tremendous increase in
research in this area. Early studies of insecticide re-
sistance resulted in relatively gross explanations of this
phenomenon based on physiological, morphological, or behav-
ioristic differences between susceptible and resistant
strains.

Out of these observations and from the information
previously obtained from vertebrates has developed the
concept that resistance may be due to biochemical modifica-
tion of the toxic substances. Many laboratories are now
engaged in studying the metabolism of insecticides by
resistant and tolerant insects, and the role of metabolism
in the resistance phenomenon seems to be well accepted.
The end result of these metabolic processes is to produce

a water-soluble product which can be excreted.

Most synthetic insecticides are insoluble in water and must, therefore, be converted to water soluble forms before they can be eliminated from the insect body. This feat is accomplished in insects by several mechanisms, some of which may involve the introduction of a hydroxyl group into the aromatic portions of the molecule. In vivo studies have shown that houseflies have the capacity to hydroxylate naphthalene and to further metabolize these hydroxylated products by conjugating them with glucuronic acid, acetylated cysteine, and sulphate. The discovery that a subcellular particulate fraction of vertebrate liver tissue is capable of the in vitro hydroxylation of naphthalene has suggested that similar activity may be found in insect cell particles. A key reaction in the metabolism of foreign aromatic compounds would then be available for further study.

The purpose of this investigation was to determine if a microsomal fraction of houseflies was capable of hydroxylating aromatic hydrocarbons such as naphthalene-1-C^{14}. If this fraction could be shown to possess such activity a further objective was to determine the optimum conditions for its use in toxicological studies.

This dissertation presents the results of experiments

conducted during the investigation of this problem and discusses the toxicological implications of the knowledge gained. It is hoped that the ideas presented will prove stimulating to other workers and that they will provide a basis for further investigations into insect biochemistry and the phenomenon of resistance to insecticides.

HISTORICAL REVIEW

Foreign organic compounds administered to living
organisms usually undergo a number of chemical changes
resulting in the detoxication of the compound. The first
evidence of detoxication was found in 1842 (96, p. 10)
when Keller discovered that cows were able to synthesize
hippuric acid from injested benzoic acid by conjugation
with the amino acid glycine. In 1854, (34, p. 18) Davy
found that caterpillars were also capable of forming
hippuric acid from ingested benzoic acid. The next de-
fense mechanism to be discovered was the oxidation of
benzene to phenol, which was then conjugated with sulfuric
acid to produce a conjugate known as an etheral sulphate
(96, p. 11). Today the scientific literature contains
thousands of reports of investigations on metabolism of
drugs, natural products, and pesticides covering the field
of medicine, biochemistry, and insect toxicology. This
review will include only those papers necessary to estab-
lish background and those closely related to the present
study.

For convenience the review will be presented in two
parts: the first will be a brief review of the known
detoxication mechanism in insects, and secondly, a review
of the metabolism of naphthalene and its derivatives.

The latter part will be concerned principally with the initial metabolic steps in naphthalene degradation.

DETOXICATION MECHANISMS IN INSECTS

Oxidation:

Oxidation is one of the most general reactions of foreign compounds within the insect body. When such a compound cannot be directly conjugated, it must be modified to provide an active site for conjugation. This may be accomplished by several reactions.

Aliphatic oxidation: The oxidation of organic phosphate insecticides by insects has been well established. An example of this is the conversion of parathion to paraoxon. Such enzymatic oxidation has also been shown to occur with methyl-parathion, malation, EPN, and other phosphate insecticides (60, p. 63-74). The over-all reaction may be summarized as follows:

$$RO-\overset{\overset{\textstyle S}{\|}}{\underset{\underset{\textstyle OR}{|}}{P}}-OR \xrightarrow{\ (O)\ } RO-\overset{\overset{\textstyle O}{\|}}{\underset{\underset{\textstyle OR}{|}}{P}}-OR$$
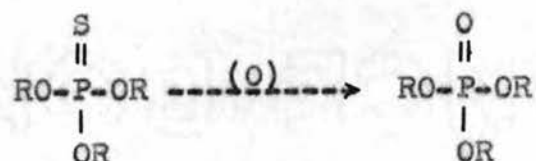
An exception to this is the oxidation of pyrophosphoramide insecticides which are modified by the addition of an oxygen atom to the amide nitrogen.

These oxidations may not be considered as detoxication

processes since the products are more toxic than their parent compounds. The products, however, are more susceptible to hydrolysis by esterases, to produce non-toxic derivatives. In the over-all sense, therefore, this type of oxidation can be considered as a detoxication process.

Epoxidation: The discovery that heptachlor is metabolized to heptachlor-epoxide by dogs has revealed a new and interesting reaction in the detoxication of insecticides. This over-all reaction amounts to the addition of an oxygen atom to a double bond. The epoxidation of heptachlor has also been shown in houseflies (72, p. 346-351). Similarly, cockroaches have been found to convert aldrin to dieldrin (41, p. 588-592), and isodrin to endrin (62, p. 128). Evidence that these epoxides are further metabolized has not been reported.

Hydroxylation: A probable primary reaction in the degradation of foreign compounds is the hydroxylation of aromatic rings. The hydroxylated product can then be eliminated in the free forms or as conjugates of glucuronic acid, glucose, acetylated cysteine or sulfuric acid. One of the few insecticides to be studied in this regard was phenothiazine, whose hydroxylated product was found by Zukel (101, p. 796-866) to be leucothionol. Kikal has also demonstrated that locusts can hydroxylate

chlorobenzene to chlorophenols. Terriere and co-workers
(90, p. 620-623) found that houseflies converted naphtha-
lene to 1:2-dihydro-1:2-dihydroxynaphthalene, 1-naphthol
and their conjugates. The hydroxylated intermediates are
generally less toxic than the parent compound, as Topposada
(92, p. 121) has shown in comparing 1-naphthol to naphtha-
lene in houseflies.

Insects have been found to metabolize DDT to several
unknown products (84, p. 214-219; 51, p. 167-172; 88, p.
829-837; 22, p. 699; 89, p. 736-739; 45, p. 151-156).
The fact that some of these products are water soluble
has led to the suggestion that they are conjugates of
hydroxylated DDT derivatives (89, p. 736-739). Tsukamoto
(93, p. 141-151) obtained evidence for another version of
hydroxylation when he found that Drosophila melanogaster
could hydroxylate DDT at the tertiary carbon to produce a
kelthane-like product. Menzel and co-workers (60, p. 9-12)
later confirmed that this hydroxylated DDT metabolite was
kelthane. Agosin and co-workers (1, p. 340-342) have
shown that housefly and cockroach microsomes can hydrox-
ylate DDT to a kelthane-like product. These authors also
obtained evidence by means of infra-red spectroscopy, that
ring hydroxylation may have occurred.

Conjugation:

Several reports in the literature indicate that insects possess a variety of conjugation mechanisms. For example, the compound, 6-amino-4-nitro-o-cresol, which is a possible metabolite of the insecticide, dinitro-o-cresol, has been shown to be acetylated by locusts (66, p. 498-503). Uyeda (94, p. 24) found that houseflies produced hippuric acid from ingestion of benzoic acid and that the most important reaction in the detoxication of phenylacetic acid was its conjugation with glycine. The larvae of Aedes aegypti have been shown (24, p. 216-221) to produce less toxic glycine conjugates from several organic acids. According to Friedler and Smith (40, p. 396-400), p-aminobenzoic acid was transformed to p-aminobenzoyl glycine by Locusta migratoria.

Myers and Smith (67, p. 498-503) reported glucoside formation after injecting locusts with three different phenols. Smith (83, p. 435-437) studying the conversion of phenolic compounds in 15 species of insects found that they were conjugated as glucosides and ethereal sulphates, rather than glucosiduronides. From these results, he concluded that insects differ biochemically from other species. Terriere and co-workers (90, p. 620-623), however, have obtained evidence which indicates that houseflies can

conjugate naphthalene and 1-naphthol with glucuronic acid.

A conjugate of some type was found by Butts and co-workers (22, p. 699) when _Periplaneta americana_ was treated with labeled DDT and also by Terriere and Schonbrod (89, p. 736-739) in their studies with DDT metabolism by houseflies. The former authors also found that this conjugation was much higher at 35° than at 25°C. This observation is quite interesting in view of the discovery that many insects are more resistant to DDT at higher temperatures. Smith (82, p. 460) suggested that the material found by Butts and co-workers was a conjugate of hydroxylated DDT.

Dehydrochlorination:

Despite the tremendous amount of work that has been done with DDT and its analogues, the mode of action of these compounds is still unknown. On the other hand, the studies on the detoxication of these insecticides, have met with more success. The _in vivo_ conversion of DDT to a nontoxic dehydrochlorinated metabolite, DDE, by houseflies was first demonstrated by Sternberg and co-workers (84, p. 214-219) and Perry and Hoskins (69, p. 600-601). Numerous insects have now been shown to possess this same dehydrochloronation mechanism (73, 119-137). Sternberg

and co-workers (85, p. 513-519) were successful in isolat-
ing the enzyme, DDT-dehydrochlorinase, which catalyzes
the dehydrochlorination of DDT in vitro. The isolation
of this enzyme has provided conclusive evidence that the
conversion of DDT to DDE is the major detoxication mechan-
ism utilized by some resistant insects. Perry (71, p. 130)
has isolated another enzyme which catalizes the breakdown
of DDT.

Sternburg and Kearns (86, p. 548-552) found that
lindane was dehydrochlorinated to a compound identified as
pentachloro-cyclohexane. However, this compound has been
considered as an intermediate in the degradative processes,
since it is further metabolized to unidentified products.
These authors also found that DDT-dehydrochlorinase was
not involved in the detoxication of lindane. Another
step in the elucidation of the metabolic pathway of benzene
hexachloride was made when Bradbury and Standen (19, p.
983-989) found that alkaline hydrolysis of its excreted
metabolites produced dichlorothiophenols.

Hydrolysis:

Insect detoxication of organophosphorus insecticides
occurs primarily through phosphoryl hydrolysis. With
dialkyl aryl phosphorothioates this hydrolysis may occur

at the alkyl phosphate group and with phosphorodithioates
and phosphorothiolates, the phosphoryl hydrolysis occur
primarily by P —⦂— S-C cleavage (25, p. 216-238). Accord-
ing to Metcalf et al (63, p. 274-279) the hydrolytic break-
down of parathion and paraoxon is due to an aromatic esterase.

THE METABOLISM OF NAPHTHALENE IN VERTEBRATES AND INSECTS:

One of the earliest studies on naphthalene metabolism
was in 1877 when Beaumont and Herter observed that when
the acidified urine of animals dosed with naphthalene was
heated, it yielded naphthalene. Lesnic in 1888 treated a
dog with naphthalene and identified a product as l-naphthyl
glucuronide (99, p. 27-39).

More recent work with vertebrates has revealed that
naphthalene is converted to seven different conjugates
(17, p. 175-181; 16, p. 440-446; 14, p. 679-683; 31, p.
132-140). These are known chemically as: 1:2-dihydro-2-
hydroxy-l-naphthyl glucosiduronic acid, l-naphthyl gluco-
siduronic acid, 1:2-dihydro-l-naphthyl glucosiduronic
acid, l-naphthyl-2-hydroxy sulfuric acid, l-naphthyl
sulfuric acid, N-acetyl-S-(1:2-dihydro-2-hydroxy naphthyl)
cysteine, and N-acetyl-S-(l-naphthyl) cysteine. Houseflies
have been shown to metabolize naphthalene to the same
seven conjugates (90, p. 620-623).

Hydroxylated Metabolic Products:

1-naphthol, 2-naphthol and 1:2-dihydroxynaphthalene:
Rabbits dosed with naphthalene were found to produce free
1-naphthol (46, p. 467-487), while Corner and Young (30,
p. 647-655) found both 1-naphthol and 2-naphthol in the
urine of rats, rabbits, guinea pigs and mice when they were
treated with naphthalene. Boyland and Wiltshire (13, p.
636-641) and Corner and Young (31, p. 132-140) found that
rats and rabbits would produce 1-naphthol when treated
with 1:2-dihydro-1:2-dihydroxynaphthalene. These authors
could not detect any 1:2-dihydro-1:2-dihydroxynaphthalene
when the animals were treated with 1-naphthol, which led
them to suggest that the dihydrodiol was a precursor of
1-naphthol, but that the reverse was very unlikely.
Terriere and co-workers (90, p. 620-623) found that house-
flies, treated with 1-naphthol would produce 1:2-dihydro-
1:2-dihydroxynaphthalene and obtained indirect evidence
that the dihydrodiol was produced by rats.

Recently, Booth and Boyland (8, p. 73-78; 9, p. 681-
688) found that the microsomal fraction of rat liver, in
the presence of TPN, TPNH-generating system, and nicotin-
amide would convert naphthalene to both 1-naphthol and
1:2-dihydro-1:2-dihydroxynaphthalene. The same system
was unable to convert the dihydrodiol to 1-naphthol.

They suggested that 1:2-dihydro-1:2-dihydroxynaphthalene was an unlikely intermediate in the formation of 1-naphthol from naphthalene.

Although 2-naphthol has been isolated as a naphthalene metabolite, it is not excreted in the conjugated form (30, p. 647-655). However, when rats were treated with 2-naphthol, its conjugates were isolated (5, p. 165-169). Another hydroxylated metabolite was discovered when Corner and Young (30, p. 647-655) isolated 1:2-dihydroxynaphthalene from urine of guinea pigs treated with naphthalene.

1:2-dihydro-1:2-dihydroxynaphthalene: This metabolic product has been found consistently in the urine of rats, guinea pigs, rabbits, and mice (30, p. 647-655; 14, p. 679-682; 80, p. 389-395; 18, p. 376-384) and also in the excreta of flies (90, p. 620-623) when treated with naphthalene. Different species have been found to produce different optical forms of this metabolite. Young (98, p. 417-422) isolated a laevorotatory isomer from the urine of rats. However, Booth and Boyland (7, p. 361-365) found both the optically inactive and the laevorotatory forms. These same workers isolated the optically inactive and dextrorotatory isomers from the urine of rabbits. From the urine of guinea pigs, only the

laevorotatory forms were isolated (30, p. 647-655).

Isolated liver slices of rats and rabbits have been shown to convert naphthalene to 1:2-dihydro-1:2-dihydroxy-naphthalene (13, p. 636-641). Naphthalene is also converted to this product by rabbit and rat liver microsomes in the presence of TPNH and oxygen (64, p. 431-441; 8, p. 73-78; 9, p. 681-688).

METHODS AND MATERIALS

Experimental Insects

One insecticide susceptible and three insecticide
resistant strains of unsexed houseflies, Musca domestica
Linn., were used in this study. The flies used were of
various ages, depending on the particular experiment.
As will be discussed later, this had a significant influ-
ence on the biochemical processes under consideration.
The flies were reared under established procedures (77,
p. 350-351) at a temperature of $27 \pm 2^{o}C$. The adults
usually emerged about 10 days after eclosion and survived
for approximately 25 days.

The susceptible strain was originally obtained from
a wild population at Corvallis, Oregon and maintained in
the laboratory without exposure to insecticides.

The dieldrin strain was obtained from the Shell
Chemical Corporation, Modesto, California, in 1959 and is
resistant to insecticides of the Cyclo-diene class of
which dieldrin is a typical example:
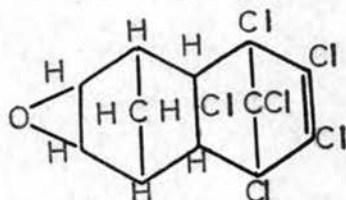
dieldrin (1,2,3,4,10,10-hexachloro-6, 7-epoxy-1,4,4a,5,6,

7,8,8a-octahydro-1,4-endo-exo-5,8-dimethanonaphthalene)
To maintain the resistance level, the adults are period-
ically exposed to dieldrin. At present the LD_{50} value
for this compound is approximately 45 micrograms per fly
compared with an LD_{50} of approximately 0.27 micrograms per
fly for the susceptible strain.

The naphthalene strain was derived from the suscept-
ible strain by selection with naphthalene vapors. At the
time of this investigation its resistance to the knock-down
action of naphthalene was approximately three-fold that of
the susceptible strain.

The DDT strain was obtained in 1955 from the Bureau
of Entomology and Plant Quarantine, USDA, Orlando, Florida,
and is resistant to DDT.
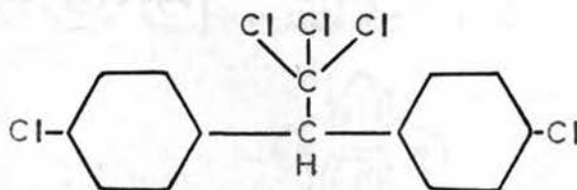
DDT (1,1,1-trichloro-2,2-bis-(p-chlorophynyl) ethane)
The resistance level is maintained by periodically expos-
ing the adults to DDT. At present the approximate LD_{50}
for this insecticide is 50 micro-grams per fly compared
to 0.14 micro-grams per fly for the susceptible strain.

