Biochemical Characteristics of Insect Microsomes

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Electron microscope studies indicate that, although insect microsomal preparations resemble those from mammalian liver, the methods of preparation must be varied from one species to another and from one tissue to another. Oxidation of xenobiotics is dependent, in *Musca domestica*, on a microsomal electron transport system which includes an NADPH oxidase and a CO-binding pigment. Preliminary studies indicate that the latter is generally similar to cytochrome P_{450} from mammalian liver. NADPH oxidation has been studied in intact microsomes and the NADPH oxidase (NADPH-cytochrome c reductase, EC 1.6.2.3) solubilized and purified 100-fold. Inhibitor studies indicate that an —SH group is essential for activity. In *Musca domestica* microsomes, both oxidase and β-esterase activity are readily inducible by chlorinated insecticides, much as they are in mammals. Whether the increase represents a total stimulation of microsomal protein synthesis or a specific increase in particular components is unknown. Many insecticide-resistant strains of houseflies possess unusually high levels of microsomal oxidase activity. Genetically, high activity is controlled by single semidominant genes on chromosomes 2 and 5. Substrate specificities vary between strains having different genes for high oxidase levels, but to date no adequate biochemical characterization of the difference has been made.

STRUCTURE, PREPARATION, AND CONSTITUENT ENZYMES

Microsomal mixed-function oxidases play a major role in the metabolism of xenobiotics in mammals (Gillette *et al.*, 1969) and insects (Hodgson, 1968). The term "microsomes" describes a high-speed sediment prepared by centrifugation of the post-mitochondrial supernatant of a homogenate of plant or animal tissue, and is composed primarily of material derived from the endoplasmic reticulum of the cell (Siekevitz, 1963).

Although valuable information has been gained from *in vivo* investigations, time limitations on this presentation and the fact that *in vitro* investigations have not previously been reviewed make it desirable to limit the material covered to that derived from reactions carried out *in vitro* which fill at least some of the following criteria: they are catalyzed by a particulate enzyme system prepared from the post-mitochondrial supernatant of an insect homogenate; they require NADPH and molecular oxygen for activity; they are inhibited by CO.

Structure. Cassidy *et al.* (1969) examined the ultrastructure of microsomal preparations from *Musca domestica* and *Prodenia eridania* by electron microscope. The microsomes from adult housefly abdomens and from the gut and fat body of sixth instar *Prodenia* larvae were prepared by homogenizing in Millonigs phosphate-buffered sucrose, and after a preliminary centrifugation to remove mitochondria and heavier particles, were sedimented by centrifugation at 100,000 G for 60 min. The pellets were fixed in glutaraldehyde, followed by OsO_4, then dehydrated and embedded in Maraglas 732. Following sectioning and staining with lead citrate and uranyl acetate, the sections were examined in the electron microscope.

The components of these enzyme preparations are similar to smooth microsomes from mammalian liver (Gram *et al.*, 1967) and consist of membranous vesicles, broken membranes, and free ribosomes. Clusters of glycogen granules were a feature of the *Prodenia* gut preparations. A preliminary centrifugation at 10,000 G for 15 min removed all mitochondria from housefly preparations, but was inadequate for this purpose when *Prodenia* gut was being used and higher speeds were necessary for this purpose. The fine structure of the membranes was intact in housefly abdomen and *Prodenia* gut preparations under these conditions, but those in *Prodenia* fat body microsomes were not, as the pellet contained disrupted membranous vesicles and discontinuities in the membrane fragments. This difference between gut and fat body microsomes became even more dramatic when phosphate buffer was substituted for buffered sucrose. It is apparent that buffering systems and centrifugation speeds must be varied, not only from one organism to another, but also from one tissue to another in the same organism. Studies of the microsomal fraction from *Periplaneta americana* fat body which activated parathion indicated that it is derived primarily from the endoplasmic reticulum (Brindley and Dahm, 1970).

Intact Microsomes. SUBSTRATE SPECIFICITY. Many xenobiotics have been shown to be metabolized by isolated insect microsomes, including most types of insecticides as well as some relatively nontoxic compounds. Although most of these reactions are detoxifying, a significant number give rise to products of greater toxicity than the substrate. Substrates metabolized are listed below.

DDT is oxidized to a more polar metabolite, possibly Kelthane (Agosin *et al.*, 1961, 1964, 1969; Dinamarca *et al.*, 1962; Gil *et al.*, 1968; Oppenoorth and Houx, 1968; Plapp and Casida, 1969; Tsukamoto, 1959; Tsukamoto and Casida, 1967a).

Cyclodienes may be epoxidized (Brooks and Harrison, 1969; Brooks *et al.*, 1970; Chan *et al.*, 1967; Khan, 1969a,b; Khan and Terriere, 1968; Krieger and Wilkinson, 1969, 1970; Lewis *et al.*, 1967; Plapp and Casida, 1969; Ray, 1967; Schonbrod *et al.*, 1968; Tsukamoto and Casida, 1967a) or hydroxylated (Brooks and Harrison, 1966, 1967a,b, 1969; Brooks *et al.*, 1970; Krieger and Wilkinson, 1970).

