

MICROSOMAL ELECTRON TRANSPORT SYSTEMS IN
THE HOUSEFLY, *MUSCA DOMESTICA*
A MODEL FOR THE STUDY OF DETOXICATION SYSTEMS IN
INSECTS

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ABSTRACT

Although the electron transport system of insect microsomes is fundamentally similar to that of mammalian microsomes, differences of some interest exist. Studies carried out in this laboratory on NADPH—cytochrome c reductase and cytochromes P—450 and b₅, as well as on the ultrastructure, oxygen uptake and NADPH oxidation of intact microsomes, are summarized and discussed.

Binding spectra of type I, II and III ligands with cytochrome P-450 from several insect species and mammals are compared, reference being made to pesticides as well as to model compounds. The differences noted indicate that quantitative and qualitative differences exist between the cytochromes from different organisms and from different genetic strains of the same organism. The genetics of cytochrome 450 variants in *Musca domestica* are discussed.

Solubilization studies have been carried out on NADPH-cytochrome c reductase from housefly microsomes and on cytochrome P—450 from the same species. The former enzyme has been purified and characterized and appears to be similar to the same enzyme from mammalian hepatic microsomes.

The importance of methodology is stressed and a critical analysis of methods currently in use is included.

INTRODUCTION

Although the study of microsomal mixed function oxidase activity in insects is, for all practical purposes, less than ten years old, we have already learned a considerable amount about this system. Even though the number of research groups has been small, the body of knowledge of this system, as it occurs in insects, is second in scope only to that drawn from mammals. In at least two areas, the biochemical genetics of mixed function oxidase enzymes and their role in resistance to toxicants, the insect studies have been more extensive than those on mammals.

Since the investigation of other complex pathways, e.g. that from glutathione transferase to mercapturic acids, is proceeding rapidly, it seems appropriate to pause and examine the current status of our knowledge of the microsomal mixed function oxidase for the oxidation of xenobiotics in the hope that both the successes and failures will help comparative toxicologists avoid pitfalls in their own endeavors. Of particular importance is an assessment of the methodological pitfalls of comparative investigations: what can we learn from prior experience with mammalian systems and when should we avoid overextending prior knowledge or use it without reference to the new circumstances.

Much of the information concerning cytochrome P-450 and the electron transport enzymes associated with it has been derived from studies of mammalian liver. It is known, however, to be ubiquitous in nature, occurring in at least 14 species of mammals, 17 species of insects and 1 or more species of birds, reptiles, fish, amphibians, bacteria, fungi and higher plants. A voluminous literature exists on microsomal mixed-function oxidases and cytochrome P-450 and several recent reviews have appeared (e.g. AGOSIN, 1976; ESTABROOK, 1971; ESTABROOK et al., 1973; HODGSON, 1974; HODGSON *et al.*, 1974; WILKINSON and BRATTSTEN, 1972).

Two microsomal electron transport systems exist, one associated with cytochrome b_5 and NADH and the other with cytochromes P-450 and NADPH. Although the latter pathway is known to be involved in the oxidation of many xenobiotics and endogenous substrates, its specificity varies from tissue to tissue and organism to organism. The former system, in addition to its role in fatty acid desaturation and other reactions, may also be involved in the reduction of cytochrome P-450, although this is still a matter of controversy.

These electron transport systems are located in both rough and smooth endoplasmic reticulum, although oxidative activity toward xenobiotics may be greater in the latter. Microsomes, the particulate fraction obtained by centrifugation of post-mitochondrial supernatant at 100,000 - 200,000 g, are usually the enzyme preparation of choice.

The effect of substrates and other ligands on the optical difference spectrum has been the principal tool used in the investigation of cytochrome P-450. The dithionite reduced cytochrome P-450-carbon monoxide difference spectrum, with its prominent peak at or about 450 nm, is the basis for the quantitative measurement of cytochrome P-450. Type I spectra, with a peak at 385 nm and a trough at 420 nm, are formed by a large variety of ligands with oxidized cytochrome P-450 and are believed to represent binding to a lipophilic site somewhat removed from the heme iron, while type II spectra, with a peak at about 430 nm and a trough at about 390 nm, represent binding directly to the ferric heme iron. The type II spectrum formed by n-octylamine is of particular interest since it occurs in two forms, one with a double trough at 410 and 392 nm and the other with a single trough at 392 nm. These forms have been used in the characterization of qualitatively different cytochromes in both mammals and insects.