Experimental Compounds

<u>Naphthalene-1-C^{14}</u> -- with a specific activity of 2.0 millicuries per millimole was obtained from the Radio-Chemical Center, Amersham, England, through the Nuclear Chicago Corporation. Its purity was confirmed by infrared spectrophotometry by comparison with standard naphthalene that had been purified by resublimation.

<u>1-Naphthol-1-C^{14}</u> -- with a specific activity of 2.3 millicuries per millimole was obtained from the same source. Its purity was confirmed by paper chromatography in two different solvent systems.

<u>SKF 525-A</u> (B-diethylaminoethyl diphenylpropylacetate) -- was obtained from Smith, Kline and French laboratories.

<u>Piperonyl Butoxide</u> -- (a-(2-(2-butoxyethoxy)-ethoxy)-4, 5-methylenedioxy-2-propyltoluene) was obtained from Fairfield Chemicals, Food Machinery and Chemical Corporation.

<u>Folic acid</u> -- obtained from Bios Laboratories Inc.

<u>Sucrose</u> -- (reagent grade) obtained from Mereck and Co., Inc.

<u>Nicotinamide</u>[1]

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[1]Obtained from the Sigma Chemical Co.

Glucose-6-phosphate (Disodium salt)[1,2]

TPN (Triphosphopyridine Nucleotide -- monosodium salt)[1,2]

Glucose-6-phosphate dehydrogenase (Zwischenferment)[1,2],

this enzyme was used from two purification levels, having

the following activity:

      Type II - 1,000 Kornberg units[3] per gram

      Type V - 90,000 Kornberg units per gram

Tris buffer -- (trishydroxymethylaminomethane)[1]

Isolation of Microsomal Fraction

Currently the microsomal fraction is usually defined
as the sub-cellular particles which can be sedimented after
the material visible in the bright field or phase contrast
microscope has been removed by centrifugation. Electron
micrographs of a microsomal fraction have revealed at least
two types of particles: a small spherical particle approx-
imately 20 millimicrons in diameter, and a flattened
pancake-like particle with a diameter of about 130 milli-
microns (81, p. 172-186). Although it has been considered
that the microsomes exist as such in the intact cell,
Potter's (74, p. 346-375) studies of the endoplasmic

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[2]Stored under desiccation at -15°C.

[3]One Kornberg unit is defined as that amount of enzyme
catalyzing the reduction of 1.0 micromole of TPN per
minute in the presence of G-6-P at pH 7.4, 25°C.

reticulum suggests that the microsomes may be part of this more complex structure.

The separation of the particulate fractions was accomplished by means of differential centrifugation, and all isolation procedures were carried out at approximately 0-3°C. to prevent retrogradation of the enzymatic activity. The houseflies were pre-chilled to 0°C. and their cells were broken up in 0.25 molar sucrose solution with a loose-fitting, fifty-milliliter capacity, Potter-Elvehjem (75, p. 495-504) type homogenizer utilizing a teflon pestle. A quarter-inch electric drill was used as the power source for the homogenizer. The resultant homogenate was filtered through cheesecloth to remove pieces of cuticle and other tissue. A second filtration through glass wool removed the remaining cell debris.

To begin the isolation of the microsomal fraction, the homogenate was first centrifuged for 10 minutes at 6,780 times gravity (x g) to sediment the nuclei and other debris. A fraction free of mitochondria was obtained by a ten-minute centrifugation at 20,200 x g resulting in a supernatant containing the microsomal and soluble fractions. The above operations were carried out in a Servall, Type RC-2, Automatic Superspeed Refrigerated centrifuge, which was maintained at a temperature of 0°C.

The supernatant obtained from the previous centri-
fugations was centrifuged for 30 minutes at 95,540 x g in
a Spinco, Model L, Preparative ultracentrifuge, with a
No. 40 rotor. The supernatant remaining at this point
constituted the soluble fraction and the sediment was the
microsomal fraction. In some experiments, the microsomal
pellets were resuspended once or twice in fresh 0.25 molar
sucrose solution (pH adjusted to 7.8), and recentrifuged
for 30 minutes at 110,800 x g to provide a more homoge-
neous microsomal fraction. Prior to their incubation with
the substrate, the microsomes were resuspended by homo-
genization in 0.25 M. sucrose in a three milliliter Potter-
Elvehjem homogenizer.

Weight was used as the criterion for determining the
number of flies to be used in a particular experiment. As
will be shown later, the microsomal fraction isolated from
two grams of flies, which usually amounted to about 85
insects, was the optimum amount for each incubation flask.
In order to provide a standard for comparison of micro-
somal preparations, the protein nitrogen present in each
fraction was estimated by the biuret colormetric method
(27, p. 30). A standard curve for the estimation of the
microsomal protein was made by using crystallized bovine
plasma albumen. As shown in Figures 2-11 and Tables 2-4,
there was very little variation in the microsomal protein

nitrogen as determined by this method.

Reduction of TPN

The hydroxylating systems located in the microsomal fraction have been shown to have a remarkable requirement for reduced TPN (TPNH) and oxygen (64, p. 431-441). In order to maintain a constant level of this constituent in the housefly microsome studies, a TPNH-generating system was employed instead of using chemically reduced TPN. The procedure used was essentially as described by Kornberg (63, p. 805-812). The over-all reaction in the reduction of TPN may be stated as follows:

$$\text{Glucose-6-phosphate} + \text{TPN}^+ \xrightarrow{\text{G-6-p dehydrogenase}}$$
$$\text{6-phospho-gluconate} + \text{TPNH} + \text{H}^+$$
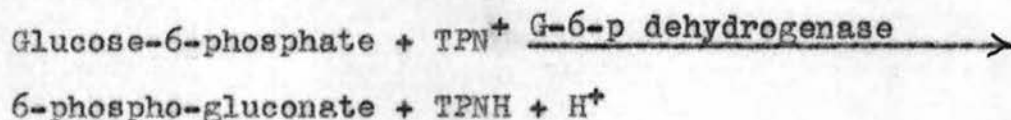
The TPNH generating system consisted of TPN, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, and nicotinamide in pH 7.8 tris buffer. Although the nicotinamide is not an essential cofactor in the reduction of TPN, it is necessary for the inhibition of the hydrolytic action of microsomal TPN-ase, which cleaves the nicotinamide moiety of oxidized TPN. A TPN-nicotinamide ratio of 1:10 was used because, according to Zatman and co-workers (100, p. 197-212), in a high nicotinamide concentration there is very little hydrolytic breakdown of oxidized TPN.

The time required for the complete reduction of TPN was determined by the rate of increase in optical density of a solution containing the TPNH generation constituents. Under the experimental conditions employed, reduction was completed after eight minutes of incubation at 23°C. (Figure 1). As a matter of convenience the reduction of TPN was begun 30 minutes prior to the addition of this constituent to the incubation flasks.

A second complication in the use of microsomes is the presence of TPNH-oxidase. To insure that this enzyme was not destroying TPNH during the experimental period, a qualitative test for the reduced form was devised. It was found that when the various constituents of the incubation mixture were exposed to long wave ultraviolet light (maximum intensity at 366 millimicrons) a brilliant blue fluorescence occurred when TPNH was present (Table 1). This was, therefore, taken to be a measure of the presence of TPNH and was used as a qualitative test in all subsequent experiments.

FIGURE I

REDUCTION OF TPN
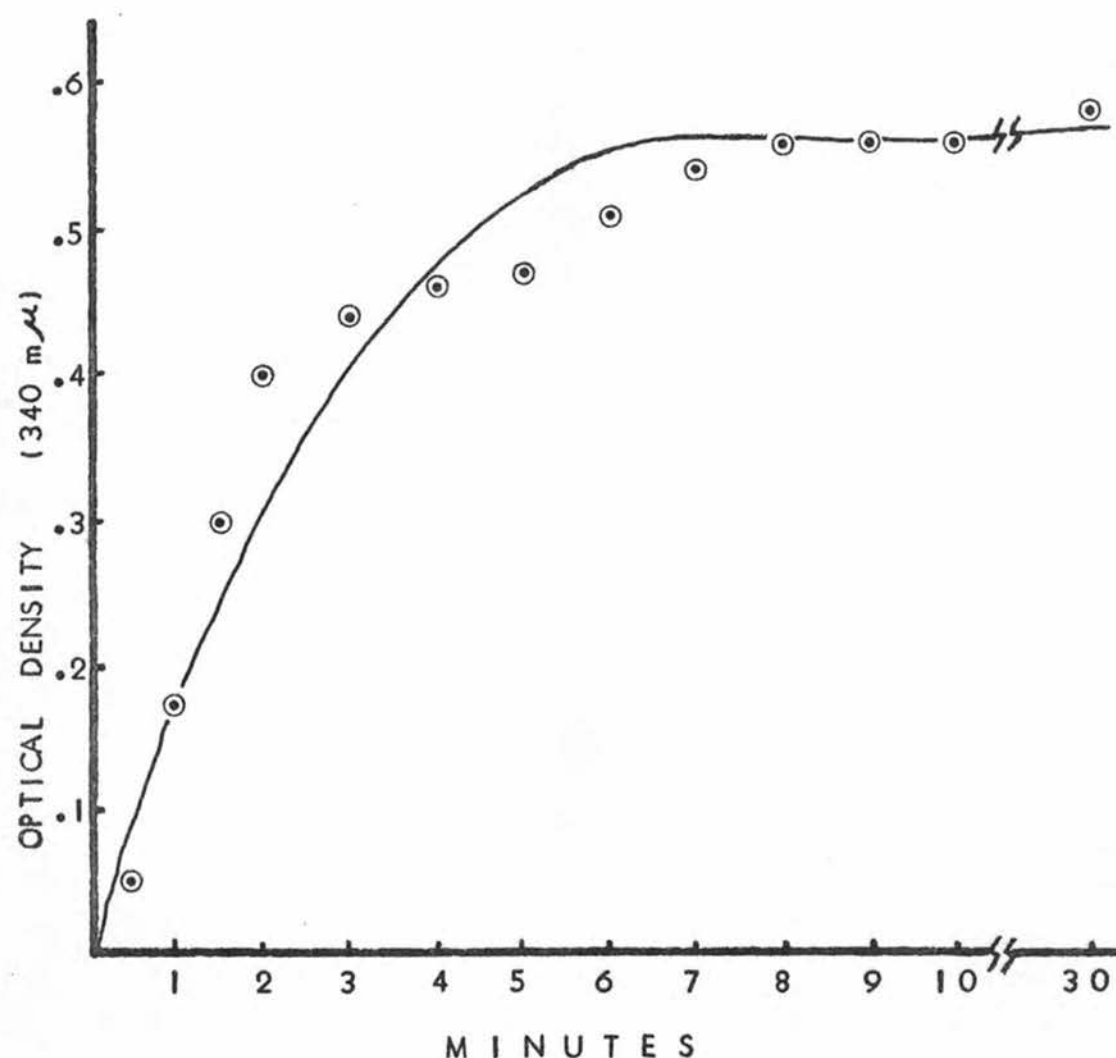
The reaction mixture consisted of: 0.6μM. TPN, 1.2μM. glucose-6-phosphate, 0.4 Kornberg units of glucose-6-phosphate dehydrogenase, 1.2μM. nicotinamide, 1 ml. tris buffer (0.3 M., pH 7.8), 2 mls. of water and was incubated aerobically at room temperature.

TABLE 1

FLUORESCENCE OF THE COMPONENTS OF THE INCUBATION MIXTURE

Component(s)	Fluorescence (UV-light)
Nicotinamide	Negative
TPN	Negative
TPN, G-6-P	Negative
TPN, G-6-P, nicotinamide	Negative
TPN, G-6-P, nicotinamide, G-6-P de-hydrogenase	Positive
TPNH (chemically reduced)	Positive
TPN, G-6-P, nicotinamide, microsomes	Negative
TPN, TPNH-generating system, microsomes	Positive

Microsomal Metabolism of Naphthalene

A typical reaction mixture consisted of: two milliliters of tris buffer solution (0.3M. at pH 8.0), one milliliter of sucrose solution (0.25M.), one milliliter of a solution which consisted of: oxidized TPN (1.2 micromoles), glucose-6-phosphate (12 micromoles), glucose-6-phosphate dehydrogenase (0.4 Kornberg units), nicotinamide (12 micromoles), 0.15 milliliters of naphthalene solution (0.375 micromoles or 0.75 microcuries), one

milliliter of the microsomal suspension, and water to make a total volume of 6 milliliters. This suspension will be referred to hereafter as the standard incubation mixture.

Double distilled water was used to make all solutions. The tris buffer was adjusted to pH 8.0, which had been determined (Figure 4) to be the optimum pH for microsomal activity. A nonbuffered isotonic sucrose was used in most of the experiments, but in later stages of the study it was adjusted to pH 7.3 before use. The substrate, naphthalene, being only slightly soluble in water, was dissolved in ethylene glycol monomethyl ether (methyl cellosolve).

The reaction constituents were incubated in 50-milliliter, glass-stoppered Erlenmeyer flasks, which were mechanically shaken in a Precision Dubnoff Metabolic Shaking incubator, and in all cases the atmosphere above incubating medium was air. In most experiments the reaction mixture was incubated for 30 minutes at which time the reaction was stopped by the addition of 15 milliliters of ethyl ether.

Extraction and Purification of Metabolites

In the initial stages of this study 30-milliliter separatory funnels were utilized for the separation of

the aqueous and ether phases of the reaction mixture. In later experiments the method was improved by using a hypodermic syringe to remove the ether phase from the aqueous phase in the original incubation flask. Four successive extractions with ether, totaling 45 milliliters were used to extract the ether soluble naphthalene metabolites. Traces of water in the ether were removed by dehydration with anhydrous sodium sulfate.

The combined ether extracts were evaporated to dryness under an air jet. This process also removed the majority of the unmetabolized naphthalene extracted by the ether. Approximately 0.5 milliliter of ethyl ether was added to dissolve the metabolic products and this solution was then quantitatively transferred with washing, to a short chromatographic paper using a one-milliliter hypodermic syringe.

The technique of Menn, et al, (59, p. 41-42) was occasionally used to chromatographically purify the metabolites, thus eliminating most of the interfering substances from the elutriate. It was necessary to make a slight modification in this technique by adding a small amount of methanol (9:1, acetonitrite-methanol) in order to quantitatively elute two of the naphthalene metabolic products: 1:2-dihydro-1:2-dihydroxynaphthalene and 1-naphthol (See Figure 13). The other metabolites, when

present, were left on the paper strips. The eluates from this preliminary chromatographic separation were collected in ten-milliliter beakers and the solvent evaporated to dryness under an air stream. Ethyl ether was used to redissolve the residue which was quantitatively transferred to a standard chromatographic strip by means of a one-milliliter hypodermic syringe.

Separation and Identification of Metabolites

Measurement of Radioactivity: Two methods were used to measure the radioactivity present in the ether extracts. In the first, one-milliliter aliquots of the extract were transferred to copper planchets, and the ether along with final traces of radioactive naphthalene was removed by evaporation under an infra-red lamp. The radioactivity of the material on the planchet was then counted in a Tracerlab manual sample changer equipped with a TGC-2, Geiger-Muller detector tube which was connected to a model SC-19, Tracerlab Utility Scaler. In the second method a model SU-3D Tracerlab laboratory monitor was used to monitor the radioactivity on the standard chromatographic strip immediately after transferring the ether extract.

Two additional methods were used for radio-assay of the radioactive chromatograms. The first was a chromatogram scanning system which consisted of three main

components: a window-less Forro gas-flow chromatographic
scanner connected to a model 1620, Nuclear Chicago Rate-
meter which was connected to a model A.W. Esterline-Angus
chart recorder. The chromatographic strip was drawn
across the scanning head by the chart drive mechanism of
the recorder so that scanning rate and chart speed were
synchronized (Plate 1--Appendix). In the above methods,
the measurements were corrected for background and count-
ing efficiency of the detectors.

Radioautography was another method occasionally used
to locate the radioactive areas on the paper chromatograms.
Medical X-ray film was exposed to the radioactive strips
for periods up to 12 days before development. This method
is capable of greater sensitivity than that possible with
the strip scanning device and also has the advantage of
showing the spot characteristics.