Carbamates may undergo *N*-dealkylation (Shrivastava *et al.*, 1969; Tsukamoto and Casida, 1967a,b; Tsukamoto *et al.*, 1968), *N*-methylol formation (Dorough and Casida, 1964; Hansen and Hodgson, 1970; Kuhr, 1969; Matthews and Hodgson, 1966; Plapp and Casida, 1969; Price and Kuhr, 1969; Tsukamoto and Casida, 1967a,b; Tsukamoto *et al.*, 1968), ring hydroxylation (Dorough and Casida, 1964; Kuhr, 1969; Plapp and Casida, 1969; Price and Kuhr, 1969;

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Shrivastava *et al.*, 1969; Tsukamoto and Casida, 1967a,b; Tsukamoto *et al.*, 1968), *O*-dealkylation (Plapp and Casida, 1969; Shrivastava *et al.*, 1969; Tsukamoto and Casida, 1967a,b; Tsukamoto *et al.*, 1968), sulfoxidation (Tsukamoto and Casida, 1967a,b), or alkyl side chain elongation (Tsukamoto and Casida, 1967a). Persuasive evidence (El-Aziz *et al.*, 1969; Metcalf *et al.*, 1967) has been presented to support the hypothesis that some carbamate ring hydroxylations are carried out by another mixed-function oxidase, phenolase. In view of the close similarity in some characteristics of the two systems and the contradictory evidence of Kuhr (1969), this interesting hypothesis must be regarded as tentative.

Organophosphates may be either activated (ElBasheir and Oppenoorth, 1969; Fenwick, 1958a,b; Fukami and Shishido, 1963; Nakatsugawa and Dahm, 1962, 1965a,b; Nakatsugawa *et al.*, 1968; Plapp and Casida, 1969; Tsukamoto and Casida, 1967b) or degraded (ElBasheir and Oppenoorth, 1969; Lewis, 1969; Nakatsugawa and Dahm, 1965a,b; Nakatsugawa *et al.*, 1968; Yang, 1970).

In our laboratories, Yang (1970) has been investigating a microsomal enzyme system in susceptible and resistant houseflies which degrades diazinon and diazoxon to diethylphosphorothioic and diethylphosphoric acids, respectively. This system is located in the microsomal fraction prepared from the abdomens of adult houseflies, requires NADPH and oxygen, and is inhibited by CO. Diazinon is a better substrate than diazoxon and the two substrates are competitive inhibitors of each other. In a diazinon-resistant strain (Folsom *et al.*, 1970) the enzyme activity is approximately four-fold higher; and this increase in activity is correlated with an increase in *N*-demethylation, *O*-demethylation, NADPH oxidation, oxygen consumption, and cytochrome P_{450} content.

Naphthalene is hydroxylated by isolated insect microsomes (Arias and Terriere, 1962; Hansen and Hodgson, 1970; Philleo *et al.*, 1965; Schonbrod and Terriere, 1966; Schonbrod *et al.*, 1965, 1968).

Methylene dioxyphenyl synergists are metabolized in a variety of ways, including oxidation at the methylene carbon resulting in a splitting of the methylene dioxyphenyl ring (Casida *et al.*, 1966; Esaac and Casida, 1969; Wilkinson, 1967; Wilkinson and Hicks, 1969). Since the original suggestion of Sun and Johnson (1960) that these compounds act by inhibiting oxidative metabolism, they have been shown to inhibit, *in vitro*, the microsomal oxidation of a number of insecticides in insects. These include cyclodienes (Brooks and Harrison, 1967a,b; Krieger and Wilkinson, 1969; Lewis *et al.*, 1967; Wilkinson and Hicks, 1969), carbamates (Shrivastava *et al.*, 1969; Tsukamoto *et al.*, 1968; Wilkinson, 1967), organophosphates (Lewis, 1969; Nakatsugawa and Dahm, 1965a,b), chemosterilants such as hempa (Akov *et al.*, 1968), rotenone (Fukami *et al.*, 1969), naphthalene (Philleo *et al.*, 1965) and methylene dioxyphenyl compounds themselves (Wilkinson and Hicks, 1969). It now seems to be generally agreed that methylene dioxyphenyl synergists function as alternate substrates for the mixed-function oxidases of the microsomes (Casida *et al.*, 1966; Philleo *et al.*, 1965; Wilkinson and Hicks, 1969) although the details of the interaction are not entirely clear. This is supported by our own (Philpot and Hodgson, 1970) observation that piperonyl butoxide forms a typical type I substrate difference spectrum with cytochrome P_{450} from houseflies. Methylene dioxyphenyl synergists are degraded more slowly by insect than by mammalian microsomes (Casida *et al.*, 1966; Wilkinson and Hicks, 1969), a factor which should enhance their selectivity as synergists.

Table I. Intracellular Distribution of Microsomal Activity

Fraction		% Total Activity		
		pNA[a]	DpNC[b]	Naphthalene
Nuclei and debris	R–2,000	17	15	18
Mitochondria	R–12,000	15	15	17
Microsomes	R–100,000	68	68	65
Soluble	S–100,000	0	2	0

[a] *p*-Nitroanisole. [b] *N,N*-dimethyl-*p*-nitrophenyl carbamate.

Rotenone is hydroxylated (Fukami *et al.*, 1967, 1969) while pyrethrins are metabolized to a number of compounds, the most important of which are carboxyl derivatives (Plapp and Casida, 1969; Yamamoto and Casida, 1966; Yamamoto *et al.*, 1969).