Type III spectra, formed by ethyl isocyanide (EtNC) and methylenedioxyphenyl insecticide synergists, result from interaction with the reduced form of cytochrome P-450. These spectra have two peaks in the Soret Region at 455 and 427-430 nm which are in pH dependent equilibrium. The pH equilibrium point, the pH at which both peaks are of equal magnitude, has been used in the characterization of different cytochromes.

Although solubilization and complete purification have proven an elusive goal, enough success has been attained to show that the oxidation of xenobiotics in mammalian liver requires cytochrome P-450, a NADPH-specific flavoprotein reductase, NADPH, O₂ and phosphatidylcholine. While the mechanism is not yet completely understood, it is apparent that the first step is the combination of the oxidized form of the cytochrome to form an oxidized cytochrome-substrate complex. This is reduced, by a reducing equivalent derived from NADPH via the flavoprotein reductase, to form a reduced cytochrome-substrate complex. This reduced complex reacts with molecular oxygen to form an oxygenated intermediate, which then reacts with a second reducing equivalent to form, via dismutation reactions not yet clearly understood,

a hydroxylated product, water and the oxidized form of the cytochrome.

Induction of microsomal enzymes by xenobiotics has been extensively studied in mammals, more recently, the same phenomenon has been observed in insects.

In the following account of microsomal electron transport systems in insects limitations of space make a comprehensive review impossible. Although emphasis is placed on investigations carried out in our laboratories at North Carolina State University, appropriate consideration is given to those from other laboratories, particularly in areas of potential controversy. More comprehensive references to investigations carried out in other laboratories can be found in the reviews cited above.

REDUCTASES

Although microsomal oxidation in insects requires both NADPH and molecular oxygen, little is known of the biochemical characteristics of their utilization. FOLSOM and co-workers (1970, 1971a, 1971b), in a series of studies demonstrated that both NADH and NADPH are oxidized by housefly abdominal microsomes, the K_m value being $1.8 \times 10^{-5} M$ for NADPH and $1.6 \times 10^{-4} M$ for NADH. NADPH oxidation was inhibited by carbon monoxide, indicating the importance of cytochrome P-450 as a terminal oxidase in the NADPH dependent electron transport pathway. Cytochrome c stimulated NADPH oxidation by accepting electrons at a point prior to cytochrome P-450. A number of sulfhydryl inhibitors, SKF-525A, sulfoxide and pyridine all inhibited electron flow from NADPH but not necessarily initial rate of NADPH oxidation.

NADPH oxidation depends on an intact electron transport chain and since it acts at a point prior to cytochrome P-450, cytochrome c could be used to indicate the locus at which the inhibition occurs. Failure of cytochrome c to reverse the effects of most of the sulfhydryl inhibitors indicates that these inhibitors act mainly at the reductase level while substantial reversal of the effects of SKF-525A, pyridine and sulfoxide indicate inhibition at the cytochrome P-450 level. Oxygen consumption is dependent on NADPH and on enzyme concentration while the effect of the above mentioned inhibitors is essentially the same as their effect on NADPH oxidation, again showing the need for an intact electron transport chain. Similar studies have shown NADPH oxidation by gypsy moth larval microsomes to be essentially identical (AHMAD and FORGASH,

1973). Recently, KULKARNI *et al.* (1976a) reported species differences up to four fold in the NADPH-oxidase activity of microsomes from several insect species.

Studies by WILSON and HODGSON (1971a, 1971b) resulted in the purification and characterization of NADPH - cytochrome c reductase from housefly abdominal microsomes. The enzyme is a flavoprotein with a molecular weight of approximately 57,000. Like the mammalian reductase, the housefly reductase is specific for NADPH, utilizes either cytochrome c, 2,6-dichlorophenol indophenol or ferricyanide as electron acceptors and is competitively inhibited by NADP. As compared to the mammalian enzyme, housefly reductase is less responsive to changes in ionic strength, has a lower affinity for substrates and is more sensitive to sulfhydryl inhibitors, thus confirming the work of FOLSOM and HODGSON relative to the locus of action of these inhibitors. In addition to the housefly, this enzyme has been studied in several insect species (KULKARNI *et al.*, 1976a).

Recent studies (KULKARNI *et al.*, 1976b) indicate that, like mammalian hepatic microsomes, housefly microsomes possess NADH-cytochrome b₅ reductase activity and transfer electrons derived from NADH to either cytochrome b₅ or artificial electron acceptors such as cytochrome c, dichlorophenol indophenol or ferricyanide. However, in contrast to the reductase of cytochrome b₅ by mammalian microsomes, housefly microsomes exhibited at least two reduction phases which are NADH-concentration dependent. Several lines of evidence suggest that contamination of housefly microsomes by phenolic compounds and tyrosinase is probably responsible for the observed complex reduction properties of the housefly microsomal cytochrome b₅ system.