Chromatographic Apparatus and Methods: The chromato-
graphic tank was a borosilicate glass jar, 12-inches square
and 24-inches high, with the usual solvent assembly:
trough, cradle, antisiphon rods and anchor rods. The
Whatman No. one chromatographic strips, 3.8 cm. x 53 cm.
were hung in the chamber and allowed to equilibrate in an
atmosphere saturated with the aqueous phase of the solvent
system for approximately four hours. The organic phase of
the solvent system was then added to the troughs through

a small hole in the glass lid, allowing the solvent to flow down the paper by gravity.

The following solvents were used to partition the metabolic products: n-butanol, absolute ethanol, distilled water (BEW), 17:3:20 (v/v); benzene (reagent grade), glacial acetic acid (reagent grade), distilled water (BAW), 5:1:4 (v/v); cyclohexane (practicle), glacial acetic acid (reagent grade), distilled water (CAW), 5:1:4 (v/v); and aqueous 0.1 N. ammonium hydroxide. The development of the chromatograms with BEW usually required about 15 hours. The other solvents took considerably less time to develop the chromatogram. After the solvent had migrated a sufficient distance, the strips were removed from the chamber and allowed to air dry.

In some cases, when a radioactive area located along the chromatograms could not be identified, due to a shift of R_f values[4] or other reasons, that area was eluted with methanol and rechromatographed on another strip using a second solvent system. This allowed the comparison of the R_f value of the unknown compound with the values of the standard compounds.

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[4] $R_f = \dfrac{\text{Distance substance travels from the origin}}{\text{Distance solvent travels from the origin}}$

RESULTS AND DISCUSSION

Several assumptions are implicit in the use of mass isolation techniques for the study of enzymatic activity in isolated cell particulate fractions. The three most important assumptions are: that rupture of the cell membrane does not in itself initiate rapid irreversible cytolytic changes; that the cytoplasm can be infinitely diluted, with the formed elements retaining their intracellular properties; and that solutions can be devised which will approximate the cytoplasmic environment sufficiently well to maintain the isolated fraction in some state of activity during the experimental period.

The experiments to be described in this dissertation were modeled at first after the work which had already been done with microsomes of vertebrate liver tissue (9, p. 681-688). Once it had been determined that housefly microsomes did function to some extent under the conditions already established, it was expedient to study the variables so as to learn their most favorable parameters and thus achieve the maximum efficiency with the system. The variables studied in this way included pH, temperature, microsome concentration, substrate concentration, time of incubation, and the requirements for certain co-factors.

Once the basic conditions under which housefly micro-somal system functioned efficiently were established, the influence of other factors of particular interest to the investigator were studied. These included the effect of age on the activity of microsomes, the differences, if any, which existed between microsomes of resistant and non-resistant insects, the possible inhibition of microsome activity, and the efficiency of the tissue isolation technique in providing an active preparation. These exper-iments are described and their results discussed in the sections to follow.

ESTABLISHMENT OF OPTIMUM CONDITIONS:

All the constituents of the incubation mixture and the conditions of incubation were kept constant except for the experimental variable being studied. Likewise, every effort was made to isolate the housefly microsomes under identical conditions. The amount of substrate changed, thus the amount of radioactivity recovered in a 30-minute period was measured by methods already discussed. When the metabolites were resolved the predominant metab-olites were 1:2-dihydro-1:2-dihydroxynaphthalene and 1-naphthol. The former metabolite will hereafter be re-ferred to as dihydrodiol. The presence of other metab-olites seemed to depend somewhat on the experimental

variable being studied.

Microsomal Concentration

The amount of substrate metabolized in a given period
by a suspension of microsomes should be proportional to
the weight of microsomes present if other conditions are
not limiting. The purpose of this experiment was to
determine the microsome level which was optimum under the
experimental conditions. The microsomes were isolated
from four-day old flies of the DDT resistant strain and
prepared by the previously mentioned methods. As can be
seen in Figure 2, there was a linear relationship between
the amount of substrate transformed, and the protein con-
centration of the microsomal suspension. This relation-
ship existed up to a concentration of approximately 12
milligrams of protein. Above this level there was no
further increase in the amount of substrate metabolized.
The microsomal protein concentration of 12 milligrams was
usually obtained from two grams of fresh fly tissue which
amounted to approximately 85 insects.

This level of microsomal protein is difficult to com-
pare with the work of others since most authors do not
state the amount of microsomal protein utilized in their
experiments. Mitoma and co-workers (64, p. 431-441) re-
ported that the microsomes incubated in each flask were

FIGURE 2

THE EFFECT OF MICROSOMAL PROTEIN LEVEL
ON THE METABOLISM OF NAPHTHALENE
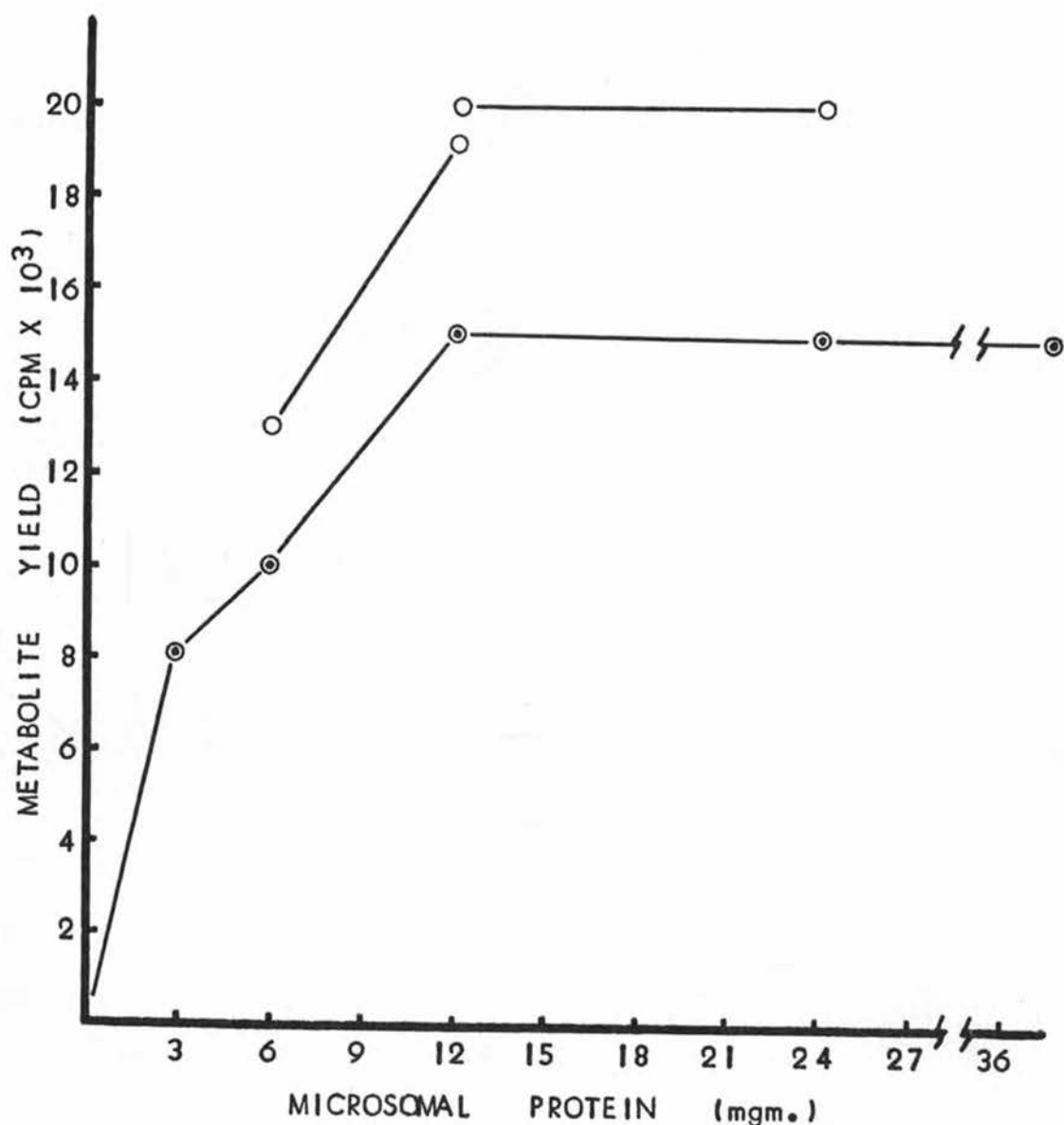
Experimental Conditions: The standard incubation mixture was incubated aerobically at a temperature of 37° C. with microsomes that were isolated from flies of the following ages: ⊚ 4 days; ○ 12 days.

equivalent to 750 milligrams of liver tissue, while Agosin and co-workers (1, p. 340-342) reported that their microsomal fractions were derived from 3.5 grams of cockroach tissue. Not much significance can be attributed to these figures since the amount of microsomes isolated from a given weight of tissue depends on the homogenization technique. On the other hand, Booth and Boyland (8, p. 361-365; 9, p. 73-78) and Booth and co-workers (10, p. 182-186) reported a concentration of 6.7 to 9.3 milligrams of protein per milliliter of microsomal suspension. However, this figure represented protein of microsomes that were isolated from liver tissue, which is more homogeneous than the tissue of intact flies.

The leveling-off of the reaction rate above a concentration of 12 milligrams of microsomal protein was not expected although it might have been due to a substrate shortage. This was difficult to conceive since the amount of substrate metabolized was approximately one per cent of the total amount introduced into the incubation flasks. The results obtained from experiments to be described later, however, suggested that exhaustion of naphthalene could have taken place.

Substrate Concentration

In many enzyme-substrate systems, the enzyme may react efficiently only with low concentrations of substrate, while in others, the rate of reaction increases with substrate. In investigations of enzymatic reactions it is important to know if the substrate becomes a limiting factor and if so, at what level. It was of interest to determine if the hydroxylase system which was under consideration, would fall into one of the above mentioned categories. The microsomes which were used in studying this variable were isolated from four-day old flies of the DDT resistant strain and prepared by the standard methods.

As can be seen in Figure 3, the amount of substrate transformed increased linearly with increasing concentration of substrate up to a level of 0.75 micromoles (0.4 ml of solvent), or 96 micrograms of naphthalene. Either the solvent or the substrate may have become inhibitory at this point. In preliminary observations, the amount of solvent that would cause a visual change of the suspension was found to be 0.5 milliliters; whereas, the concentration of standard naphthalene could be increased to 3.5 micromoles without causing any noticeable effects. It was, therefore, concluded that the solvent was the

FIGURE 3

THE EFFECT OF SUBSTRATE CONCENTRATION
ON MICROSOMAL METABOLISM OF NAPHTHALENE
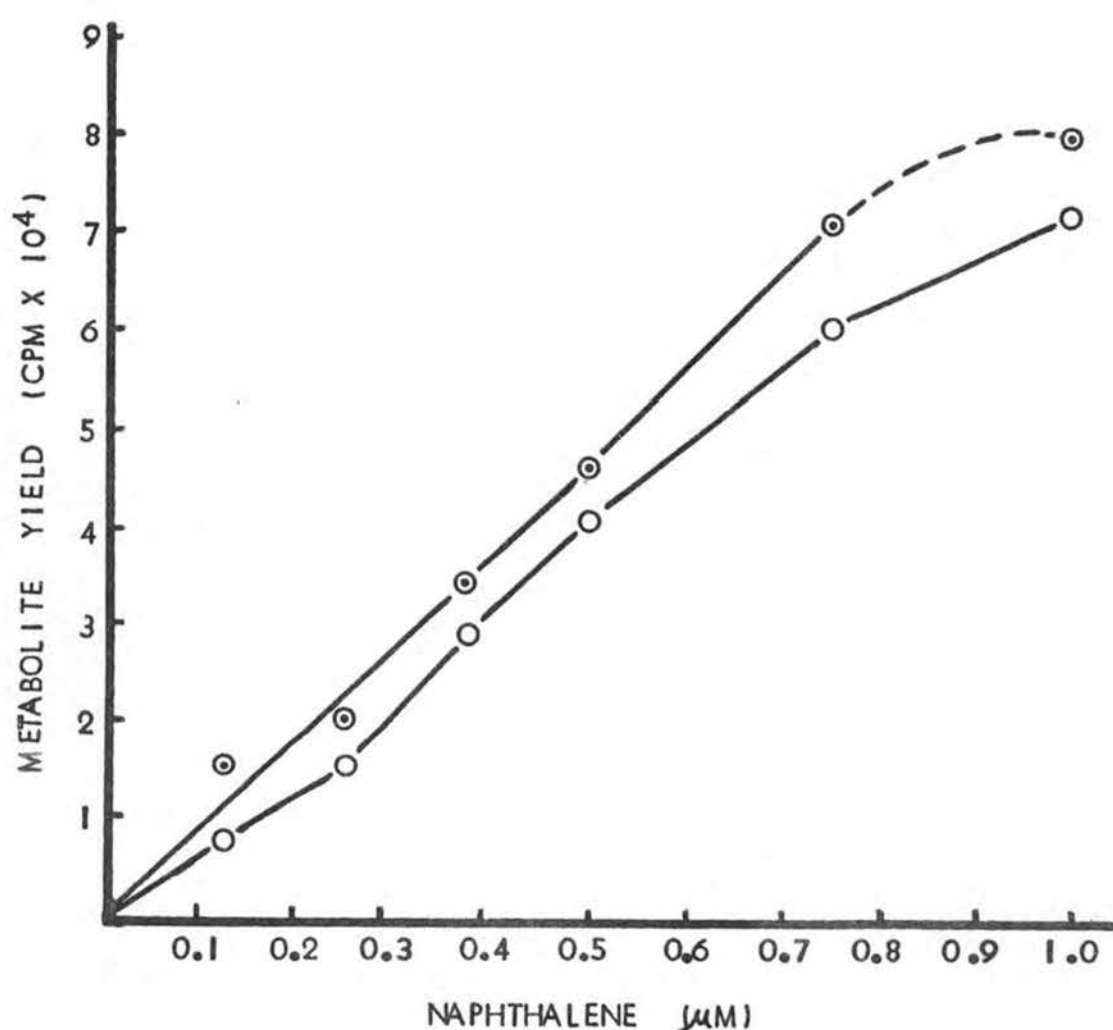
Experimental Conditions: The standard incubation mixture was incubated aerobically at a temperature of 37° C. with microsomes that were isolated from flies of the following ages and having a protein equivalent of: ◉ 9 days— 12.6 mgm.; ○ 7 days— 12.1 mgm.

limiting factor.

If indeed the leveling-off effect was due to the solvent, then the results of the above experiments indicated that the enzyme(s) present in the microsomal preparation was not saturated with the substrate at any one time, an assumption which was confirmed by the increasing yield of metabolites as substrate was increased. These results help to explain those found in the previous experiment. If one compares the substrate levels in Figures 2 and 3, a substrate deficiency is seen as a possible limitation in the former experiment. The deficiency of substrate could be aggravated by the high volatility of naphthalene. The results shown in Figures 2 and 3 were substantiated several times in additional experiments.