Other reactions catalyzed, *in vitro*, by insect microsomes include the *N*-demethylation of the chemosterilant, hempa (Akov and Borkovec, 1968; Akov *et al.*, 1968), *O*-demethylation of *p*-nitroanisole (Hansen and Hodgson, 1970), oxidation of *p*-nitrotoluene to *p*-nitrobenzoic acid (Chakraborty and Smith, 1964, 1967; Chakraborty *et al.*, 1967) and alkyl side chain oxidation of *n*-propylbenzene and *i*-propylbenzene (Chakraborty and Smith, 1967).

Methods of Preparation and Assay Conditions. There is almost as much variation among preparative methods as there are pertinent publications in the literature. With certain notable exceptions, the optimum conditions are seldom clearly established nor is the essentiality of added cofactors. In many cases fractions other than the microsomes are not checked, so that the reaction in question could, conceivably, occur in other subcellular fractions.

Our approach to this problem (Hansen and Hodgson, 1970) has been to select a group of relatively simple substrates, each of which undergoes a different type of reaction, and to characterize the preparative methods and assay conditions for maximum activity using microsomes from the housefly. The substrates used are *N,N*-dimethyl-*p*-nitrophenyl carbamate (*N*-dealkylation), *p*-nitroanisole (*O*-dealkylation), and naphthalene (hydroxylation). Initial experiments (Table I) established that the reactions are all microsomal, the reduced activity in the other particulate fractions probably being due to microsomal contamination, as they were not washed. Preliminary experiments on the method of homogenization confirmed the earlier observation of Schonbrod and Terriere (1966) that a Potter-Elvehjem homogenizer with a Teflon pestle is superior to blender-type homogenizers.

A comparison of several homogenizing media used by other workers, namely 0.25*M* phosphate buffer, 0.71*M* KCl, 0.25*M* sucrose with 0.15*M* phosphate buffer, 1.15% KCl, and 0.25*M* sucrose revealed that the first, 0.25*M* phosphate buffer, gave maximal activity with all three reactions. Subsequent examination of this effect revealed that the critical feature was the ionic strength, and that 0.20*M* phosphate buffer gave the best results. It was apparent, however, that KCl was essentially as good if the ionic strength was the same (Table II), while the pH was not critical between 7.0 and 8.0. The stability of microsomes is also dependent on the ionic strength of the preparative medium, while bovine serum albumin, necessary in the assay medium, is without effect in the preparative medium.

Examination of the conditions for assay showed that if the ionic strength and pH are carefully controlled, Tris and phos-

Table II. Effect of Ionic Strength of Preparative Medium

Ionic Strength	KHPO$_4$ (pH 7.8) M	% Maximum Activity pNA	DpNC	KCl (pH 6.9) M	% Maximum Activity pNA	DpNC
0	0	...	...	0	74	57
0.14	0.05	63	80	0.14	76	...
0.28	0.10	68	80	0.28	79	64
0.43	0.15	85	90	0.43	87	69
0.57	0.20	100	100	0.57	89	74
0.71	0.25	96	99	0.71	95	83
0.85	0.30	93	82	0.85	90	81
1.13	0.40	81	75	1.13	86	72
1.42	0.50	72	53	1.42	82	...
1.70	0.60	58	38	1.70	75	69
2.83	1.00	...	...	2.83	52	...

phate buffers give the same results, and that the optimum pH for all three reactions shows a broad peak between 7.7 and 8.1. This compares with the following: 7.0 to 7.5 for organophosphate activation in microsomes from the fat body of *Periplaneta americana* (Nakatsugawa and Dahm, 1965a,b); 7.8 for aldrin epoxidation by microsomes from the gut of sixth instar larvae of *Prodenia eridania;* 7.8 to 8.0 for hydroxylation of naphthalene by housefly microsomes (Arias and Terriere, 1962); 8.5 to 8.8, 8.5, and 9.0 for DDT oxidation in the German cockroach, the housefly, and *Triatoma infestans*, respectively (Agosin *et al.*, 1961, 1969; Gil *et al.*, 1968); 8.0 for hempa demethylation by housefly microsomes (Akov *et al.*, 1968); 8.0 for carbaryl metabolism by microsomes from the larval fat body of *Calliphora erythrocephala* (Price and Kuhr, 1969); 8.2 for aldrin epoxidation and dihydroaldrin hydroxylation in housefly microsomes (Brooks and Harrison, 1969; Ray, 1967).

The effect of temperature was examined and a peak of activity was apparent between 30° and 33° C. This compares with 30° C for DDT hydroxylation in *Triatoma infestans* microsomes (Agosin *et al.*, 1969), 34° C for naphthalene hydroxylation in housefly microsomes (Arias and Terriere, 1962), and 37° C for DDT hydroxylation in housefly microsomes (Gil *et al.*, 1968).

Most of the above experiments were carried out using a glucose-6-phosphate-glucose-6-phosphate dehydrogenase system for generating NADPH. This system, however, offered no significant advantage over added NADPH. As indicated by Tsukamoto and Casida (1967), added bovine serum albumin was stimulatory.

A striking feature of the above investigation was that all parameters appear to be closely similar for all three reactions, suggesting a common enzyme system, or at least systems with a rate-limiting step in common. This is in agreement with the work of Khan (1969b).