The eye pigment, xanthommatin, functions as an inhibitor of microsomal mixed-function oxidases in whole body homogenates of houseflies by accepting electrons from the NADPH cytochrome reductase. Since it is readily autooxidizable, this forms an efficient mechanism for diverting electrons from cytochrome P-450 (SCHONBROD and TERRIERE, 1971; WILSON and HODGSON, 1972).

PHOSPHOLIPIDS AND THE MICROSOMAL MEMBRANE

Although mammalian and insect endoplasmic reticulum and microsomal enzyme preparations appear morphologically similar (CASSIDY *et al.*, 1969) it is clear that differences in their biochemical properties exist. Evidence for this may be cited as follows:

1. The phospholipid composition is quite different, the ratio of phosphatidylethanolamine to phosphatidylcholine being higher in the case of the housefly (KHAN and HODGSON, 1967) than in mammals.

2. Solubilization of NADPH by isobutanol extraction, effective with housefly microsomes, is ineffective with mammalian microsomes, even though the enzymes, after extraction and purification, appear almost identical (WILSON and HODGSON, 1971a, 1971b).

3. The preparation of subparticles using subtilisin yields P-450 containing particles from mouse microsomes with any P-420 formed becoming soluble, while in the housefly the particles formed contain particulate cytochrome P-420 (PHILPOT and HODGSON, 1971b).

4. The low speed preparation of microsomes, by precipitation with CaCl_2 , is much more effective using mammalian microsomes than in identical experiments with housefly microsomes (BAKER *et al.*, 1973).

The available data are inadequate to determine whether there is a specific requirement for a phospholipid in the electron transport chain, as in mammals, but it is apparent from studies of the effect of phospholipases that they are required for overall activity of the microsomes (FOLSOM *et al.*, 1971a). Capdevila *et al.* (1975) were unable to demonstrate a phospholipid requirement with partially purified enzymes from housefly microsomes.

CYTOCHROME P-450

Cytochrome P-450 has not been extensively characterized in insects, although a start has been made in the case of the housefly, *Musca domestica* (see HODGSON, 1974, for references). The principal tool used has been optical difference spectroscopy. The CO-reduced difference spectrum is the basis for the quantitation of cytochrome P-450 and its denatured form, cytochrome P-420. The most reliable estimates of these hemoproteins are obtained when CO treatment of microsomes is followed by dithionite reduction. Reversal of this sequence (CAPDEVILA *et al.*, 1973a,b) or the use of NADPH as a reductant (TERRIERE and YU, 1973) results in erroneously lower values especially for cytochrome P-420 (KULKARNI and HODGSON, 1975, 1976f). Although oxidized cytochrome P-420 does not exhibit normal type I and II difference spectra, it does interact with EtNC and its presence seriously affects the

evaluation of the EtNC type III difference spectrum and its pH equilibrium point (KULKARNI and HODGSON, 1975, 1976a). When present in relatively large amounts, cytochrome P-420 may even cause a shift in the absorption maximum of the cytochrome P-450 difference spectra and be responsible for a 10-50% underestimation of cytochrome P-450 (KULKARNI and HODGSON, 1976a). Thus the implications of underestimating cytochrome P-420 are obviously manifold. To a considerable extent, denaturation of cytochrome P-450 in housefly microsomes can be avoided by employing buffered sucrose as a suspension medium. Such a medium was also found to be most efficient for storage of microsomes for up to 6 months (KULKARNI and HODGSON 1976a). Type II spectra appear to be caused by direct binding of ligands to the Fe^{3+} heme iron and are due, primarily, to nitrogen compounds such as pyridine and n-octylamine with spatially accessible sp^2 or sp^3 non-bonded electrons. In some cases organic oxygen atom may act in a similar way, that is as nucleophiles interacting at the fifth or sixth ligand position of the heme (MAILMAN *et al.*, 1974; KULKARNI *et al.*, 1974). Such spectra have been seen in all insect species examined (KULKARNI and HODGSON, 1976c, KULKARNI *et al.*, 1976a) and it appears that heme in cytochrome P-450 from insects is more accessible to ligand binding than that from mammalian source.