Effect of pH

Enzymes, in general, are active over a limited pH range, and most have a definite optimum pH level. It was desirable, therefore, to determine the optimum pH of the system under consideration. The microsomes were isolated from five-day old flies of the DDT resistant strain, and the pH values tested ranged from 6.8 to 8.6. The effect of increasing the pH is shown in Figure 4 which indicates that the pH of 7.8 to 8.0 was optimum for maximum activity,

FIGURE 4

THE EFFECT OF PH
ON MICROSOMAL METABOLISM OF NAPHTHALENE
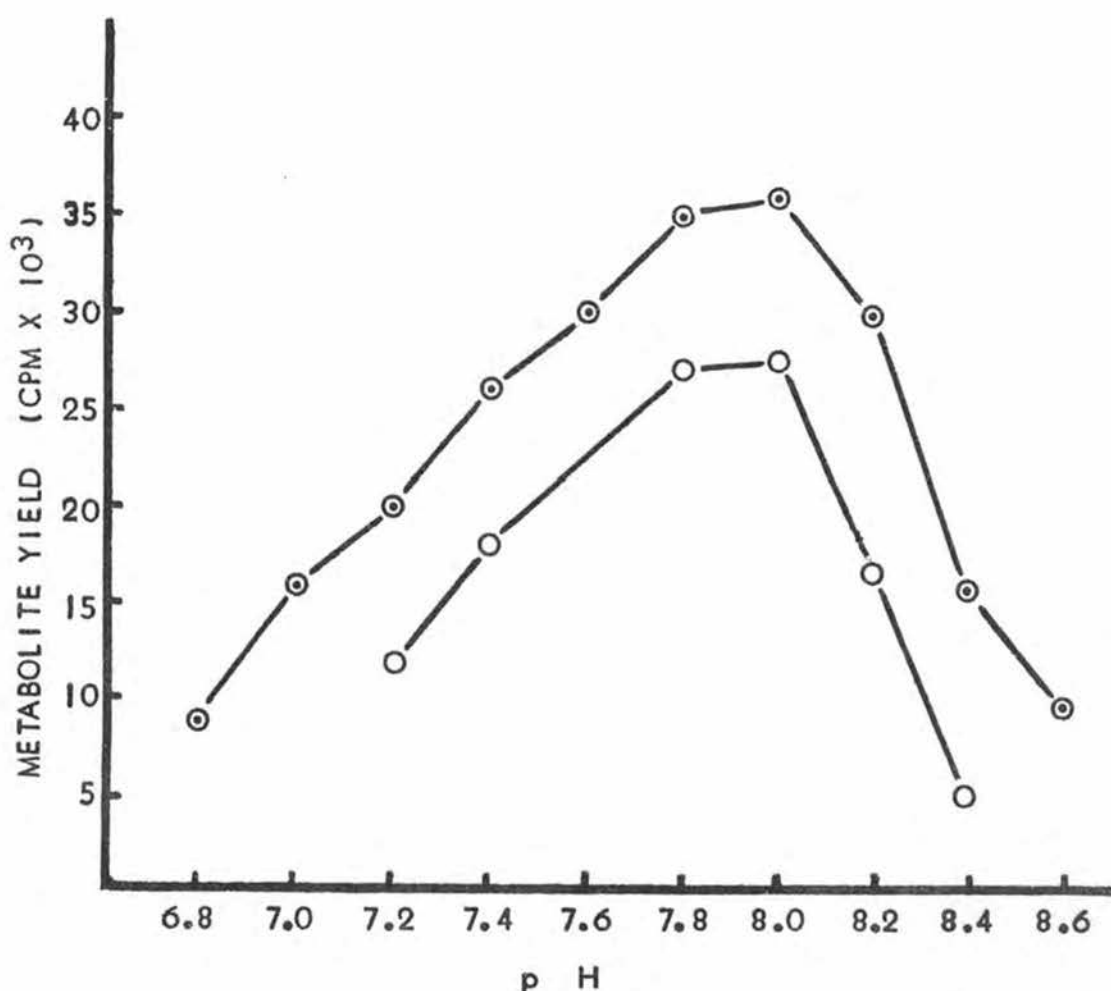
Experimental Conditions: The standard incubation mixture was incubated aerobically at a temperature of 37°C. with microsomes that were isolated from flies of the following ages and having a protein equivalent of: ⊙ 6 days- 12.6 mgm.; ○ 4 days- 12.2 mgm.

with low enzymatic activity at pH 6.8 and 8.6 which were the extremes of the pH range tested. The two experiments were conducted under identical conditions, and the central portions of the curves were substantiated in still other experiments.

Although the pH optimum of an enzyme is a useful datum in its characterization, it is well known that the value found for one substrate may not necessarily apply to all substrates of that enzyme. Microsomal preparations from rabbit liver have been incubated at pH 8.2 for studies on naphthalene metabolism (64, p. 431-441), while preparations from rat liver have been found to have an optimum pH of 8.2 for 1:2-dihydro-1:2-dihydroxynaphthalene formation and 7.6 for 1-naphthol formation (9, p. 681-688). Agosin and co-workers (1, p. 340-342) in an investigation conducted simultaneously with the present study, incubated their housefly and cockroach microsomal preparations at pH 8.7, but presented no evidence that this factor was studied in detail.

According to Williams (96, p. 68), at pH 7.0 microsomes readily lose their ability to catalyze aromatic hydroxylation. On the contrary, Booth and Boyland (9, p. 681-688), in their studies with rat liver microsomes, found that even at pH as low as 6.4, some hydroxylation of naphthalene occurred. No attempts were made in the

present study to distinguish the pH optima for naphtha-
lenediol and 1-naphthol; therefore, the optimum found
could be a compromise with those found by Booth and Boy-
land. These variations in pH optima and inactivating pH
values of hydroxylating systems, reported by the various
workers, suggest that this metabolic step may be performed
by different enzymes, each somewhat unique to the species
involved.

Effect of Temperature

The effects of temperature on enzymatic reactions
are extremely complex; however, in the characterization
of the enzyme system under consideration, it was desirable
to determine the temperature which was optimum for the
experimental conditions. These determinations were con-
ducted on microsomes isolated from 12-day old flies of the
DDT resistant strain. The flasks were incubated at the
following temperatures: 15°, 23°, 27°, 34°, and $37^\circ C$.

When the temperature of incubation was plotted against
the yield of metabolites (Figure 5), it was observed that
up to $34^\circ C$. there was a definite increase in substrate
transformation. The amount of naphthalene metabolized
dropped sharply when the incubation of the microsomal
preparation was carried out above this temperature. Addi-
tional experiments have confirmed the general shape of

FIGURE 5

THE EFFECT OF TEMPERATURE
ON MICROSOMAL METABOLISM OF NAPHTHALENE
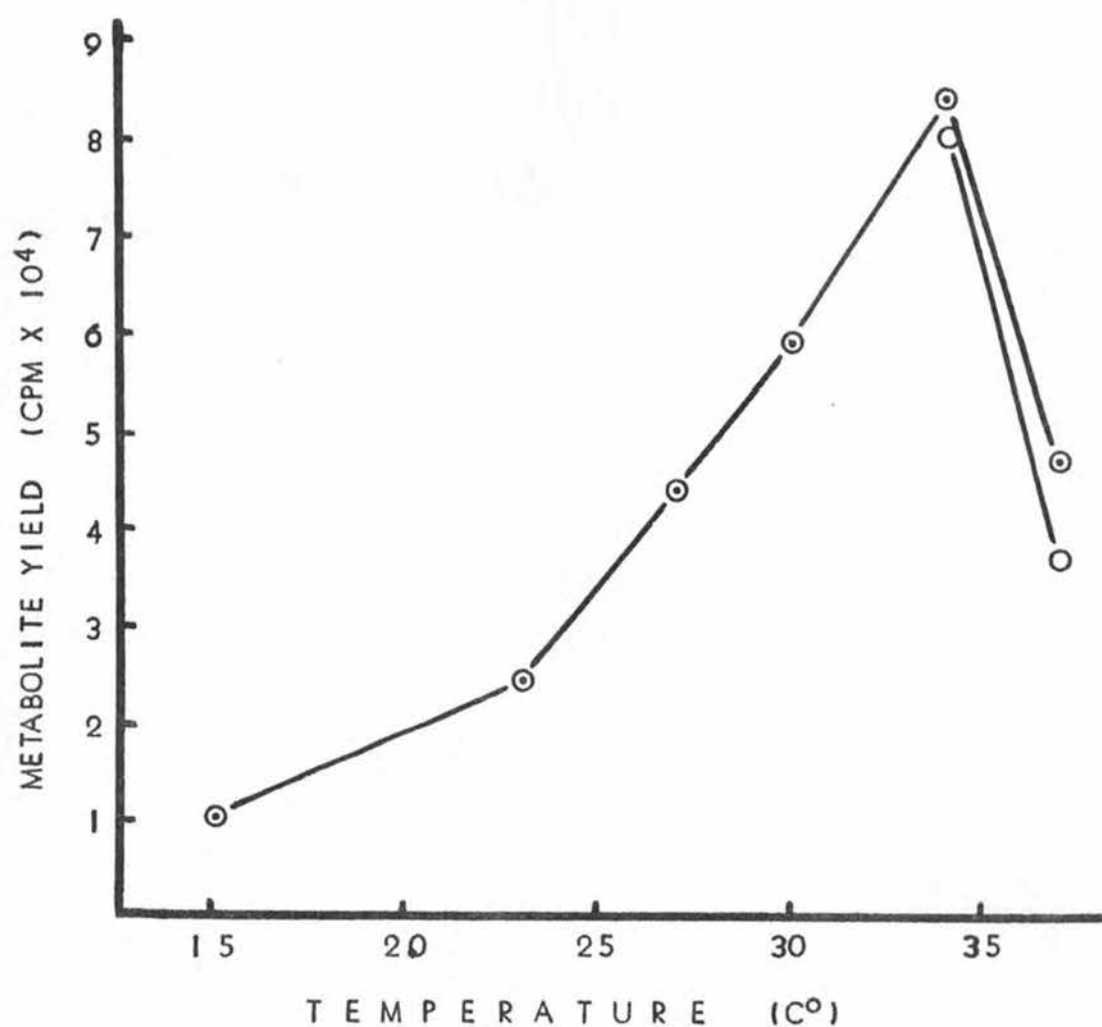
Experimental Conditions: The standard incubation mixture
was incubated aerobically with microsomes that were
isolated from 12-day old flies having a protein equivalent
of: ⊙ 12.3 mgm.; O 11.8 mgm.

this curve.

It is believed that the over-all enzyme reaction consists of at least three successive stages, namely, formation of the enzyme-substrate complex, conversion of this into the enzyme-product complex, and finally the dissociation of the product. The influences of temperature on the reaction is the resultant of its separate effects on these stages. Other authors working with the microsomal fractions isolated from rat liver (8, p. 73-78; 9, p. 681-688; 17, p. 175-181), rabbit liver (28, p. 55-63; 38, p. 480-485; 42, p. 532-540; 64, p. 431-441; 47, p. 571-572; 48, p. 164-165), and insects (1, p. 340-342), have carried out their experiments at a temperature of $37^{\circ}C$. None of these authors appear to have made a systematic investigation of this variable, and thus the present results may represent a more reliable temperature optimum. On the other hand, since most of these studies have involved homoiothermic animals, it is logical to conduct such experiments at approximately body temperature. Insects, however, being poikilothermic might be expected to possess enzymes of different temperature optima.

There may be some connection between the temperature optimum and the observed negative temperature coefficient of insecticide action. Wilson (97, p. 423-428) found, for example, that flies are less sensitive to pyrethrum

poisoning at high temperatures than at lower temperatures. Similarly, Vinson and Kerns (95, p. 484-496), Tahori and Hoskins (88, p. 829-837), in studies with DDT, found that severely affected insects recovered in a few minutes when moved to a higher temperature, but quickly reverted to the intoxicated condition if cooled again. Menn and co-workers (58, p. 67-74) in their extensive work with DDT also found similar negative coefficient of toxicity, but they found that at higher temperatures DDT was being metabolized at a faster rate. Previously, Butts and co-workers (22, p. 699) had shown that when Periplaneta americana were treated with C^{14} labeled DDT, a conjugated metabolite was produced in higher amounts when the insects were kept at a temperature of $35^\circ C$. than when maintained at $25^\circ C$.

As a result of in vivo studies of this negative temperature coefficient of DDT, two theories have been advanced. One proposes the storage of DDT at higher temperatures and its crystallization at lower temperatures with DDT in this form being more toxic. The other suggests that a more rapid detoxication takes place at the higher temperatures. The manner in which symptoms of poisoning by DDT can be reversed by alternate cooling and warming has led to doubt that detoxication of the chemical could be a factor. It is conceivable, however, that both theories are valid. According to the results of the

present study, the detoxification reactions were indeed more rapid at higher temperatures. The more rapid hydroxylative activity in conjunction with storage of DDT in a non-crystalline form would explain the negative temperature coefficient. This dual action theory would seem to be valid if the assumption can be made that there is always a surplus of toxicant at or near the sensitive site. Since only the DDT family and not all chlorinated hydrocarbon insecticides exhibit this negative temperature coefficient of toxicity, it is difficult to make this a general hypothesis.

Co-factor Requirements

Foreign compounds have been found to be oxidized along a variety of metabolic pathways by enzyme systems of liver microsomes. All these enzyme systems have been shown to require TPNH. It was of interest, therefore, to determine if this co-factor was required by the housefly microsomal system for the metabolism of naphthalene. Due to the observed age effect, to be discussed later, it was desirable to study all stages of the same generation.

All insects used for these experiments were obtained from a single colony of the DDT resistant strain. The desired weight of larvae, pupae, or adults were obtained and the microsomes were isolated and prepared as before,

except that the larvae, due to their slimy condition were ground-up in a mortar before proceeding with the normal homogenization. TPNH requirements were tested with the following combination of co-factors: (a) reference flask with complete TPNH-generating system; (b) oxidized TPN only; (c) folic acid only; and (d) no co-factors added.

An explanation of the results shown in Figures 6 and 7 indicates that reduced TPN is not essential for naphthalene metabolism by the microsomes isolated from tissue of the larvae, pupae, or one-day old adults. There was a definite indication that reduced TPN is required for these reactions to proceed in the older adults. The adult age at which TPNH becomes essential was not apparent from these studies because of the lack of intermediate reference points. The curves (Figure 7) also indicated that the TPNH requirement of older flies cannot be replaced by oxidized TPN or by folic acid.

The efficacy of folic acid as an electron donor was tested in view of Kaufman's (60, p. 2677-2682; 51, p. 2683-2688) results, in which he has shown that tetrahydrofolate and several tetrahydropteridines can replace TPNH in the hydroxylation of phenylalanine to tyrosine. The ineffectiveness of folic acid in the system used in the present study could be due to several factors. It was possible, for example, that the microsomal fraction lacked the