DISTRIBUTION BETWEEN TISSUES. In vertebrates, the liver always has a higher level of microsomal oxidation than any other tissue. In insects there is no analogous organ, as an examination of the literature shows the gut to have the highest activity in sixth instar *Prodenia eridania* larvae (Krieger and Wilkinson, 1969), in *Heliothis zea* and *Anthaerea polyphemus* (Khan, 1969a), and in the housefly (Tsukamoto and Casida, 1967b), while the fat body is highest in *Calliphora erythrocephala* larvae (Price and Kuhr, 1969) and the malpighian tubes in the cockroach, *Periplaneta americana* (Nakatsugawa and Dahm, 1962).

Isolated abdomens of houseflies are usually used for the preparation of microsomes because of the presence of endogenous inhibitors in the head and thorax (Lewis *et al.*, 1967; Matthews and Hodgson, 1966; Tsukamoto and Casida, 1967a, b). Inhibitors have also been found in the locust, *Schistocerca gregaria* (Chakraborty *et al.*, 1967), *Prodenia eridania*, the southern armyworm (Krieger and Wilkinson, 1970) and the cockroach, *Periplaneta americana* (Nakatsugawa and Dahm, 1965a; Fukami *et al.*, 1969). These inhibitors may inhibit microsomal oxidations in vertebrate preparations (Chakraborty *et al.*, 1967; Kukami *et al.*, 1969; Matthews and Hodgson, 1966). They are not, however, all similar compounds. While the inhibitor from *Prodenia* has been shown to be a proteolytic enzyme (Krieger and Wilkinson, 1970) and one of those from the cockroach to be a small molecular weight protein (Fukami *et al.*, 1969), that from the housefly is heat stable and dialyzable (Matthews and Hodgson, 1966).

VARIATIONS WITH AGE AND SEX. Both *N*- and *O*-demethylation are dependent on age and sex in the housefly (Hansen and Hodgson, 1970). Activity increases with age following emergence from the puparium, becoming higher in females than in males. The peak activity occurs at 4 days in the males and 8 days in the females. This is in line with the small amount of data available, which generally shows a post-emergence increase (Arias and Terriere, 1962; Schonbrod *et al.*, 1965; Tsukamoto and Casida, 1967b) although no differences were seen between the sexes in hydroxylation of naphthalene (Schonbrod *et al.*, 1965). In larval insects, the maximum activity for aldrin epoxidation is seen in the sixth instar of *Prodenia eridania* (Krieger and Wilkinson, 1967) and the maximum activity for carbaryl metabolism is seen about halfway through the larval life of *Calliphora erythrocephala* (Price and Kuhr, 1969).

NADPH OXIDATION AND OXYGEN CONSUMPTION. Even though NADPH and oxygen appear to be required for all microsomal oxidations, NADPH oxidation and consumption have not previously been measured in insect material. Folsom and Hodgson (1970a,b) have recently carried out investigations on both of these aspects using microsomes prepared from housefly abdomens. These investigations indicate that NADPH is readily oxidized and that the oxidation is stimulated by the addition of exogenous cytochrome c. The K_m for NADPH is $1.8 \times 10^{-5}M$. This is not changed by cytochrome c although the maximum velocity is.

Although accurate CO/O_2 ratios were not determined, comparisons of CO, Air, N_2, and $CO:O_2$ (1:1) showed oxidation of NADPH to be inhibited by carbon monoxide, indicating that electron flow proceeds via a CO-binding pigment, presumably cytochrome P_{450}. As one might expect, this inhibition is partially reversed by an exogenous electron acceptor such as cytochrome c. It should be emphasized that all of these experiments were carried out with isolated microsomes, shown by enzyme assay and electron microscopy to be free from cytochrome oxidase and mitochondria. In this context cytochrome c is an artificial exogenous electron acceptor which is not subject to enzymes of the mitochondrial electron transport chain. In this system cytochrome c is reduced by enzymes which oxidize reduced pyridine nucleotides and thus accepts electrons before they can reduce cytochrome P_{450}. Thus, whether or not cytochrome c reverses an inhibitor of the overall oxidation of NADPH by microsomes is an indication of whether that inhibitor is acting before the point at which cytochrome c accept electrons (NADPH oxidation level) or after that point (cytochrome P_{450} level).

Metal ions such as Cu^2 and Zn^2 are inhibitory, as are several sulfhydryl inhibitors. *p*-Chloromercuribenzoate and *p*-chloromercuriphenyl-sulfonate are the most active at levels of 1 ×

Table III. Comparison of NADPH-Cytochrome c Reductase from Houseflies and Mammals

Property	Housefly[a]	Pig Liver[b]	Rat Liver[c]
Solubilizing agent	Isobutanol Trypsin Lipase	Lipase	Trypsin
Molecular weight	57,000	68,000	57,700
Flavoprotein	Yes	Yes	Yes
pH optimum	8.1	8.3	7.7
Sensitivity to ionic strength:			
Cytochrome c reduction	insensitive	. . .	sensitive
DCIP reduction	sensitive	. . .	sensitive
Rate with NADH	1%	low	1%
Electron acceptor substrates	cytochrome c DCIP ferricyanide	cytochrome c DCIP ferricyanide neotetrazolium inorganic iron	cytochrome c DCIP
Michaelis constants:			
NADPH (μM)	10.5	3.8	1–8
Cytochrome c (μM)	17.2	5.4	2–6
DCIP (μM)	44	1.5	. . .

[a] Wilson (1970). [b] Williams and Kamin (1962). [c] Phillips and Langdon (1962).

