control, may already have an inherent capacity for resistance to them. It is worth noting, however, that even though cross-resistance has been shown with several insect species, the most active juvenile hormones appear to be more toxic in these strains than many of the presently used pesticides. High juvenile hormone activity coupled with specificity may still offer considerable hope for the future.

The work with juvenile hormone mimetic compounds to date indicates that these compounds may well be degraded by insects in much the same way as our present insecticides. The results further suggest that juvenile hormone mimetic compounds will be subject to all the problems associated with other xenobiotics used for the control of insects, namely the development of resistance. These juvenile hormone mimics will also apply pressure in favor of the development of cross-resistance by interacting with the microsomal enzyme system through induction.

Although insect juvenile hormone mimetic compounds still appear to offer promise as third generation insecticides, they are not the panacea originally predicted. We must continue our efforts to find new ways to control insect pests. We must not be deluded into the hope that juvenile hormone and its mimics will reach the marketplace devoid of the same problems besetting our present day insecticides.

ACKNOWLEDGMENT

Appreciation is expressed to Dr. L. L. Keeley for helpful suggestions.

LITERATURE CITED

Ajami, A. M., Riddiford, L. M., *J. Insect Physiol.* **19,** 635 (1973).
Benskin, J., Vinson, S. B., *J. Econ. Entomol.* **66**(1), 15 (1973).
Berkoff, C. E., *J. Chem. Educ.* **48**(9), 577 (1971).
Bigley, W. S., Plapp, F. W., *Ann. Entomol. Soc. Amer.* **53,** 360 (1960).
Bowers, W. S., *Bull. W. H. O.* **44,** 381 (1971).
Brown, A. W. A., *Bull. Entomol. Soc. Amer.* **14,** 3 (1968).
Cerf, D. C., Georghiou, G. P., *Nature (London)* **239,** 401 (1972).
Cox, J., *Org. Gard. Farm.* **19,** 110 (1972).
Dyte, C. E., *Nature (London)* **238,** 48 (1972).
Harris, F. A., Graves, J. B., Nemec, S. J., Vinson, S. B., Wolfenbarger, O. A., "Southern Cooperative Series Bulletin 169," 1972, p 92.
Hodgson, E., Ed., "Enzymatic Oxidations of Toxicants," North Carolina State University Press, 1968, p 229.
Hoyer, R. G., Plapp, F. W., Jr., *J. Econ. Entomol.* **59,** 495 (1966).
Hoyer, R. F., Plapp, F. W., Jr., Orchard, R. D., *Entomol. Exp. Appl.* **8,** 65 (1965).
Kopec, S., *Biol. Bull.* **42,** 323 (1922).
Meyer, A. S., Schneiderman, H. A., Hanzmann, E., Ko, J. H., *Proc. Nat. Acad. Sci. U. S.* **60,** 853 (1968).
Perry, A. S., Buckner, A. J., *Life Sci.* **9,** 335 (1970).
Philpot, R. M., Hodgson, E., *Chem. Biol. Interact.* **4,** 399 (1971).
Plapp, F. W., Jr., *J. Econ. Entomol.* **63,** 138 (1970a).
Plapp, F. W., Jr., *J. Econ. Entomol.* **63**(6), 1768 (1970b).
Plapp, F. W., Jr., *J. Econ. Entomol.* **64**(5), 999 (1971).
Plapp, F. W., Jr., Casida, J. E., *J. Econ. Entomol.* **62**(5), 1174 (1969).
Plapp, F. W., Jr., Vinson, S. B., *Pestic. Biochem. Physiol.* **3,** 131 (1973).
Roller, H., Dahm, K. H., Sweely, C. C., Trost, B. M., *Angew. Chem.* **6**(2), 179 (1967).
Schmialek, P., *Z. Naturforsch.* **16,** 461 (1961).
Slade, M., Zibitt, C. H., in "Insect Juvenile Hormones," Menn, J. S., Beroza, M., Ed., Academic Press, New York, N. Y., 1972.
Slama, K., *Annu. Rev. Biochem.* **40,** 1079 (1971).
Suchy, M., Slama, K., Sorm, F., *Science* **162,** 582 (1968).
Tate, L. G., Plapp, F. W., Hodgson, E., *Chem. Biol. Interact.* in press (1973).
Wiggelsworth, V. B., *Quart. J. Microsc. Sci.* **77,** 191 (1934).
Wiggelsworth, V. B., *Quart. J. Microsc. Sci.* **79,** 91 (1936).
Wiggelsworth, V. B., *J. Insect. Physiol.* **7,** 73 (1961).
Wigglesworth, V. B., *J. Insect. Physiol.* **9,** 105 (1963).
Williams, C. M., *Nature (London)* **178,** 212 (1956).
Williams, C. M., *Sci. Amer.* **217**(1), 13 (1967).
Williams, C. M., International Symposium on New Perspective on the Control of Injurious Insects, Rome, Italy, Sept 16–18, 1968.