FIGURE 6

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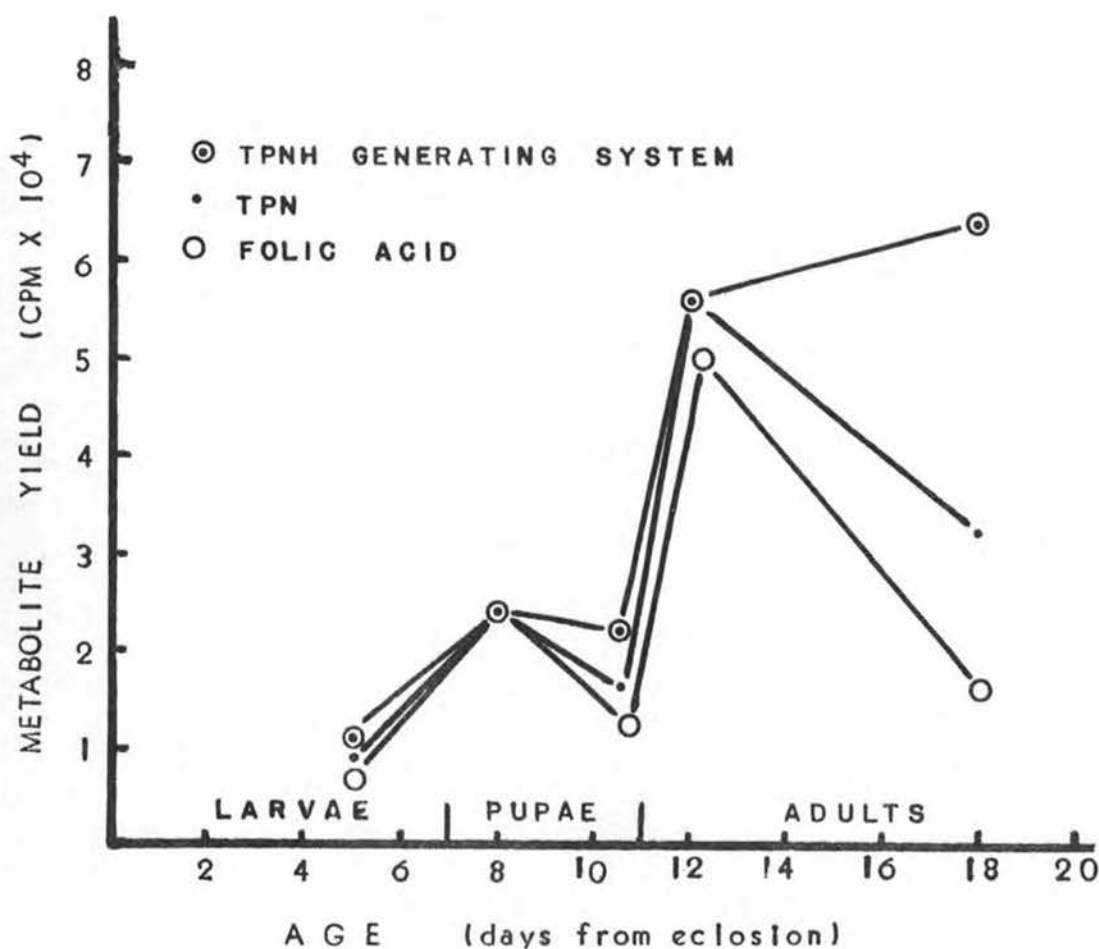# THE EFFECT OF TPNH AND INSECT DEVELOPMENT
ON MICROSOMAL METABOLISM OF NAPHTHALENE
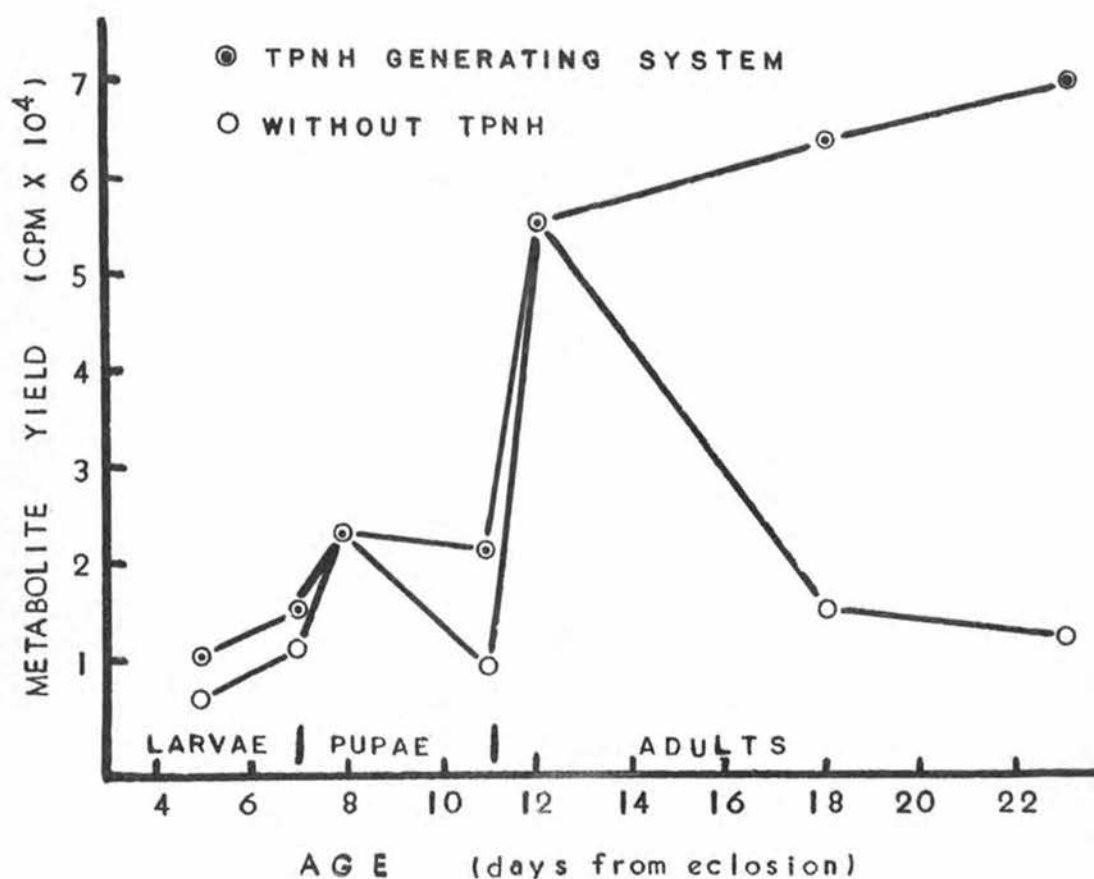
Experimental Conditions: The standard incubation mixture was incubated aerobically at a temperature of 37° C. with microsomes that were isolated at the following ages and having a protein equivalent of: 5 days- 11.5 mgm.; 7 days 12.1 mgm.; 8 days- 11.4 mgm.; 11 days- 12.8 mgm.; 18 days 12.3 mgm.; 23 days- 13.1 mgm.

FIGURE 7

THE EFFECT OF TPN, FOLIC ACID
AND INSECT DEVELOPMENT
ON MICROSOMAL METABOLISM OF NAPHTHALENE

Experimental Conditions: The standard incubation mixture was incubated aerobically at a temperature of 37° C. with microsomes that were isolated at the following ages and having a protein equivalent of: 5 days- 11.5 mgm.; 8 days 11.4 mgm.; 11 days- 12.8 mgm.; 18 days- 12.3 mgm.

necessary enzymes to reduce folic acid. Another possibility is that the absence of reduced TPN could have been the limiting factor since Kaufman believes that TPNH is essential for the reduction of folic acid.

All previous studies including those with insect microsomes (1, p. 340-342), have indicated the indispensability of reduced TPN for the metabolism of foreign compounds by microsomes. Still, as can be seen in Figure 6, only the microsomes of the aging adult houseflies require TPNH. This is not necessarily a contradiction of previous work since other authors have not mentioned the use of immature forms. An explanation for the present results is that mature insects are no longer able to provide a sufficiently active TPNH generating system.

The slight decrease in the amount of substrate metabolized during the last larval instar (Figures 6 and 7) may be correlated with the availability of reduced TPN. Even when TPNH was added, this effect was seen. As pupation approaches at the last larval instar there is a marked increase in the concentration of tyrosine (43, p. 48-62) which could possibly reduce the amount of TPNH present since this co-factor is essential during the production of tyrosine from alanine (51, p. 2683-2688). This could create a competition for introduced TPNH as well as that generated by the microsomes. This idea is substantiated

by observations made on the color of the late larval stage
which turned from white to a dark gray color during the
centrifugation processes, while no other fraction in this
series showed this melanic process.

Another interesting observation was made during this
phase of the study. As shown in Figures 6 and 7, naphtha-
lene was metabolized to a limited extent by the larvae,
and pupae, and appreciably by one-day old adult flies, in
the presence or absence of the various co-factors. The
radio-active metabolites produced during this period were
quite volatile, however, and would soon disappear from
the chromatographic strips or planchets on which they were
deposited. When the microsomal preparation was isolated
from older adult flies and incubated with the substrate
in the presence of reduced TPN, the radioactivity extract-
ed did not volatilize appreciably; although when incubated
in the absence of TPNH the volatility of the metabolites
was again apparent. This prevented chromatographic resolu-
tion of the unknown material.

It was suspected that the observed volatility was
due to the presence of unmetabolized naphthalene which had
persisted to this point in the experiment. This assump-
tion was tested by incubating naphthalene without any
particulate fraction or with boiled microsomal fraction,
for the same period and under identical conditions. No

radioactivity could be detected after the extraction and ether evaporation procedures. This indicated that the compound(s) was a new metabolite of the substrate, not naphthalene or any of its known metabolites.

The transitory compound observed during this study could possibly be 1:2-epoxy-1:2-dihydronaphthalene which was originally proposed by Boyland (12, p. 27-29) as an intermediate in the metabolism of naphthalene. This structure has also been postulated by Davidov and Radomski (33, p. 259-265), as a key intermediate in the formation of phenols. These authors assumed that aromatic hydrocarbons are oxidized to epoxides, and could then be hydrated to dihydrodiols, which may be dehydrated to phenols.

Additional evidence for an epoxide intermediate was obtained by Boyland and Sims (17, p. 175-181) and Booth, et al., (10, p. 182-186). These authors studied the epoxide, 1:2-epoxy-1:2:3:4-tetrahydronaphthalene, and its metabolites. Another possible intermediate, 1:2-dihydronaphthalene, was also studied by the same group. The results obtained by in vivo studies (17, p. 175-181) indicated that both compounds were converted to 1:2-dihydroxy-1:2:3:4-tetrahydronaphthalene and two other metabolites. The in vitro studies by Booth and co-workers (10, p. 182-186) of microsomal action on 1:2 dihydronaphthalene suggested that an intermediate was formed, which was dependent

upon the presence of reduced TPN. They concluded that the likely structure for the intermediate compound was 1:2-epoxy-1:2:3:4-tetrahydronaphthalene, which was shown to give products indistinguishable from those of 1:2-dihydronaphthalene. The hypothesis that TPNH is required for this initial reaction is not supported in the current studies with houseflies since the volatile intermediate(s) was produced by adult fly microsomes in the absence of TPNH. This intermediate may thus be a product of a "mixed-function" oxidase system, in which one atom of oxygen is added to a double bond of the substrate, according to the mechanism proposed by Mason (56, p. 85; 57, p. 211-214). Its further conversion to non-volatile metabolites of naphthalene such as Diol and Naphthol might then require TPNH.

The concept of biological epoxidation is not unique since epoxides are a naturally occurring class of compounds (6, p. 5767-5768). Davidov and Radomski (33, p. 259-265) have shown that heptachlor is converted to heptachlor epoxide by dogs, and this was also confirmed in houseflies by Perry and co-workers (72, p. 346-351). Similarly cockroaches have been shown to epoxidate aldrin to dieldrin (41, p. 588-592) and also isodrin to endrin (62, p. 300).

The failure to detect non volatile metabolites of

naphthalene after the incubation of microsomes of immature insects in the presence or absence of TPNH and of mature insects without added TPNH requires some explanation. One possibility is that a key enzyme(s), required in the transfer of hydrogen from TPNH to the volatile intermediate, is not available in the young developing forms. As the insect matures, this enzyme may become active and aid in the conversion of the opoxide form to the hydroxylated metabolic products of naphthalene. This concept is supported by the following arguments: no naphthol or dihydrodiol are formed by microsomes of immature insects even in the presence of TPNH; two types of radioactive metabolites appear to be produced by mature insects, if TPNH is present, the normal non-volatile metabolites and the volatile compound; and, only the volatile metabolite is produced by the older microsomes if TPNH is absent. This hypothesis assumes that the volatile intermediate is produced by microsomes of all stages and that it does not require reduced TPN; but, as the hypothetical hydrogen "transfer" enzyme becomes active, the unknown compound is further converted to known non-volatile products.

Stability of Enzymatic Activity:

Under in vitro conditions the period in which the activity of enzymes remains stable varies considerably.

This period may extend from a few minutes to several hours and in some cases the enzymatic preparation will remain active for days. Useful information could be gained by determining the period in which the microsomal suspension would retain its oxidative activity under the present experimental conditions.

The microsomes were isolated from six-day old adult flies of the DDT resistant strain and the pellets were resuspended in fresh isotonic sucrose solution. The suspension was then divided into two equal portions, one was recentrifuged in the ultra centrifuge for 30 minutes, and the resulting microsomal pellets were again resuspended in fresh sucrose solution. The other portion, not further centrifuged, was referred to as the "unwashed" microsomal preparation. The two suspensions were stored at a temperature of $1^{o}C$. during the eight-day experimental period and were tested for their hydroxylating activity by incubation of one-milliliter aliquots with the normal mixture.

As can be seen in Figure 8 the microsomal oxidative system was reasonably stable. The enzymatic activity of of the washed microsomal preparation decreased slightly in two days and lost approximately 66 per cent of its initial activity in four days and 96 per cent in eight days. The activity of the unwashed microsomes increased 24 per cent at the two day period but decreased very

FIGURE 8

THE EFFECT OF STORAGE OF A MICROSOMAL
SUSPENSION ON METABOLISM OF NAPHTHALENE
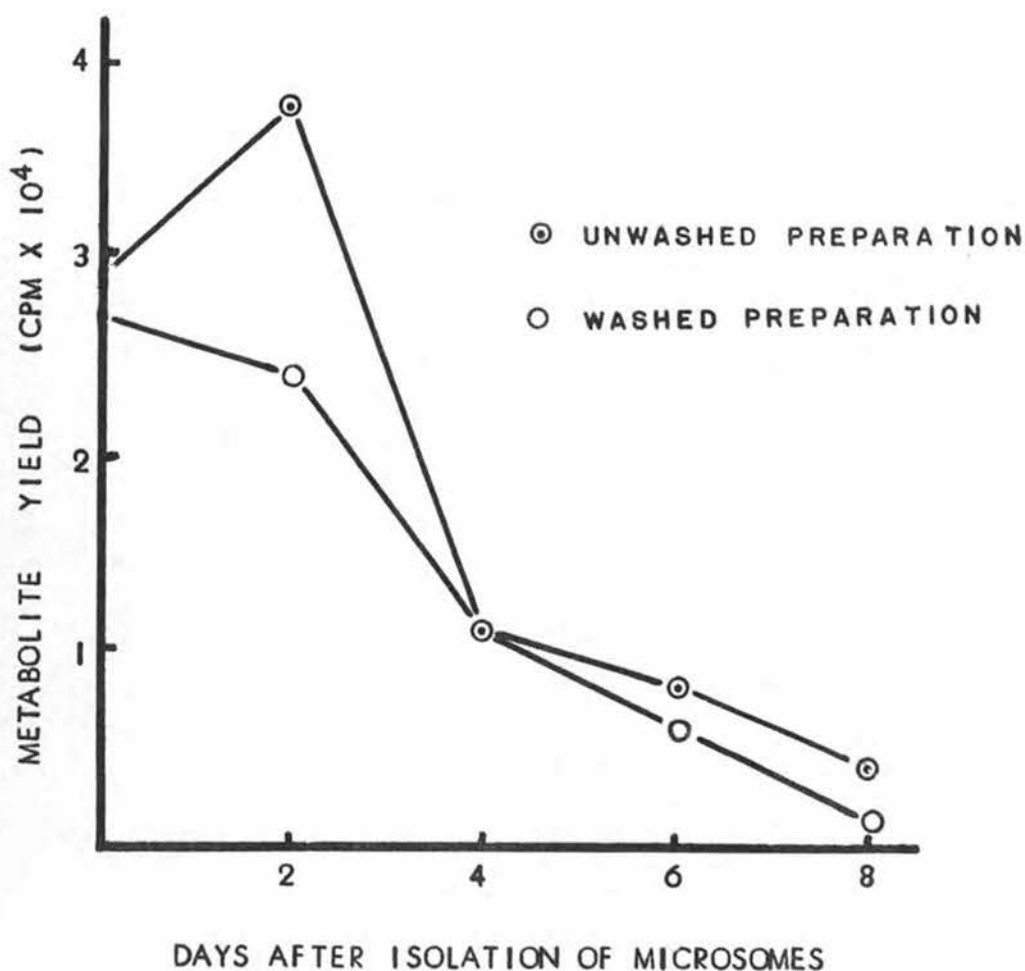
DAYS AFTER ISOLATION OF MICROSOMES

Experimental Conditions: The standard incubation mixture
was incubated aerobically at a temperature of 37° C. with
microsomes that were isolated from six-day old flies
having a protein equivalent of 13.2 mgm.

rapidly thereafter.

The increase in activity of the unwashed microsomal preparation, after storing the suspension for two days, is not fully understood. When the metabolites produced by these microsomes were resolved chromatographically however, radioactive metabolites not found in the parallel experiments with washed microsomes were present (Figure 24, Appendix). Two of these metabolites were mercaptates which were not detected in any other experiment during investigation. These could have arisen due to the release of cysteine from protein upon aging of the microsomal preparation, which could account for the increased radio-activity in the two-day old, unwashed microsomal suspension. This result may be correlated with the observations of Imai and Sato (48, p. 164-165) who found that after aging of rabbit liver microsomes for 20 hours, they were more readily solubilized than fresh microsomal preparations.

Length of Incubation Period:

The progress curves of most enzymatic reactions generally show that the velocity falls with time. It was of interest to determine the incubation period in which housefly microsomes would give maximum transformation of naphthalene. This factor was studied by isolating

microsomes from seven-day old adult flies of the DDT
resistant strain and incubating them for 2,4,8,16,32, and
47 minutes.

The results (Figure 9) showed an increasing amount of
substrate metabolized up to 32 minutes of incubation, with
the rate of increase leveling at this point. When these
conditions were repeated, the results confirmed those of
the first experiment. Booth and Boyland (1958) found the
optimum time for 1-naphthol and dihydrodiol formation by
liver microsomes to be 15 minutes, but the addition of
cysteine or EDTA (Eythylene diamine tetracetic acid) in-
creased this time to 30 minutes in the case of dihydrodiol
and to 20 minutes for 1-naphthaol. Agosin and co-workers
(1, p. 340-342) in studying the hydroxylation of DDT re-
ported that their microsomal preparations were incubated
for two hours, but they did not indicate the reason for
the long incubation period.