$10^{-6}M$ and $1 \times 10^{-5}M$, respectively. This inhibition could not be reversed by cytochrome c, indicating that the inhibition was probably at the level of the NADPH-cytochrome c reductase. The inhibition caused by SKF-525-A, sulfoxide {1,2-(methylenedioxy)-4-[2-(octylsulfinyl)propyl]benzene} and pyridine was substantially reversed by cytochrome c, indicating that the inhibition is probably at the cytochrome P_{450} level.

These preparations were also used for the measurement of oxygen consumption, and the relationship between oxygen consumption and NADPH is shown in Figure 1. The relationship between oxygen uptake is further supported by the effect of inhibitors on oxygen uptake in the presence of NADPH. The inhibitors tested, CO, *p*-chloromercuriphenylsulfonate, SKF-525-A and sulfoxide, also inhibited oxygen uptake.

Components of the Electron Transport Chain. NADPH-CYTOCHROME C REDUCTASE. The NADPH-cytochrome c reductase from microsomes prepared from housefly abdomens has been purified in our laboratory (Wilson, 1970). The enzyme is solubilized by extraction in the cold with isobutanol, and purified by chromatography on DEAE cellulose and hydroxyl apatite. Examination of the best preparations by disk gel electrophoresis indicates that the enzyme is substantially pure.

A comparison of the properties of this enzyme and preparations from pig and rat liver is shown in Table III. The enzyme can be solubilized by isobutanol extraction, whereas this method is completely ineffective with mammalian microsomes. The trypsin or lipase treatments used for the mammalian enzymes do, however, effect some solubilization of the insect enzyme.

All three enzymes shown are flavoproteins and have similar molecular weights. All will react with several electron acceptors, including cytochrome c, 2,6-dichlorophenolindophenol, and ferricyanide, while NADH is ineffective as an electron donor. Some differences are apparent with respect to the sensitivity to ionic strength when cytochrome c is an electron acceptor, and in the K_m values for NADPH, cytochrome c, and 2,6-dichlorophenolindophenol, all of which show a lower affinity for the enzyme in the case of the insect enzyme.

CYTOCHROME P_{450}. This cytochrome, the carbon monoxide-binding pigment of microsomes, has been clearly identified as the locus of microsomal oxidations in mammals (Remmer *et al.*, 1969) but has been studied little in insects. Ray (1967) noted its presence in housefly microsomes, as did Fukami *et al.* (1969) in the cockroach fat body and midgut. Recently, Perry (1970) and ourselves (Folsom *et al.*, 1970a) noted its presence in susceptible and resistant houseflies. Perry (1970) further noted that P_{450} is absent in the egg, rises to a peak about midway through larval life, to fall to 0 in the pupa. Following emergence from the pupa there is a rapid increase to a maximum at 4 to 5 days post-emergence. Of considerable interest was the finding that the CO-difference spectrum of the P_{450} in the microsomes could no longer be demonstrated within 2 to 4 hr of topical applications of methylene dioxyphenyl synergists, but reappeared over the next 72 hr. This was correlated with an increase in the toxicity of administered Baygon.

Our studies (Philpot and Hodgson, 1970) have demonstrated P_{450} in microsomes from housefly abdomens. A typical CO-difference spectrum with a peak at 450 mμ is shown in Figure 2. Figure 3 shows a typical type II substrate difference spectrum obtained in this case with pyridine. Figure 4 is a substrate difference spectrum obtained with piperonyl butoxide. It is a typical type I spectrum with a peak at 390 mμ and a trough at

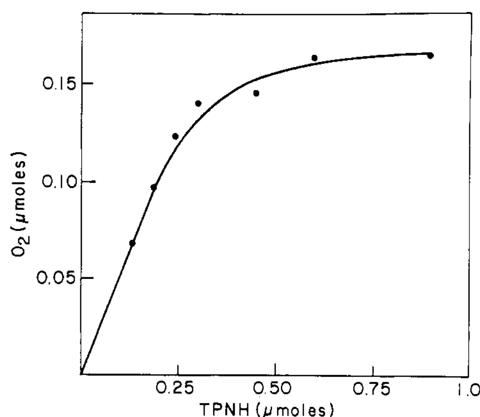

Figure 1. Relationship between oxygen uptake and added NADPH for microsomes prepared from housefly abdomens

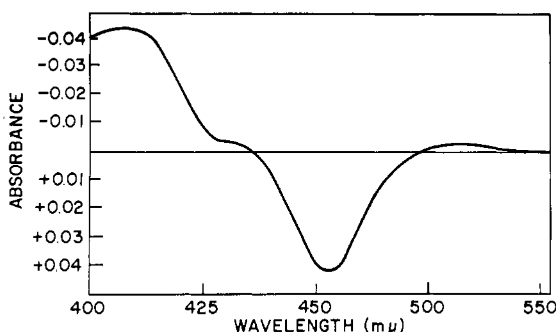

Figure 2. CO-difference spectrum for cytochrome P_{450} in housefly abdomen microsomes

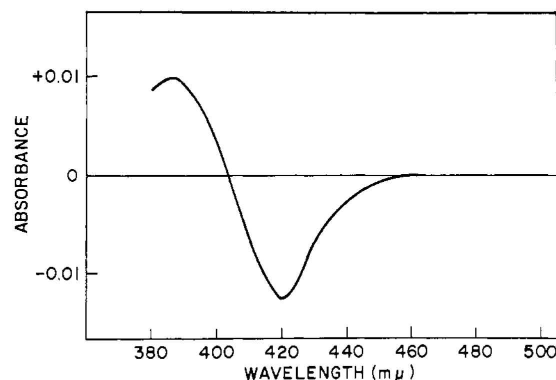

Figure 4. Type I substrate difference spectrum obtained with piperonyl butoxide and housefly abdomen microsomes