Received for review May 7, 1973. Accepted September 13, 1973. Approved for publication as TA-10485 by the Director, Texas Agricultural Experiment Station, in cooperation with the U. S. Department of Agriculture. Presented at the Division of Agricultural and Food Chemistry, Symposium on Biochemistry of Insect Resistance, 165th National Meeting of the Americal Chemical Society, Dallas, Texas, April 1973.

Microsomal Cytochrome P-450: Characterization and Possible Role in Insecticide Resistance in *Musca domestica*

Ernest Hodgson,* Lawrence G. Tate, Arun P. Kulkarni, and Frederick W. Plapp, Jr.[1]

Cytochrome P-450 plays a central role in oxidative metabolism of xenobiotics. Recent studies of insecticide-resistant housefly strains have shown that cytochrome P-450 is qualitatively and quantitatively different from that in susceptible strains. Cytochrome P-450 was characterized through the following difference spectra: carbon monoxide; type I substrate; type II (including *n*-octylamine); type III; and ethyl isocyanide. It is probable that more than one cytochrome P-450 occurs in the housefly resembling, in some respects, the cytochromes P-450 from normal and induced mammals. The type I spectrum characteristic of mammalian microsomes cannot be demonstrated in the insecticide-susceptible CSMA housefly strain, although it is apparent in some resistant strains. Current work concerns the correlation of cytochrome P-450 types with the genetics of resistance and on structure–function relationships in difference spectra. Genes on chromosomes II and V appear to control qualitative differences between cytochrome P-450 from different strains, the same chromosomes associated with resistance involving high oxidase activity.

The microsomal mixed function system, which includes cytochrome P-450, plays a major role in the metabolism of xenobiotics in mammals (Gillette *et al.*, 1969) and insects (Hodgson, 1968; Hodgson and Plapp, 1970). An excellent review by Wilkinson and Brattsen (1972) has recently summarized the information on insect microsomal oxidations available through early 1972. The present article deals particularly with two areas, methodology and interpretation of optical difference spectra as applied to cyto-

Department of Entomology, North Carolina State University, Raleigh, North Carolina 27607.
[1] Department of Entomology, Texas A&M University, College Station, Texas 77840.

chrome P-450 from *Musca domestica* L. and the occurrence and genetics of qualitative variants of cytochrome P-450 from insecticide-susceptible and resistant strains of the same organism. Although particular emphasis is placed on publications and on-going research from this laboratory, the literature is reviewed to the extent necessary to place this work in proper perspective.

Cytochrome P-450 was first demonstrated in insects, including the housefly, by Ray (1965, 1967). Since that time it has been demonstrated in a number of other insect species from several different families (see Wilkinson and Brattsen, 1972, for references).

DIFFERENCE SPECTRA

The diagnostic feature most used by investigators of insect cytochrome P-450 is the carbon monoxide optical difference spectrum. This results from an interaction between reduced cytochrome P-450 and CO, giving an absorption peak at or about 450 nm in the difference spectrum. Cytochrome P-450 is relatively labile, breaking down to an enzymatically inactive pigment, cytochrome P-420. This change has long been known in mammals (Omura and Sato, 1964a,b; Omura *et al.*, 1965) and the insects appear to be similar in this regard (Philpot and Hodgson, 1971a). Other spectra of importance, as yet little used by investigators of insect microsomal oxidations, are the substrate difference spectra, types I, II, and III, the ethyl isocyanide (EtNC) spectrum, and the *n*-octylamine spectrum. It should be noted that type I, type II, and *n*-octylamine spectra represent interactions with the oxidized form of the cytochrome, while the remainder are with the reduced cytochrome. The pH dependence and the pH equilibrium point of the type III and EtNC spectra are of some importance and will be discussed later.