Age of Adult Flies:

It has been conventional in investigations of insect
biochemistry and toxicology to make use of young adult
insects. The reasons for this choice are not always clear,
although they probably include the belief that younger in-
sects are physiologically more active. As far as can be
determined there are no published reports in which the

FIGURE 9

THE EFFECT OF INCUBATION TIME
ON MICROSOMAL METABOLISM OF NAPHTHALENE
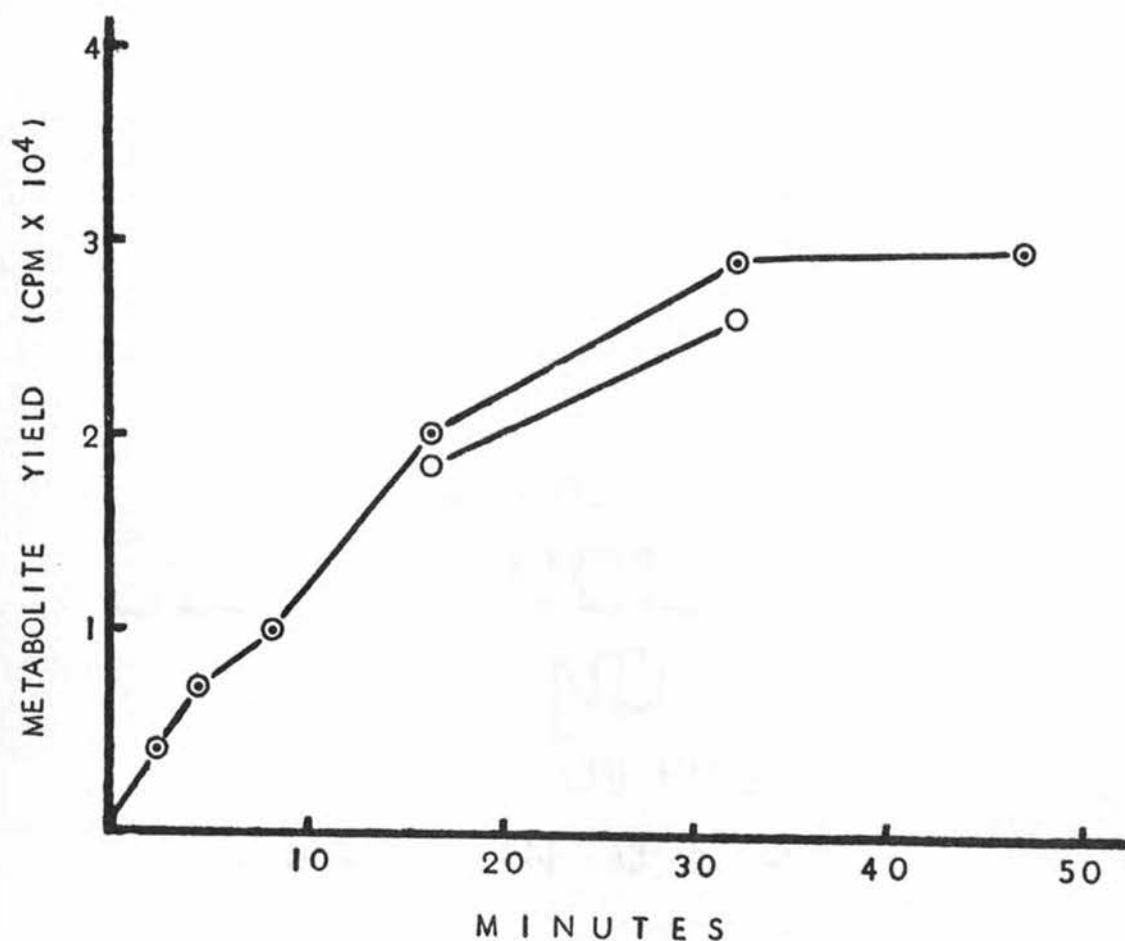
Experimental Conditions: The standard incubation mixture was incubated aerobically at a temperature of 37° C. with microsomes that were isolated from seven-day old flies having a protein equivalent of: ◉ 11.9 mgm.; ○ 12.1 mgm.

age of the test insects has been considered as a factor in the activity of sub-cell particles. In studying the effect of age on the hydroxylase activity of housefly microsomes, it was considered desirable to prepare the microsomal fraction from a single generation of insects. This was assured by isolation of a large number of pupae from the DDT resistant strain, and conducting the experiments on random samples of flies as this generation aged. The desired weight of adult flies was obtained from the colony at each sampling age, which range from one to 17 days.

When the ether extracts of these experiments were counted for radioactivity and compared from one age group to the next, a surprising trend was seen (Figure 10) in that there was a definite increase in the amount of substrate metabolized as the flies aged. The same aging effect was seen in another experiment, when the microsomes were isolated from six and 12-day old flies of resistant and susceptible strains (Table 2). It should be noted also that the outcome of these experiments was similar, although more striking, than that observed in earlier tests of co-factor requirements (Figures 6 and 7).

Reports in which insect age has been a consideration include those of Rockstein (78, p. 282-285) who found that the concentration of beta-glycerophosphatase and ATP-ase declined gradually with aging in adult houseflies.

FIGURE 10

THE INFLUENCE OF HOUSEFLY AGE
ON MICROSOMAL METABOLISM OF NAPHTHALENE
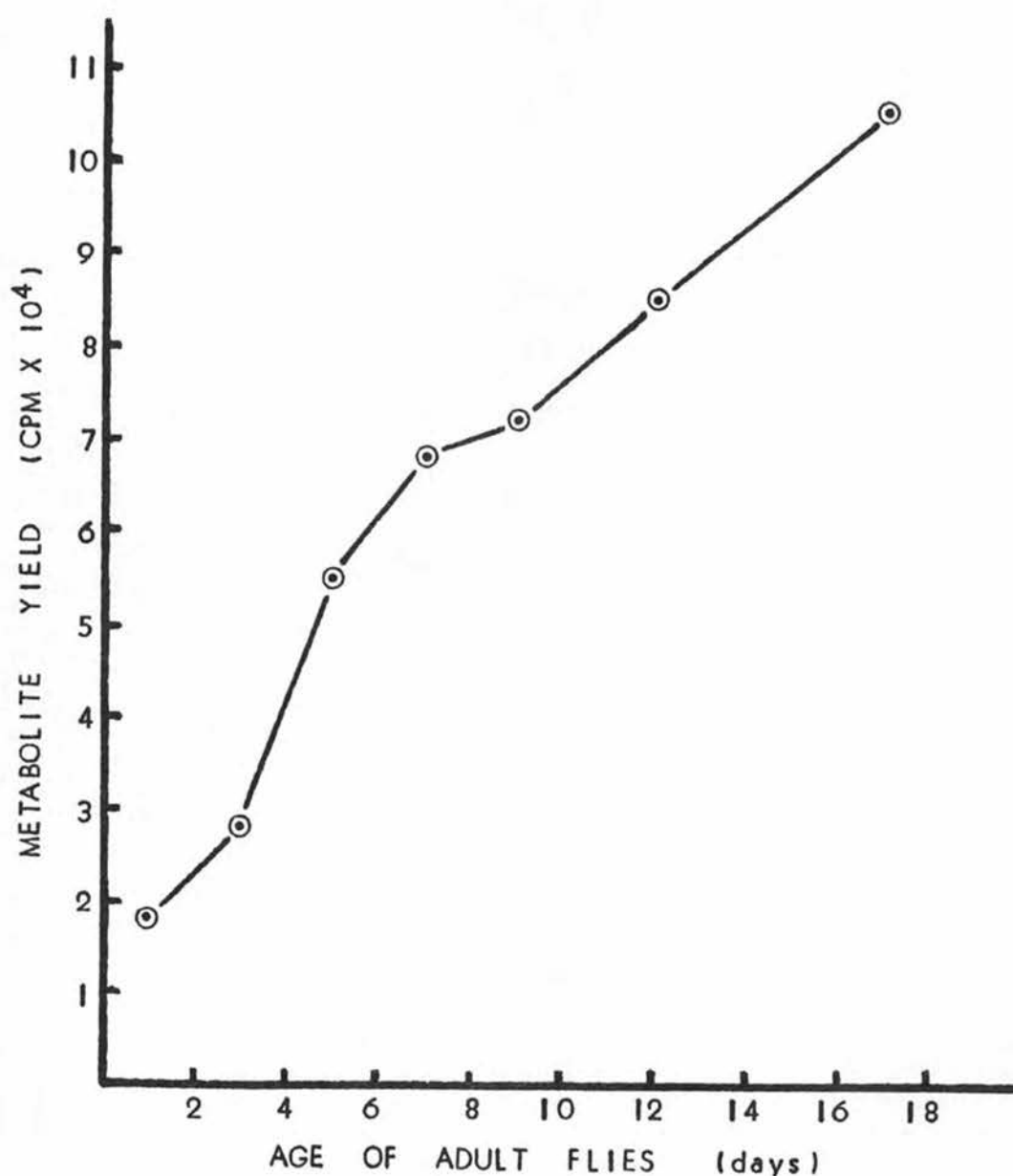
Experimental Conditions: The standard incubation mixture
was incubated aerobically at a temperature of 37° C. with
microsomes that were isolated from flies of the following
ages and having a protein equivalent of: 1 day - 11.5 mgm.
3 days- 12.1 mgm.; 5 days- 11.9 mgm.; 7 days- 12.6 mgm.;
9 days- 12.3 mgm.; 12 days- 13.2 mgm.; 17 days- 12.8 mgm.

Similarly in aging flies, he (79, p. 1476-1477) found a decline of 66 per cent in the activity of enzymes dephosphorylating organophosphorous compounds. In contrast to this, Tobias and co-workers (91, p. 287-293) reported that newly emerged flies had an LD_{50} for DDT of 2 micrograms per gram, while with older flies, the LD_{50} increased to 8-21 micrograms per gram of fly tissue. Barker (4, p. 499-500) reported that with flies treated with DDT, the dehydrochlorination product, DDE, was found in increasing quantities with aging of the adults. Thus other investigators present conflicting evidence regarding the affect of age with Rockstein showing a decrease in certain metabolic enzymes as the test insect ages, and Tobias and Barker finding an increase in the titer of detoxifying enzymes.

Although it is recognized that comparisons between in vitro and in vivo studies should be made with extreme care, the results found in the present work seem to merit a reconsideration of the age factor for such studies. As Figure 10 indicates, the investigator would obtain a system much more active in hydroxylation, and possibly in other biochemical transformations, by the use of older flies. Another point to consider is the implications concerning the biochemistry of aging. If aging is due to an acceleration of biochemical degradation, as is sometimes

indicated, hydroxylation processes may be one of those
involved. The housefly, or insects in general might be
ideal animals for basic studies on the processes involved
in aging. Not only are life cycles short, thus allowing
more rapid experimentation, but immature forms are easily
obtained and their growth control by endocrine systems is
at least partially understood.

Metabolite Yields:

One of the main objectives of this study was to
establish conditions which could provide the best yield
of metabolites. In the initial experiments, approximately
one per cent of the substrate was metabolized (Figures 2,
3 and 4) but as the study progressed, yields were increased
to eleven per cent (Figure 10, Table 2). Compared to pub-
lished data, the yields achieved with this system were
good. For example, Mitoma and co-workers (64, p. 431-441)
working with a microsomal preparation of rabbit obtained
yields of approximately one per cent, while Booth and Boy-
land (9, p. 681-688) using rat liver microsomes also achiev-
ed yields of one per cent. The major experimental con-
ditions contributing to these improved yields were the
use of older flies (Figure 10) and incubation at more
optimum temperatures (Figure 5).

Microsomal Activity of Resistant and Susceptible Strains:

Insect species differ in their susceptibility to insecticides possibly due to differing rates of metabolizing the toxicants. These species differences are also sometimes observed within a single species after the development of insecticide resistant strains. In the initial phases of this study, similar differences were observed among the four housefly strains exposed to naphthalene. It was of interest to further investigate the metabolism of naphthalene by microsomes of these strains. This was done by experiments with 6 and 12-day old flies of 3 resistant and 1 susceptible strain.

Table 2 indicates a marked difference in the amount of substrate transformed by the microsomes of the 6-day old flies with the DDT strain being more active. Except for the latter strain, these yield differences had disappeared at 12 days. This age effect had been previously noted only in the DDT resistant strain, but with these results, it appears to be a characteristic of all strains studied and is quite likely a general trait of this species. Similar strain differences have been obtained in vivo by exposing adult flies to saturated atmosphere of naphthalene, and observing the time required to narcotize 50 per cent of the sample.

TABLE 2

NAPHTHALENE METABOLISM BY MICROSOMES
OF SUSCEPTIBLE AND RESISTANT HOUSEFLY STRAINS

Strain	Metabolite Yield (cpm x 10^5)	
	6 day flies	12 day flies
Susceptible	5.52	12.42
Dieldrin resistant	6.96	11.84
Naphthalene resistant	8.55	12.77
DDT resistant	11.25	20.70

Experimental Conditions: The standard incubation mixture
was incubated aerobically at a temperature of 34°C. with
microsomes that were isolated from the following fly
strains and having a protein equivalent of: Susceptible--
12.6 and 13.0 mgms.; Dieldrin R.--11.9 and 12.4 mgm.;
naphthalene R.--13.1 and 12.8 mgm.; DDT R.--12.1 and
12.6 mgm.

The significance of these observations in explaining

the resistance phenomenon is not clear; although, one is

tempted to correlate the increased hydroxylating ability

of microsomes of resistant insects with their survival

when treated with insecticides. This concept is supported

by the report of Perry (73, p. 119-137) who attributes the

high level of resistance of houseflies to chlorinated-

hydrocarbons to larger titres of the enzyme, DDT dehydro-

chlorinase.

The role of hydroxylation in explaining resistance to insecticides such as DDT may be indicated by the work of Tsukamoto (93, p. 141-151) who noted that a hydroxylated by-product of DDT in resistant Drosophila was the compound, kelthane. Using the same species, these observations were confirmed by Menzel and co-workers (60, p. 9-12). Several workers investigating the metabolism of DDT by various resistant and tolerant species have reported the presence of water-soluble metabolites of this insecticide. Such products, sometimes found to be conjugated (89, p. 736-739; 22, p. 699), might be the result of combinations with other hydroxylated metabolites of DDT.

The recent report by Agosin and co-workers (1, p. 340-342), working with housefly and cockroach microsomes indicated that a kelthane-like product was formed when DDT was the substrate. These workers also noted a difference in the activity of housefly microsomes of different resistant strains but in contrast to the present work only a parathion resistant strain was more active. The microsomes of DDT and lindane resistant strains showed no more hydroxylating activity than the susceptible strain.

As will be discussed later in greater detail, the experiments with flies of different ages produced additional results which were of interest in regard to the aging effect. Chromatographic resolution of the

metabolites produced during these experiments revealed gross differences in the type of conjugates found, with some being absent in later stages of development in the resistant strains but not in corresponding stages of the susceptible strain.

Inhibition of Microsomal Activity:

Two compounds were tested in order to study the effect of inhibitors on microsomal metabolism of naphthalene. A commonly used insecticide synergist, piperonyl butoxide, was selected because although nontoxic by itself, will increase the effectiveness of insecticides. Similarly, the other compound, SKF 525-A has no pharmacologic activity of its own but has been shown to prolong the action of a wide variety of drugs by interfering with the biochemical mechanisms by which they are detoxified. To study the effects of these two compounds, the microsomal fraction of 12-day old flies of the DDT resistant strain was isolated and prepared by the standard methods. The inhibitors were added to the standard incubation mixture at concentrations of 2.8×10^{-3}M. (piperonyl butoxide) and 5.3×10^{-3}M. (SKF 525-A).

The two compounds markedly reduced the effectiveness of the microsomal system in metabolizing the substrate (Table 3). As the data indicate, piperonyl butoxide is

TABLE 3

THE EFFECT OF PIPERONYL BUTOXIDE AND SKF 525-A
ON THE MICROSOMAL METABOLISM OF NAPHTHALENE

Inhibitor	Metabolite yield (cpm x 10^4) Experiment	
	A	B
None	23.40	24.40
SKF 525-A (5.3×10^{-3}M.)	14.70	15.20
Piperonyl butoxide (2.8×10^{-3}M.)	6.45	7.80

Experiment Conditions: The standard incubation mixture was incubated aerobically at a temperature of 34°C. with microsomes that were isolated from 12-day old flies leaving a protein equivalent of: A - 12.5 mgm.; B - 12.8 mgm.

approximately four times more potent as an inhibitor of this system than SKF 525-A.

The compound, SKF 525-A, has been shown to block a number of microsomal reactions including N-demethylation, side chain oxidation, deamination, hydroxylation, and ether cleavage (28, p. 55-63). It also inhibits the microsomal formation of morphine glucuronides and blocks the function of nitro reductase in the soluble fraction (38, p. 480-485). The inhibition of nitro-reductase in the soluble fraction indicates that its action is not due to a physical effect on the microsomes, but, that it is a

true enzyme inhibitor. It is interesting to note that most of the drug detoxication mechanisms that are inhibited by SKF 525-A have certain factors in common--localization in the microsomal fraction and a requirement for both TPNH and oxygen (20, p. 603-604). Gillette and co-workers have considered that this inhibitor might block a reaction, common to a number of the oxidative metabolic pathways, although they have shown that the inhibiting action is not due to the interference with TPNH oxidase activity.