420 mμ (Remmer *et al.*, 1969) similar to those obtained with mammalian microsomes, although to our knowledge the first published from insects. This spectrum, plus the observation that methylene dioxyphenyl compounds are more stable to enzymatic attack by insect microsomes than those from mammals (Casida *et al.*, 1966; Wilkinson and Hicks, 1969) leads to the speculation that the disappearance of cytochrome P_{450} observed by Perry (1970) is, in fact, due to the binding of substrate in such a way that it cannot be displaced by CO to give rise to the typical CO-difference spectrum. In mammals, however, the presence of a type I substrate does not interfere with the formation of a CO complex.

In summary, although we know little of the fundamental nature of microsomal oxidations in insects, what we do know would indicate that the concept of the unity of biochemistry has been conserved. Broadly speaking, they appear to resemble those of mammals, although some differences are apparent, and we may hope, as comparative toxicologists, that more will become apparent, particularly those which may be exploited is the design of future insecticides.

BIOCHEMICAL GENETICS

The utilization of insects as a tissue source for microsome biochemistry did not occur for several years after similar work was initiated with mammals. There are a number of important reasons, however, both practical and theoretical, for employing insect tissues in this work. Of major importance is the fact that, in terms of insect control, we need to understand the ways in which insects degrade pesticides; in insects as in mammals, it is undoubtedly true that microsomal enzymes are of primary importance in this respect. Secondly, we need to understand the ways in which insects become resistant to insecticides. Here, too, the role of microsomal systems is frequently of major importance.

The occurrence of microsomal detoxifying systems in a wide range of insect species is now quite well documented (Agosin *et al.*, 1961; Chakraborty and Smith, 1967; Schonbrod *et al.*, 1965). The first major research in this area was that of Terriere *et al*, in which naphthalene hydroxylation was studied in insects as a model detoxification system (Arias and Terriere, 1962; Terriere *et al.*, 1961). The results of these investigations indicated a basic similarity in the processes involved between insects and mammals.

Much early difficulty in studying microsomal reactions in insects was occasioned by the occurrence of "endogenous inhibitors" which interfered with measurements of enzyme activity (Chakraborty *et al.*, 1967; Matthews and Hodgson, 1966; Ray, 1967). This difficulty has been overcome, either by utilizing purified microsomal preparations from appropriate tissue sources (Krieger and Wilkinson, 1970; Nakatsugawa and Dahm, 1967) or by adding BSA to crude tissue preparations (Tsukamoto and Casida, 1967a), a process which allows the measurement of microsomal oxidative reactions without extensive preliminary preparations of tissues.

Of major importance in the study of microsomal detoxifying systems in insects is the fact that the level of these enzymes may vary from population to population within a species. The housefly is the most studied insect of this group, ever since the first suggestion that "high levels" of microsomal enzymes may be involved in insecticide resistance (Schonbrod *et al.*, 1965). The occurrence of changed levels of microsomal enzymes in resistant strains of insects has been amply documented in the past several years, both in terms of high levels of oxidative enzymes (Oppenoorth and Houx, 1968; Plapp and Casida, 1969; Schonbrod *et al.*, 1965; Tsukamoto and Casida, 1967a; Tsukamoto *et al.*, 1968) and also in decreased levels of aliesterases (Asperen and Oppenoorth, 1959; Bigley and Plapp, 1960). Interest is now focusing on the genetics of these differences and on the biochemical nature of the differences involved. It is in this area where work with insects should prove extremely useful in the study of microsomal biochemistry. Gaining an understanding of the nature of the differences between insect strains should lead to a better understanding of the nature of the various processes involved in insecticide metabolism and resistance.

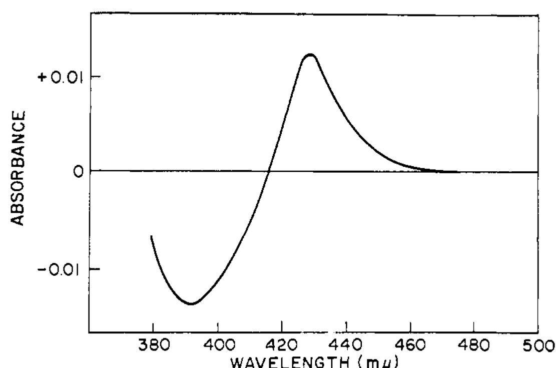

Figure 3. Type II substrate difference spectrum obtained with housefly abdomen microsomes and pyridine

An additional important area involving insect microsomes relates to growing realization that in insects, as in mammals, microsomes are inducible or capable of undergoing adaptive responses following exposure of the intact organism to appropriate inducers under *in vivo* conditions. A number of

difficulties were met in demonstrating microsomal induction on a gross scale in insects; these have been overcome in the case of the housefly, as reported recently by several researchers (Plapp and Casida, 1970; Walker and Terriere, 1969).

Evidence at the chromosomal level of the responses of insects to DDT as an inducer has recently been obtained (Balazs and Agosin, 1968; Ishaaya and Chefurka, 1968; Litvak and Agosin, 1968; Litvak *et al.*, 1968). This work has indicated that increased RNA and protein synthesis occurs in *Triatoma* and *Musca in vivo* following exposure to DDT. These results strongly suggest that in insects DDT acts as an inducer of protein synthesis through its effects on RNA.