Difference spectra are used rather than absolute spectra because of the problems caused by light scattering in highly turbid microsomal suspensions. Such suspensions scatter a great deal of light and, since the amount of such scattering varies with the wavelength, absolute spectra would be seen as small changes in the absolute absorbance on a constantly changing baseline. By putting the microsomes suspension in both cuvettes of a high-resolution spectrophotometer, the light scattering can be balanced and a flat baseline recorded. By placing the material to be investigated in the sample cuvette, the changes it causes in the absolute spectrum, rather than the absolute spectrum itself, can be recorded. This principle is illustrated in Figure 1.

There are still a number of problems remaining; a high-resolution instrument is required, if possible, with a short light path between cuvettes and photocells in order to minimize the effect of light scattering. In addition, changes may occur in both cuvettes due to the presence of other cytochromes as well as endogenous substrates, which may interfere with spectra. All spectra recorded in this laboratory are obtained with a Beckman Acta V spectrophotometer.

The problem of baseline drift, occurring after the ligand under investigation has been added, is always of concern when working with the difference spectra of turbid samples. To minimize misinterpretation of data, we have recently attempted (Mailman *et al.*, 1973) to establish formal arbitrary guidelines which will indicate perturbations caused by differences in turbidity occurring after additions have occurred or when an added ligand has native absorbance in the visible–near uv region which is affecting the difference spectrum. These guidelines are: increasing concentrations of a ligand should show concomitant increases in maxima and decreases in minima; the isosbestic point must remain constant as the concentration of the ligand is changed; there must be no noticeable absorbance inflection between 500 and 470 nm; and the absorbance

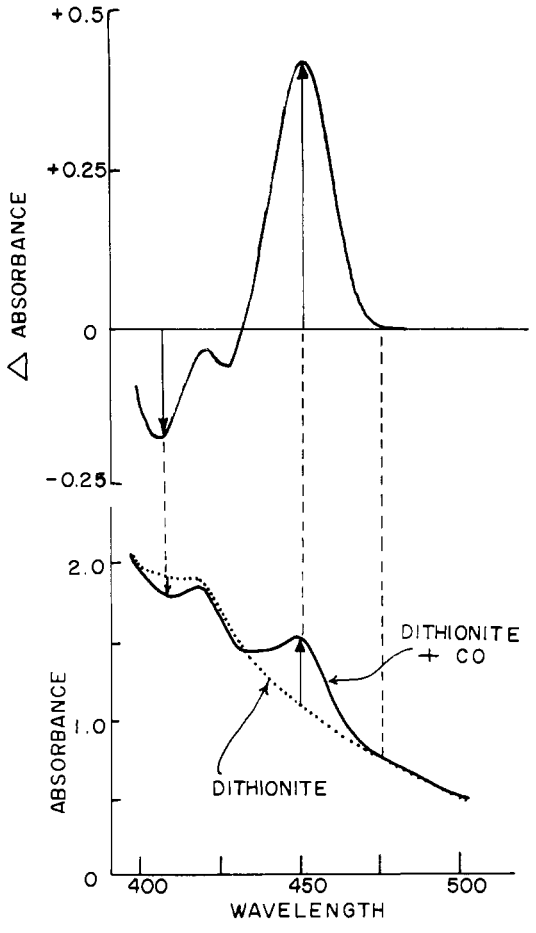


Figure 1. Principle involved in optical difference spectroscopy with reference to the carbon monoxide difference spectrum of microsomal cytochrome P-450.

change at 365 nm must be minimal and always be positive for type I spectra and negative for type II spectra.