Type I spectra are caused by ligand binding to a lipophilic site adjacent to the heme and are believed to be the manifestation of (oxidized) enzyme-substrate complex formation. Such difference spectra can be clearly discerned by standard techniques using oxidized microsomes from a number of insecticide-resistant housefly strains. Typically these strains give results similar to those with mammalian liver preparations. The lack of type I binding in the insecticide susceptible CSMA strain (PHILPOT and HODGSON, 1971a; TATE *et al.*, 1973) first demonstrated with benzphetamine has since been confirmed using a large number of pesticidal (KULKARNI *et al.*, 1975) and non-pesticidal chemicals (KULKARNI *et al.*, 1974). This absence is not confined to the housefly, having also been observed, at least on the basis of the benzphetamine spectrum, in three species of *Hippelates* and in *Manduca sexta* (KULKARNI *et al.*, 1976a). However, in subsequent studies, a significant type I difference spectra was observed with the insecticide-synergist sulfoxide and small spectra with other methyldioxyphenyl compounds. (KULKARNI and HODGSON, 1976b). Moreover, benzphetamine gave a type I spectra with some microsomal subfractions of susceptible houseflies (KULKARNI and HODGSON, 1976f). These results suggest that at least in this regard, the differences between susceptible and resistant housefly strains are quantitative rather than qua-

litative and that a small amount of type I binding cytochrome is present in susceptible strains.

CAPDEVILA *et al.*, (1973a,b, 1974) failed to observe normal type I spectral interactions with oxidized cytochrome P-450 from insecticide-resistant strains of housefly. These authors also reported that type I difference spectra could be detected in microsomes pretreated with EtNC or reduced either by dithionite or NADPH. Difference spectra obtained under these conditions exhibited peak at 410 nm and trough at 428 nm, wavelengths which are not typical of normal type I difference spectra with either mammalian (SCHENKMAN *et al.*, 1967) or insect (KULKARNI and HODGSON, 1975, 1976a-f, KULKARNI *et al.*, 1974, 1975, 1976a) cytochrome P-450. The spectral magnitudes of type I difference spectra in the presence of NADPH are different to those of oxidized cytochrome P-450 and may represent the binding of both the parent compound and its metabolite(s). Mammalian microsomal cytochrome P-450 has been found to be only partially reduced when NADPH is added under aerobic conditions (OMURA and SATO, 1964; HLAVICA, 1972). In addition, either acceleration or inhibition of cytochrome P-450 reduction by NADPH has been noted in the presence of different ligands (HLAVICA, 1972). In view of these facts, the data obtained from difference spectra using reduced microsomes would have a questionable bearing on the understanding of ligand interactions with oxidized cytochrome P-450. Type I spectra in the presence of EtNC may at best represent displacement of the oxidized EtNC spectrum in the case of certain ligands, and at worst they are uninterpretable.

Type III difference spectra caused by insecticide-synergists of the methylenedioxyphenyl group and EtNC have been demonstrated using either NADPH or dithionite reduced housefly microsomes respectively. The former case has been related to the mechanism of synergism (HODGSON and PHILPOT, 1974) while the magnitude of 455 nm peak in the latter case has been used to characterize strain or species differences in cytochrome P-450 (PHILPOT and HODGSON, 1971; TATE *et al.*, 1973, KULKARNI *et al.*, 1976a). Accurate estimation of the magnitude of 430 nm peak of type III difference spectra of EtNC is significantly affected by several factors such as concentration of EtNC, time, and cytochrome P-420 content (KULKARNI and HODGSON, 1975). Furthermore, dithionite reduction prior to CO treatment results in underestimation of cytochrome P-420 and increased denaturation of cytochrome P-450 to cytochrome P-420 at low pH conditions results from addition of excess dithionite to poorly buf-

ferred microsomal suspensions. In view of these factors, data reported (CAPDEVILA *et al.*, 1973a,b, 1974, AGOSIN, 1976) on EtNC pH equilibrium points to demonstrate qualitative differences between control and induced cytochrome P-450 from houseflies should be regarded as tentative until confirmed by other methods. The use of a correction factor representing the contribution due to cytochrome P-420 to the 430-433 nm peak would be essential to resolve this problem. Because of these problems relative to the 430-33 nm peak, we have reported spectral data on this ligand only with regard to 455 nm peak (PHILPOT and HODGSON, 1971a; TATE *et al.*, 1973; KULKARNI and HODGSON, 1976a; KULKARNI *et al.*, 1976a) and have avoided the calculation of pH equilibrium points.

Examination of a number of insecticide resistant strains of the housefly have led to the conclusion that those which show high oxidase activity generally have an increased titer of cytochrome P-450 and moreover, this cytochrome differs qualitatively from that of susceptible strains (TATE *et al.*, 1973). The "resistant cytochrome P-450" as found in diazinon-R, Fc and dimethoate-R strains differs from the susceptible as follows: 1. The λ max of the CO-reduced cytochrome P-450 spectrum is shifted several nm toward the blue. 2. Type I binding is present. 3. Type II binding is increased relative to the CO spectrum. 4. The ethyl isocyanide spectrum is reduced relative to the CO spectrum. Other resistant strains may have some, but not all, of these characteristics. It is also apparent (CHANG and HODGSON, 1975) that the catalytic properties of these cytochromes toward xenobiotic substrates are different.