The finding that microsomal drug detoxification systems in insects are inducible by chlorinated pesticides is of tremendous practical significance. If chlorinated hydrocarbons are inducers, then prior exposure of an insect population to the chlorinated compounds may explain the rapid development of resistance to organophosphates and carbamates, insecticides where resistance mechanism are frequently primarily concerned with microsomal enzymes. Thus, exposure of an insect population to DDT may render control by organophosphates and carbamates more difficult than if the chlorinated material had not been used in the first place.

GENETICS OF HIGH OXIDASE ACTIVITY. An important question in the area of microsome biochemistry is how many enzyme systems are involved in the apparently quite complex and variable array of reactions which occur. Thus, many types of insecticide chemicals are metabolized by NADPH—requiring oxidases; the reactions include phosphorothioate oxidation; *S*-alkyl, *O*-alkyl, and *N*-alkyl oxidations, ring hydroxylations, epoxidations, and aryl-phosphate bond hydrolyses among others. It is widely assumed that these reactions contribute to resistance and may in fact be the major cause of resistance with many insecticide chemicals. However, evidence on this point is quite limited.

In work done recently in Berkeley (Plapp and Casida, 1969), we attempted to deal with the problem of number of microsomal detoxification systems through a combination of biochemical and genetic techniques. In preliminary experiments we determined the resistance spectra and oxidase spectra of two insecticide resistant housefly strains in comparison with a susceptible strain. In one strain, R-Baygon, the ability to metabolize pyrethrins, carbamates, organophosphates and aldrin, but not DDT, was markedly higher than in a susceptible strain. In the other resistance strain, R-Fc, high ability to metabolize DDT, expoxidize aldrin, and metabolize certain organophosphates, but not other insecticide chemicals was present. Thus, two distinct patterns of high oxidase activity occurred.

The question was whether or not we were dealing with a large series of resistance and oxidative mechanisms, or only a few. To investigate this problem, we crossed the resistant strains with a susceptible marker strain carrying visible recessive mutants on chromosomes II, III, and V. F_1 populations were intermediate in resistance and in oxidase activity. We backcrossed the F_1 males to the mutant strain females and measured oxidase levels and resistance to insecticides in the backcross progenies. The results indicated that in each resistant strain gene(s) on only one chromosome, II, in the R-Baygon strain and V in the R-Fc strain were primarily involved in controlling high levels of oxidative microsomal enzymes activity. These findings suggested, but could not prove, that only one genetic factor was involved in high oxidase activity in each strain.

Evidence that in fact only one gene per strain was involved was obtained from the assays of substrains selected with only a single insecticide from the backcross populations. These substrains possessed an oxidase pattern closely similar to that of the resistant parent strain. However, it is well to note that the resistance pattern differed enough to show that only a portion of the resistance present in the parent strains was controlled by genes on the same chromosomes as the major high oxidase genes.

Table IV. Induction of NADPH-Dependent Oxidase Activity in Houseflies Fed Dieldrin and DDT

Insecticide Substrate	% substrate metabolized/30 min/3 housefly abdomens + S.E.		
	Control diet	Dieldrin-100 ppm	DDT-1000 ppm
Aldrin	37 ± 5	71 ± 5	54 ± 1
Allethrin	40 ± 9	77 ± 1	57 ± 2
Baygon	14 ± 3	21 ± 3	17 ± 4
DDT	1.8 ± 1.2	2.5 ± 1.1	3.4 ± 1.2
Diazinon	11 ± 1	17 ± 2	16 ± 2

That genes on chromosomes II and V are important in the housefly in controlling levels of oxidase activity is indicated by other work. Thus, Plapp and Terriere (1968) in tests with several previously described high oxidase strains (Schonbrod *et al.*, 1968) studied the inheritance of oxidative genes conferring resistance to methoxychlor in the housefly. In all cases the major genetic factors conferring oxidative resistance were located on chromosomes II and V. Similar results were obtained when a genetic study was made of the inheritance of naphthalene hydroxylation activity in the housefly (Schafer and Terriere, 1968). Other genetic and biochemical work with the housefly, as recently reviewed (Georghiou, 1969; Tsukamoto, 1967), has also indicated the primacy of the same two chromosomes in controlling resistance to organophosphate, carbamate, and pyrethroid insecticides. Portions of this work (Plapp, 1970; Tsukamoto and Casida, 1967a; Tsukamoto *et al.*, 1968) have suggested that in many resistant strains both chromosomes II and V contributed to resistance by means of oxidative processes. The possible significance of these findings in determining the number and nature of the oxidative systems involved is in need of additional evaluation.

INDUCTION OF INSECTICIDE METABOLISM IN THE HOUSEFLY. Biologically speaking, a major property of certain insecticide chemicals is that they are inducers of microsomal enzymes. This phenomenon has been extensively studied in mammals, but until recently induction of enzyme activity has not been demonstrated definitively in the housefly. Clear evidence of induction of microsomal oxidase enzymes has been obtained in experiments in which resistant houseflies were either exposed to films of insecticide inducers for several days (Walker and Terriere, 1969) and in tests where flies were fed diets containing chlorinated insecticides known to be microsomal inducers for a period of several days or more (Plapp and Casida, 1970). Table IV lists the results of tests in which the effect of two insecticide inducers on insecticide metabolism in the housefly were studied. The results show that the metabolism of all test substrates was increased by exposure to insecticide chemicals. In some cases, prolonged treatment resulted in at least a doubling of enzyme activity.