The effect of the synergist, piperonyl butoxide when used in conjunction with insecticides has been attributed to different factors; increased penetration of the lipoprotein nerve sheath by the toxicant (50, p. 426-427), or inhibition of lipase action (26, p. 153-155). Sun and Johnson (87, p. 261-266) have postulated that the synergistic action appears to be mainly due to the inhibition of certain biological oxidations which either activate or detoxify the insecticides. These workers found that piperonyl butoxide inhibited the epoxidation of aldrin to dieldrin and also the oxidation of methyl parathion to its oxygen analog. These ideas of Sun and Johnson appear to be confirmed in the present study in that the hydroxylation of naphthalene is definitely inhibited by piperonyl butoxide.

Influence of the Soluble Fraction on the Microsomal Activity

The soluble fraction of liver tissue from rats (20, p. 603-604), and rabbits (64, p. 431-441) has been reported as lacking the mechanism to hydroxylate acetanilide. Agosin and co-workers (1, p. 340-342) also found that the soluble fraction of cockroach tissue did not have the necessary enzymes to hydroxylate DDT. In view of these results it was decided to determine the hydroxylating activity of the soluble fraction obtained during the isolation of housefly microsomes. For this phase of the study, the microsomes were isolated from 12-day old flies of the DDT resistant strain and prepared as before except that the microsomal and the soluble fractions were each centrifuged three times at 110,800 x g. The fractions were centrifuged at a higher rate than normal in order to assure complete separation of the microsomal from the soluble fraction. The pellets were resuspended in fresh isotonic sucrose solution after each centrifugation. Aliquots of the microsomal suspension and of the soluble fraction from the first centrifugation were incubated separately in order to compare them with those receiving the more thorough centrifugations.

The results shown in Table 4 indicate that the soluble fraction has the mechanisms necessary for limited

TABLE 4

NAPHTHALENE METABOLISM BY CELL
PARTICULATE FRACTIONS OF HOUSEFLY TISSUE

Particulate fraction	Metabolite Yield (cpm x 10^4)
Microsomes (centrifuged 3x)	4.68
Microsomes (centrifuged 1x)	4.73
Microsomes plus soluble fraction (centrifuged 3x)	3.96
Soluble fraction (centrifuged 3x)	1.53
Soluble fraction (centrifuged 1x)	1.56

Experimental Conditions: The standard incubation mixture was incubated aerobically at a temperature of 34°C. with microsomal and soluble fractions isolated from 12-day old flies and leaving a protein equivalent of: microsomes-- 12.9 mgm.; soluble fractions--8.9 mgm.

metabolism of naphthalene. The additional centrifugations of the two particulate fractions did not alter their naphthalene metabolizing activity.

No qualitative differences in the metabolites pro- duced were revealed when the radioactive products of these experiments were resolved chromatographically. The metab- olism of naphthalene by the soluble fraction was unexpect- ed in view of the work of others, and is not readily explained, although it could reflect a species difference. Previous workers have used rats, rabbits, and cockroaches

in the preparation of microsomes for their studies. That
such a species difference might exist is indicated by the
work of Toppozada (92, p. 120) who reports that cock-
roaches treated with 1-naphthol excrete 11 different
metabolites while houseflies convert this drug into six
products. Another possible explanation is that during the
preparation of the microsomes used in the current studies
the tissue was homogenized so severely that hydroxylating
enzymes were released into the soluble fraction. There
also seems to be a slight possibility that a species dif-
ference in microsome size might have prevented the com-
plete centrifugation of the housefly microsomes used in
these experiments. Published reports would not support
this suggestion since it is generally agreed that centri-
fugations of 100,000 x g and above are sufficient to remove
all microsomes from a homogenate.

In a preliminary experiment designed to test the
effect of the soluble fraction on the metabolic activity
of the microsomes a slight decrease in metabolite yields
was noted (Figure 11). As can be seen in Table 4, the
combination of microsomes and soluble fraction resulted in
a similar slight decrease in radioactive products. The
only explanation that can be advanced is that the two
fractions combined contained sufficient protein to cause
a physical interference with hydroxylative activity. The

FIGURE II

THE EFFECT OF SOLUBLE FRACTION
ON MICROSOMAL METABOLISM OF NAPHTHALENE
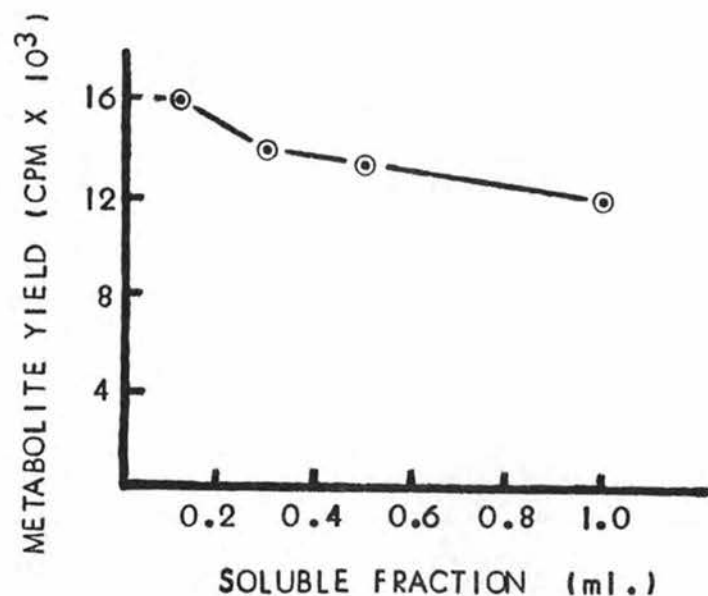
Experimental Conditions: The standard
Incubation mixture was incubated with
microsomes that were isolated from 12-day
flies, having a protein equivalent of 12.1
mgm. and with the soluble fraction which
had a protein equivalent of 9.8 mgm. per
milliliter.

biuret protein determination revealed that 1 ml of the
soluble fraction contained approximately 10 mgs of pro-
tein.

The Effect of Doubling the Concentration of Incubation Constituents

Throughout this study the contents of the incuba-
tion mixture were dissolved or suspended in a total volume
of 6 milliliters. It was desirable to determine if an
increase of substrate metabolism would result by reducing
this volume to one-half its normal level, thus doubling
the concentration of the constituents. Theoretically, the
increase in concentration, should increase the probability
of the substrate-enzyme interaction, if other complications
do not result.

The microsomes were isolated from 12-day old flies
of the DDT resistant strain. The results (Table 5) indi-
cated that the reduction in volume resulted in a slight
decrease in the amount of substrate metabolized. These
findings were confirmed in experiment 2 conducted under
identical experimental conditions. These findings cannot
be readily explained, however, it is possible that a
physical interference by protein occurred as in the pre-
vious section.

TABLE 5

THE EFFECT OF DOUBLING THE CONCENTRATION
OF THE CONSTITUENTS OF THE INCUBATION MIXTURE

Volume of incubation mixture	Metabolite yield (cpm x 10^4) Experiment	
	1	2
3 milliliters	3.66	3.28
6 milliliters	4.68	4.52

Experimental conditions: The standard incubation mixture
was incubated aerobically at a temperature of 34°C. with
microsomes that were isolated from 12-day old flies and
having a protein equivalent of 13.3 mgm. The aqueous
volume of the standard incubation mixture was reduced by
half in one set of flasks.

Color Variations in Housefly Microsomes:

An interesting phenomenon observed throughout this
study, was that the color of microsomal pellets varied
according to the age and strain of housefly used. Micro-
somal pellets of the susceptible strain were usually
yellow with a light tinge of brown, while those of the
dieldrin resistant strain were light brown. The naphtha-
lene resistant strain produced pellets of a dark brown
color with a light tinge of red at the border, and adults
of the DDT resistant strain consistently produced micro-
somes of a deep red color. These color changes were also

noted in the DDT resistant houseflies as the light-red color of the microsomes of young adults gradually changed to dark red with older insects. The larvae of the DDT resistant strain had microsomes of an olive-drab color, which changed to white in the last instar, and to a dull yellow color in the pupae stage.

Characterization of Naphthalene Metabolites:

An important part of each of the experiments described in the previous section was the paper chromatographic resolution of the metabolic products. This resulted in the detection of seven different metabolites when naphthalene was the substrate and five when 1-naphthol was used. In all experiments utilizing naphthalene, the predominant, and in some cases, the only metabolites were 1:2-dihydro-1:2-dihydroxynaphthalene and 1-naphthol. The former metabolite had an average R_f value of 0.87 in the BEW solvent system and 0.38 in BAW. The latter metabolite had an average R_f of 0.92 in BEW and 0.94 in the BAW. The former compound gave a dark blue fluorescence and the latter a black fluorescence under an ultraviolet light when exposed to NH fumes. The separation of these two metabolites in the BAW system can be seen in Figure 14 (Appendix).

Some workers have found 2-naphthol as a product of

naphthalene metabolism in living animals. The R_f values
of this metabolite and 1-naphthol are identical in the two
solvent systems mentioned above, making it necessary to
use a third system, 0.1 N NH_3 to separate them. Using
this system, the production of 2-naphthol was checked
occasionally during the investigation, always with nega-
tive results. These results indicate that this metabolite
is not a regular product of naphthalene metabolism by
housefly microsomes.

Other metabolites were not detected as consistently
as dihydrodiol and 1-naphthol, with their presence seeming
to depend somewhat on the experimental variable being
tested. A typical example of the metabolites produced
from naphthalene when incubated with the microsomes
isolated from the four strains of houseflies can be seen
in Plate 2 (Appendix). Figures 15 to 18 and Plate 2 show
that microsomes isolated from 6-day old flies of the four
strains converted the substrate to the same five metab-
olites. The results shown in Plate 2 are illustrated in
a different manner in Figures 15 to 22 (Appendix). It can
be seen that the radioactive region near the solvent
front of the chromatograms actually consists of two
metabolites when examined by the X-ray technique (Figures
15-22 and Plate 2). These are 1-naphthol and dihydrodiol.

The material seen at the origin of the chromatograms

has not been encountered in in vivo studies and is un-known at present. The other two metabolites corresponded chromatographically to 1-naphthyl glucosiduronic acid (R_f - 0.15 in BEW) and 1:2-dihydro-1-naphthyl glucos-iduronic acid (R_f - 0.22 in BEW). When the microsomes were isolated from 12-day old flies, only those from the susceptible strain retained the ability to produce the original five metabolites. The three resistant strains produced metabolites corresponding to dihydrodiol, 1-naphthol and the unknown material at the origin of the chromatographic strip (Figures 19 to 22 and Plate 2).

The relative amount of each metabolite can be seen by comparing the intensity of its radiometric spot on the X-ray film (Plate 2). The increased yield of radioactive metabolites indicated in the radioauthographs of the 12-day old flies substantiates the data shown in Table 3.

The radioautographs (Plate 2) and the radiochromato-grams (Figures 15 to 22) show that 12-day old flies of the three resistant strains lack the capacity to conjugate the hydroxylated products of naphthalene although this ability was present in the younger insects and in the 12-day old susceptible flies. These findings are difficult to ex-plain since there are very definite indications that the hydroxylating ability of housefly microsomes increases with aging of the adults. One would expect that the

titres or the ability of the conjugating enzymes to increase correspondingly as shown by the susceptible strain.

When the unwashed microsomal suspension was stored for two days two additional metabolites were produced upon incubation with naphthalene, (Figure 23) chromatographically, these correspond to N-acetyl-S (1,2-dihydro-2-hydroxy naphthyl) cysteine (R_f - 0.38 in BEW) and N-acetyl-S (1-naphthyl) cysteine (R_f - 0.58 in BEW). A possible explanation for the presence of these mercaptates has been advanced in an earlier section, "Stability of Enzymatic Activity."

In parallel experiments, 1-naphthol was used as the substrate instead of naphthalene in an attempt to establish the position of this metabolite in the metabolic sequences. Under these conditions, no metabolite corresponding to 1:2-dihydro-1:2-dihydroxynaphthalene could be detected in the three solvent systems BEW, BAW, and CAW. Two new metabolites not found in naphthalene studies were produced however. Chromatographically, these two compounds corresponded to 1:2-dihydro-2-hydroxy-1-naphthyl glucosiduronic acid (R_f - 0.07 in BEW) and an unknown metabolite with an R_f value of 0.74 in the BEW system (Figure 24, Appendix). The presence of the former compound is confusing since dihydrodiol is considered an intermediate in its formation, and therefore must have

had at least a transitory existence in the mixture. Such traces of diol could have been present but obscured by the relatively large amounts of 1-naphthol present when this compound was being tested as a substrate for the microsomes. In *vivo* studies with houseflies (90, p. 620-623) have indicated that 1-naphthol is converted to the diol by these insects.

A summary of the metabolites produced by the housefly microsomes is shown in Table 6. The R_f values obtained from standard compounds and those that have been published as a result of in *vivo* studies are also presented. It is of interest that the present in *vitro* investigation resulted in the detection of all metabolites observed in in *vivo* studies with the same species (90, p. 620-623) except the sulphate conjugates 2-hydroxy-1-naphthyl sulphate and 1-naphthyl sulphate.

The results obtained in the present study disagree with those reported by Mitoma and co-workers (64, p. 431-441) and Booth and Boyland (9, p. 681-688) who were unable to detect any metabolites other than dihydrodiol and 1-naphthol when naphthalene was incubated with their microsomal preparations. Furthermore, Booth and Boyland could not detect any degradative products of 1-naphthol when it was used as the substrate. These differences cannot be easily attributed to the species used. Since Terriere

TABLE 6

PUBLISHED AND EXPERIMENTAL R_f VALUES OF
NAPHTHALENE AND 1-NAPHTHOL METABOLITES

(Solvent System BEW)

Metabolites	Reference R_f values		Experimental R_f values	
			in vitro	
	Stand-ards	House-flies[1]	Naphtha-lene	1-Naph-thol
		in vivo		
Unidentified	-	-	Origin	Origin
1:2-dihydro-2-hydroxy-1-naphthol glucosidur-onic acid	.07	.07	-	.07
1-naphthyl glucosidur-onic acid	.12	.13	.15	.15
1:2-dihydro-1-naphthyl glucosiduronic acid		.24	.22	.22
N-acetyl-S (1,2-dihdro-2-hydroxy naphthyl) Cysteine	.39	.38	.38	-
2-hydroxy-1-naphthyl sulphate	.56	.45	-	-
N-acetyl-S (1-naphthyl) cysteine	.56	.54	.58	-
1-naphthyl sulphate	.68	.66	-	-
Unidentified		.77	-	.74
1:2-dihydro-1:2-dihydroxy naphthalene	.86	.86	.87	-
1-naphthol	.93	.93	.92	.93

[1](90, p. 620-623)

and co-workers (90, p. 620-623) have shown that in vivo, houseflies produce the same metabolites as those found in vertebrates (31, p. 132-140; 14, p. 679-683; 15, p. 38-40; 16, p. 440-446). A species difference at the microsome level might explain these results.

The detection of metabolites not heretofore reported by others working with microsomes can probably be attributed to the sensitivity of the methods used. In the present study 48 micrograms of radioactive naphthalene were incubated with the housefly microsomal preparations and the analytical methods used were capable of detecting as little as 0.02 micrograms of the metabolic products. Mitoma, et al. (64, p. 431-441) on the other hand, incubated 512 micrograms while Booth and Boyland (9, p. 681-688) utilized 51,000 micrograms of naphthalene in their systems and both authors detected their metabolites by relatively less sensitive colormetric methods.

Suggested Hydroxylation Pathways:

The metabolism of naphthalene by houseflies obviously depends upon a primary hydroxylation. With the evidence obtained during the present investigation indicating that an intermediate compound precedes the final hydroxylated form, a discussion of the structure of such a

compound and its place in the biosynthetic sequence seems worthwhile. Compounds 2 to 5 (Figure 12) might be considered candidates for such a role.

Compound No. 2, 1:2-dihydroxynaphthalene, has been isolated from urine of pigs. While its occurrence in the free form in other species has not been reported, it is considered a probable precursor to the well known naphthalene conjugate, 2-hydroxy-1-naphthyl sulphate. Although this compound could be a precursor to dihydrodiol (compound No. 6), it is more likely that the reverse is true. Furthermore this compound is not volatile and hence could not be the suspected transitory compound of this investigation.