Qualitative differences in housefly cytochrome P-450 may also occur on induction by phenobarbital or naphthalene (CAPDEVILA *et al.*, 1973a,b) but this does not occur in all cases of induction (TATE *et al.*, 1973). Attempts have been made to resolve the question of whether the qualitative differences noted in cytochrome P-450 from resistant and susceptible houseflies are related to differences between single cytochrome P-450 species or are due to different preparations of multiple forms of cytochrome P-450. An examination of apparent absolute absorption spectra (KULKARNI and HODGSON, 1976d) revealed the presence of at least two types of cytochrome P-450 absorbing at about 392 nm and 414-416 nm in the microsomes of susceptible as well as resistant houseflies. Controlled trypsin digestion of microsomes of both resistant and susceptible houseflies resulted in a differential destruction of several cytochrome P-450 species (KULKARNI and HODGSON, 1976e). Type I, II and III difference spectra of microsomes from resis-

tant houseflies exhibited an increased resemblance to those of susceptible houseflies as trypsinolysis proceeded, indicating that the susceptible cytochrome is present in resistant flies and that it is comparatively resistant to tryptic digestion.

CAPDEVILA *et al.*, (1975) and SCHONBROD and TERRIERE (1975) have reported procedures for detergent-solubilization and isolation of two forms of cytochrom P-450 species from houseflies of different strains. Unfortunately, they have not yet achieved high yields or a high level of purification. Further, the isolated cytochrome P-450 is not free from detergent, cytochrome b_5 or cytochrome P-420 and the reported CO absorption maxima of isolated cytochrome P-450 forms should be considered approximate in view of cytochrome P-420 contamination. CAPDEVILA *et al.*, (1975) report that purified cytochrome P-450 does not exhibit normal type I difference spectra with typical type I substrates and on the basis of cytochrome P-450 content, their reconstitution studies indicate that the purified hemoprotein is only 20-30% as active as microsomes. SCHONBROD and TERRIERE (1975) did not study these aspects and did not determine whether the isolated cytochrome P-450 was enzymatically active. However, in view of the large body of evidence for multiple forms of cytochrome P-450 in the housefly, such purification studies must surely lead to the isolation of soluble forms of cytochrome P-450 in which differences can be unequivocally demonstrated.

KULKARNI and HODGSON (1976f) isolated various cytochrome P-450 species by subjecting microsomes from resistant and susceptible houseflies to subfractionation by two step discontinuous sucrose gradients in which the sucrose concentration of lower layer was varied from 1.0 M to 1.4 M. This procedure has already proven to be efficient in isolation of enzymatically active multiple forms of cytochrome P-450 from mammalian sources (MAILMAN *et al.*, 1975). Using this technique, the light fractions isolated contained cytochrome P-450 species which differed in spectral characteristics from that of heavy fractions. Type I difference spectrum of benzphetamine was observed with certain subfractions of susceptible houseflies.

Genetic studies of resistant cytochrome P-450 (TATE *et al.*, 1974) using the diazinon-R and Fc strains show that these differences are under genetic control. In the former the qualitative characteristics of the "resistant cytochrome" are inherited as semi-dominants on chromosome II, the chromosome primarily responsible for resistance. In the Fc strain in which resistance is associated with chromosomes V and

II, type I binding was associated with chromosome V and the other characteristics with chromosome II. Current genetic studies indicate that the characteristics associated with chromosome II are controlled by more than one gene since they can be separated by crossing over.

CONCLUSIONS

Microsomal electron transport systems have yet to be studied in insects with the same intensity as they have been in mammals, although a good start has been made in the case of one species, *Musca domestica*. On this limited basis certain general conclusions can be drawn. The two principle pathways, the NADH dependent cytochrome b_5 system and the NADPH dependent cytochrome P-450 system, are present in both cases. Although the fundamental plan of organization and the component enzymes appear to be similar, there are differences in the insect associated both with the structure of the microsomal membrane and the biochemical characteristics of the individual enzymes.

The NADPH pathway is of central importance in xenobiotic metabolism and in such related areas as resistance to toxicants. Many problems remain, the solutions to which may depend on solubilization and purification of the component enzymes and their reconstitution into simpler, but still functional systems.

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