The question arises as to whether induction as a measurable phenomenon in the housefly is limited to normal low oxidase level strains, or whether it occurs in strains possessing the high oxidase levels characteristic of numerous insecticide resistant

Table V. Induction of Aldrin Epoxidase in Housefly Strains with Low and High Levels of NADPH-Dependent Oxidase Activity

	Age of flies	Control diet Units	Control diet % of Control	Expoxidase Activity[a] Dieldrin-100 ppm Units	Dieldrin-100 ppm % of Control	DDT-1000 ppm Units	DDT-1000 ppm % of Control
Orlando DDT							
	4 days	41	100	49	119	67	164
	9 days	28	100	42	150	66	234
	16 days	24	100	31	127	58	239
	23 days	23	100	30	133	46	202
R-Baygon							
	7 days	68	100	58	86	69	102
	14 days	52	100	40	78	67	128
	21 days	32	100	30	94	74	237

[a] Activity as % of 10 mμmoles C^{14} aldrin metabolized/30 min/3 fly abdomens. Each value is the average of two replicates.

strains. In work currently under way (Plapp and Hickson, 1970), we are measuring the phenomenon in both types of strains. The test reaction we employed as an indicator is the conversion of aldrin to dieldrin. The methods were identical to those described previously (Plapp and Casida, 1970).

Results of these tests are presented in Table V. In the low oxidase Orlando DDT strain, enzyme activity was highest in younger flies and declined steadily with age. This was true for flies on the control diet and for flies fed either DDT or dieldrin. With the insecticide containing diets, the level of induction (defined as the ratio of activity in flies fed diet-containing inducer to flies on a control diet) was nearly constant at all ages. Thus, maximum levels of inductive effects are apparently reached within a few days, and no additional increases were noted as the age of the flies increased.

Results of tests obtained with the high oxidase R-Baygon strain are also presented in Table V. Again, oxidase activity decreased with age in flies fed the control diet or the dieldrin-treated diet. Apparently dieldrin was not an inducer of microsomal enzyme activity in the R-Baygon strain, and may actually be acting as an inhibitor. When flies of this strain were fed DDT, enzyme activity did not decrease with age, but remained constant. Thus, by the time the flies were 2 days old, DDT was apparently highly active as an enzyme inducer.

These results point out some of the complexities in trying to evaluate the role of insecticides as enzyme inducers in insects. Age and strain of flies, as well as inducer used, are all variables that affect the results obtained in attempting to delineate the phenomenon of microsomal induction in the housefly.

RESISTANCE AS A PROBLEM IN BIOCHEMICAL GENETICS. The work described here suggests that only a limited number of genetic factors are involved in controlling levels of microsomal enzyme activity in the housefly. When induction of the microsomal system occurs, the metabolism of many substrates, not just particular substrates, is induced. This finding is in keeping with the many reports of the very broad nature of the responses observed within these systems in mammals to drugs of various sorts.

Two characteristics of microsomal enzyme systems in houseflies should be noted. One of these is that resistance associated with microsomal enzyme systems is always semidominant; it is never recessive to the genetically wild-type or low microsomal enzyme level conditions (Plapp, 1969). The second characteristic to be noted is that the differences between resistant and susceptible strains are quantitative, not qualitative. The reactions are the same in both types of strains, only the rates are different. Thus, both susceptible and resistant strains epoxidize aldrin, oxidize phosphorothioates, and hydroxylate carbamates. This is perhaps best shown by the nature of the synergistic responses obtained with microsomal enzyme inhibitors such as piperonyl butoxide. Synergism occurs in both susceptible and resistant strains; the difference is that more synergism occurs in resistant strains.

The evidence suggests that the genetic factor(s) involved in controlling high or low levels of apparently complex arrays of microsomal metabolizing systems may not be specific structural genes directing the synthesis of particular enzymes. Rather, the genes we measure in resistance studies may be operator genes and the microsomal metabolizing systems may be considered as operons, genetic units of coordinated transcription (Jacob *et al.*, 1960). The mutations we have observed may then represent mutations at operator sites—the sites that switch on a sequence of genes. The observed high levels of microsomal enzyme activity may occur as a consequence of mutations at operator sites which render these genes less susceptible than normal to the effects of either regulator genes or the repressor substances produced by them. Such a concept was first proposed by Tomkins and Yielding (1961), who showed that changes at a regulator site affected the activity and specificity of an amino acid dehydrogenase.

In summary, the experiments reported here demonstrate several interesting characteristics of microsomal enzymes in the housefly. First, the genetic evidence is that only a very limited number of enzyme systems may be involved in the oxidative metabolism of insecticide chemical substrates. Secondly, this metabolism is inducible in the housefly, with a generally greater effect occurring in low oxidase strains. A consequence of this inductive effect may be to confer increased detoxifying ability on cases where insecticide degradation by microsomal enzymes is important. This effect may also be related to the development of resistance to these same insecticides. Lastly, it is possible that the genetic events observed which control the high levels and low levels of microsomal enzyme activity in insects may be mutants of operator genes which control the level of detoxification enzymes by regulating the rate of synthesis of these enzymes.

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