The compound, 1:2-dihydro-1-hydroxynaphthalene (compound No. 3), has been included as a possible intermediate since its conjugated from, 1:2-dihydro-1-naphthyl glucosiduronic acid has been found by others and was indicated in the present study. The conversion of this compound to dihydrodiol would require further hydroxylation by processes for which there is still no evidence. The volatility of this compound is difficult to predict, although it is suspected to be less volatile than naphthalene.

Compound No. 4, 1:2-dihydronaphthalene, is presented as a possible intermediate since _in_ _vivo_ studies have

shown that it is converted by rats to small amounts of dihydrodiol. Its vapor pressure would be suspected to be similar to that of naphthalene and thus eliminates it from consideration at this time.

The formation of dihydrodiol from an epoxide precursor (compound No. 5) suggests another route for the metabolism of naphthalene. It is considered possible that the voktile metabolite observed during the present study could be such a compound, which on hydroxylation would yield the dihydrodiol. However, this does not explain the observed requirement for TPNH in the hydroxylation of naphthalene. The requirement for this co-factor suggests the possibility that 1:2-epoxy-1:2-dihydronaphthalene (compound No. 5) is reduced to 1:2-dihydro-1-hydroxynaphthalene (compound No. 3) which can then be oxidized by some unknown mechanisms to dihydrodiol. Evidence presented by other workers also suggests that an epoxide is a likely intermediate in the formation of dihydrodiol.

There is little doubt that both dihydrodiol and 1-naphthol are produced when naphthalene is the substrate. Whether they appear through independent reactions or in a sequence is still a mystery. In the present investigation, when 1-naphthol was used as the substrate, no dihydrodiol could be detected, possibly due to experimental difficulties.

Other workers have also failed to show the in vitro
formation of dihydrodiol from 1-naphthol, although the
sensitivity of their detection methods was low. Thus the
information available from in vitro studies is at present
inconclusive. Other evidence, however, suggests that
dihydrodiol is converted to 1-naphthol. The dihydrodiol,
for example, is readily converted to 1-naphthol by chem-
ical means. Also, in vivo studies have shown that rats
treated with dihydrodiol will produce 1-naphthol and its
conjugates.

Compound No. 7, 1:2-naphthoquinone, is shown here
because of its chemical derivation from dihydroxynaph-
thalene and the demonstration that it arises in bacteria
exposed to dihydrodiol. Its natural metabolic occurrence
in other species has not been demonstrated.

On the basis of the estimated relative volatility
of the four compounds and the expected TPNH requirement
in the formation of compounds 2, 3, and 4 and its probable
nonessentiality in the formation of compound No. 5, it
can be argued that the latter is the first intermediate
in the conversion of naphthalene to the dihydrodiol.
Naphthalene could be epoxidated by the direct addition
of oxygen to a double bond. The epoxide on hydration would
yield dihydrodiol or on reduction could yield the inter-
mediate No. 3 which could lead to dihydrodiol formation.

The latter compound could be converted to naphthol by dehydration. The place of an epoxide in such a scheme is given further support by the well documented conversion of several cyclodiene insecticides to their epoxide counterparts by various insect species.

FIGURE 12

PROPOSED METABOLIC PATHWAYS
IN THE HYDROXYLATION OF NAPHTHALENE
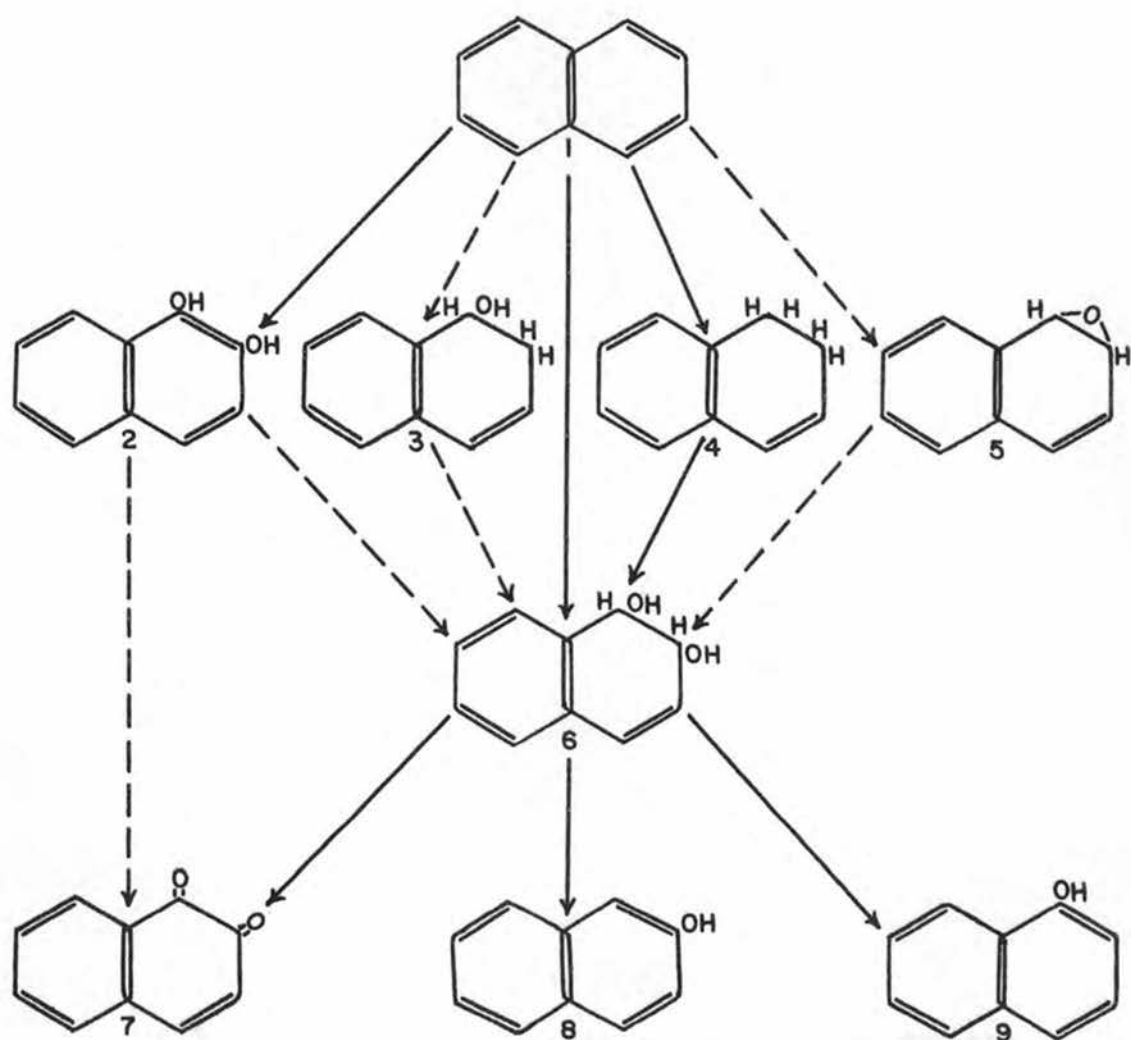
solid line- confirmed; broken line- hypothetical reactions

(1) naphthalene; (2) 1:2-dihydroxynaphthalene; (3) 1:2-dihydro-1-hydroxynaphthalene*; (4) 1:2-dihydronaphthalene; (5) 1:2-epoxy-1:2-dihydronaphthalene*; (6) 1:2-dihydro-1:2-dihydroxynaphthalene; (7) 1:2-naphthoquinone; (8) 2-naphthol; (9) 1-naphthol   * has not been isolated

ENTOMOLOGICAL IMPLICATIONS OF FINDINGS

The research results reported here touch upon several interesting aspects of insect toxicology and biochemistry. An appraisal of these results would not be complete without some consideration of the inferences that can be made.

The documentation that insect microsomes can hydroxylate aromatic organic compounds such as naphthalene proves the insect's ability to modify foreign molecules and pinpoints the cellular site of such processes. Hydroxylation could very well be the key to the phenomena of cross tolerance, the development of resistance to entire classes of insecticidal compounds. The introduction of hydroxyl groups as sites for conjugation helps greatly in explaining the long known conversion of compounds such as DDT to water soluble excretory products. These results also reconfirm the idea that high level resistance is primarily due to the selection of detoxication enzymes.

The demonstration that a well known synergist inhibits the hydroxylation reaction implies that synergism, which has long baffled entomologists, is a matter of the interference with key biochemical reactions in the modification of insecticides.

Whether the young adult insect is always the ideal organism for toxicological or biochemical studies is open

to question in view of the current findings. Another implication regarding further research with insects or insect tissue is that temperature may well have an important influence on the biochemical processes under consideration.

The success achieved in isolating active cellular particles demonstrates that a new type of "dissection" technique can be used by entomologists interested in the functional as well as the structural aspects of insect tissue.

The finding that microsomal biochemistry is influenced by age of the organism has additional implications. Research on the processes involved in aging might well be accelerated by the use of insects as experimental animals.

Finally, on a comparative biochemical scale, these results indicate that at the sub-cellular level insect tissue and that of higher animals utilize similar, if not identical, biochemical processes. This leads to the implication that the selective toxicity of insecticides is a matter of some action other than interference with fundamental processes. These implications can now be more thoroughly substantiated using the techniques and procedures established in the present investigation.

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A P P E N D I X

APPENDIX

Plates

Figures

Figures

PLATE I

PLATE   2

FIGURE 13

$R_F 0.87$ $R_F 0.93$

FIGURE 14

$R_F 0.38$ $R_F 0.94$

FIGURE 15

R_F 0.15   R_F 0.22

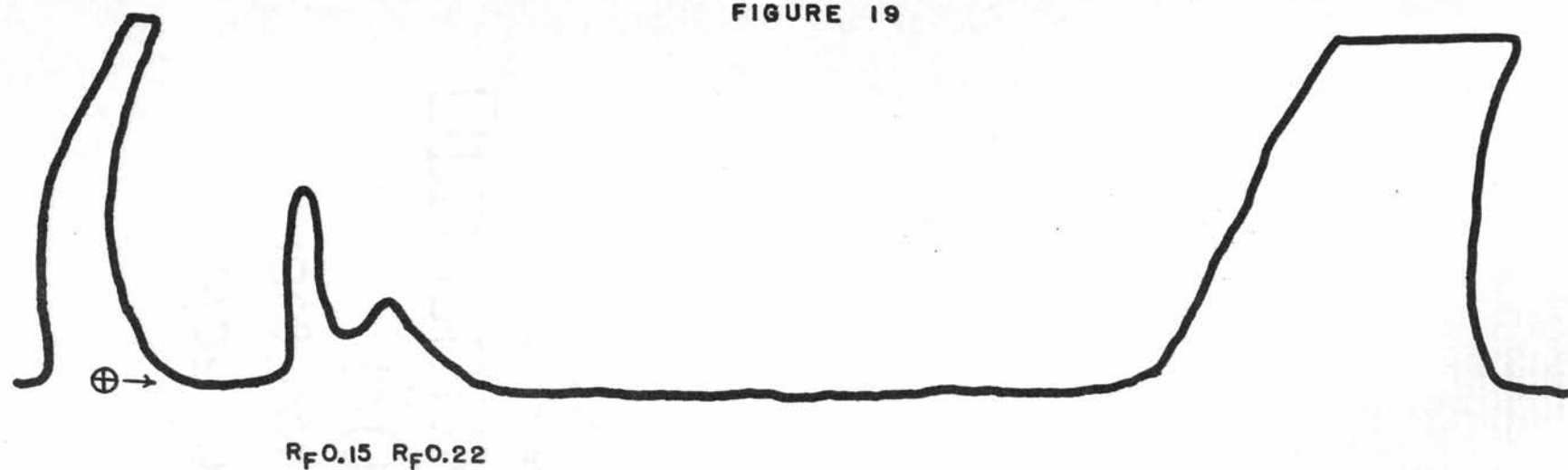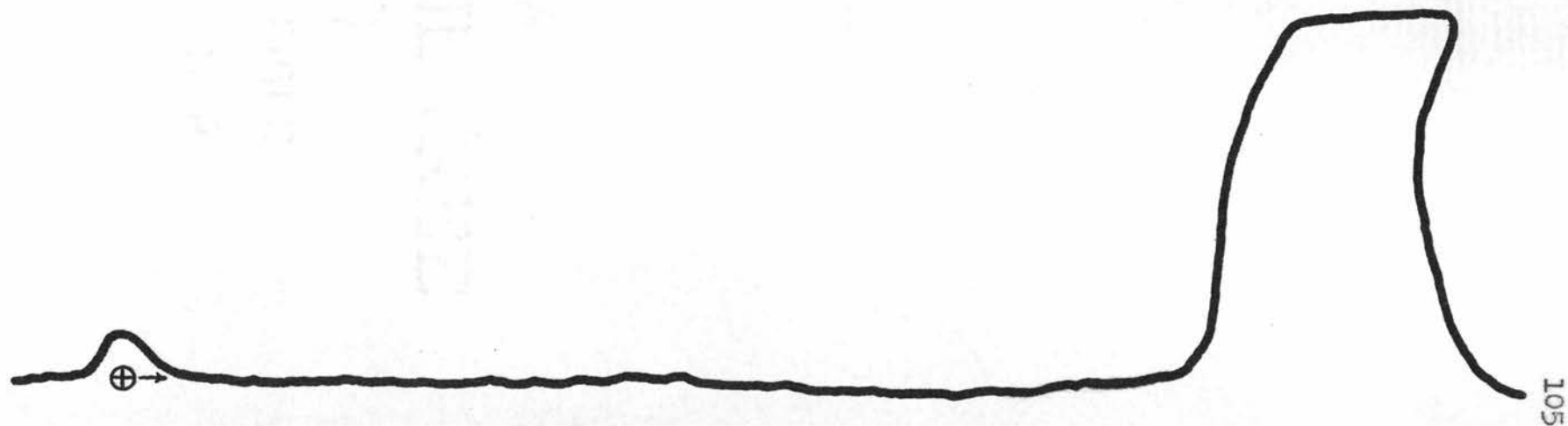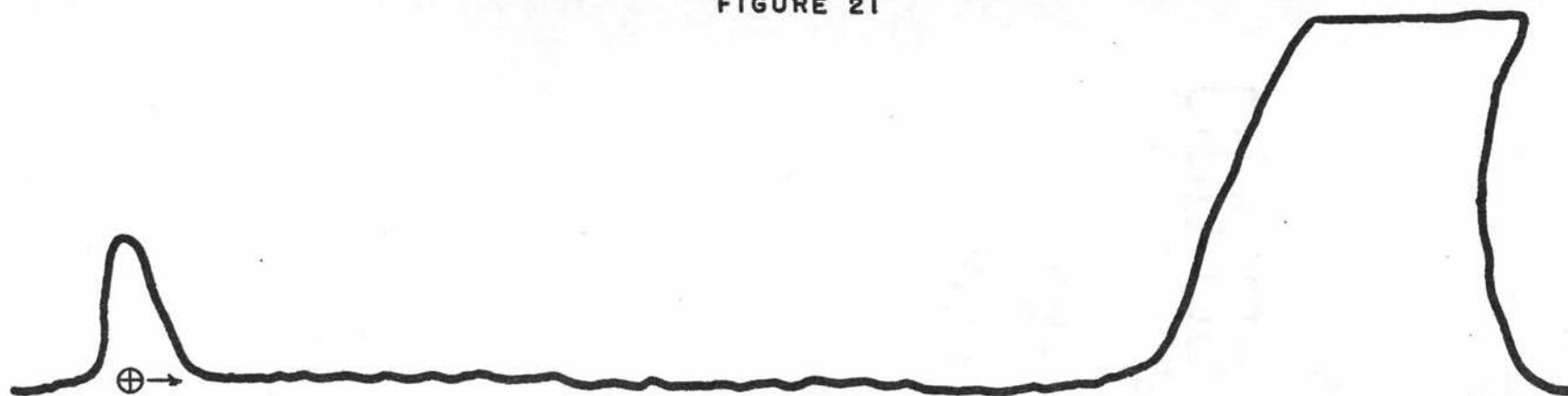FIGURE 16

FIGURE 17

$R_F 0.15$ $R_F 0.22$

FIGURE 18

FIGURE 19

$R_F 0.15$ $R_F 0.22$

FIGURE 20

FIGURE 21

FIGURE 22

FIGURE 23

R_F 0.22 R_F 0.38 R_F 0.58

FIGURE 24

R_F 0.07   0.15   0.22 R_F 0.74 R_F 0.93