For example, the aniline spectrum shown in Figure 2 is a type II spectrum obtained with mouse liver microsomes, with a maximum at 427 nm and a minimum at 395 nm. As the ligand concentration is increased, an isosbestic point is noted at about 418 nm which does not change. Concomitantly, the maximum increases, the minimum decreases, the absorbance between 470 and 500 nm remains almost unchanged, and that at 365 nm decreases only slightly with each addition of aniline. If these characteristics were not attained interpretation would be diffi-

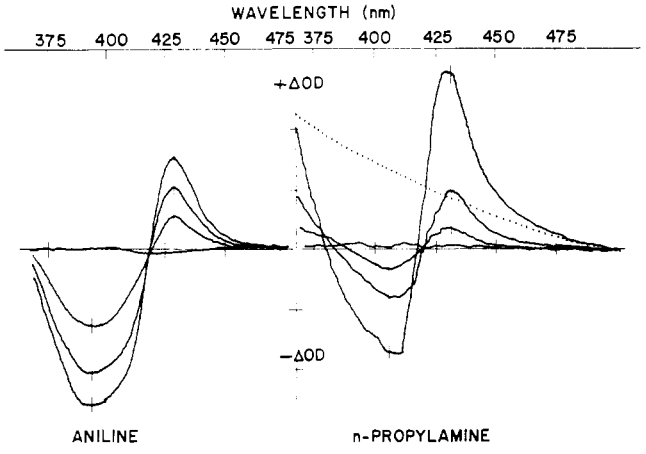


Figure 2. Effect of baseline drift on the type II difference spectrum as determined with microsomal cytochrome P-450 from the mouse (aniline) and Fc-resistant flies (*n*-propylamine). ----, estimated position of baseline following drift.

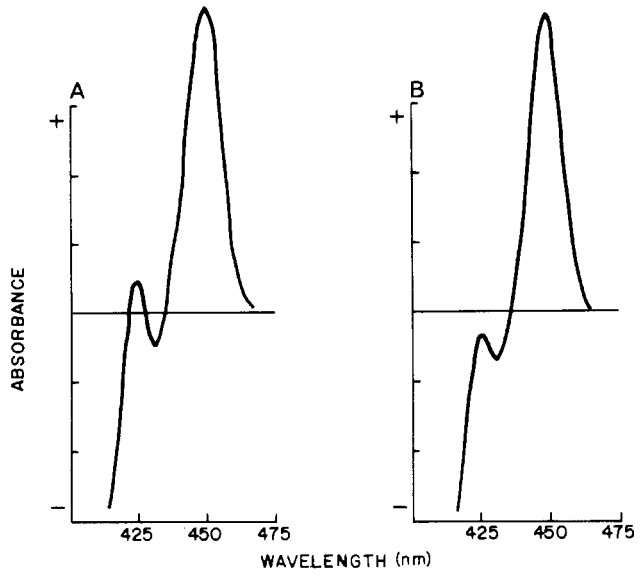


Figure 3. The effect of adding dithionite after (A) or prior to (B) the addition of carbon monoxide on the apparent level of cytochrome P-420 in microsomal enzyme preparations from the housefly.

cult, since a more complex situation must exist. Namely, a baseline change may have taken place due to turbidity changes greater in one cuvette than the other or mixed interactions of type I and type II may be occurring or denaturation of cytochrome P-450 to cytochrome P-420 may have occurred.

The type II spectrum formed by *n*-propylamine and microsomes from Fc-resistant houseflies (Figure 2) illustrates the effect of baseline drift. That drift has occurred is shown by the absorption change between 500 and 470 nm, the 5-nm shift in the isosbestic point, and the positive absorbance at 365 nm. The effects of the drift are: an apparent shift in peak position of 3–4 nm; an apparent increase of peak height of approximately 30%; and a decrease in trough size of 40% at the highest concentration added.

Several problems are encountered when working with cytochrome P-450 in housefly preparations. Observations in our laboratory indicate that contamination by cytochrome P-420 occurs more readily in preparations from insects than in those from mammals (Philpot and Hodgson, 1971a). This could be due to the presence of proteolytic enzymes, since homogenates of whole abdomens are used. Krieger and Wilkinson (1970) have reported a proteolytic enzyme from *Prodenia eridania* which acts as an inhibitor of the mixed-function oxidase system, possibly by direct attack on the microsomal protein. The modification of cytochrome P-450 to cytochrome P-420 by added proteolytic enzymes has been thoroughly established in both mammalian and insect systems (Folsom *et al.*, 1971; Omura and Sato, 1964a,b; Orrenius *et al.*, 1969; Nishibayashi and Sato, 1968).

The suggestion by Perry and Bucknor (1970) that a significant level of "apparent cytochrome P-420" in the 12,000 × *g* supernatant fraction from houseflies is due to the presence of cytochrome b_5 and CO-bound hemoglobin seems unlikely, since cytochrome b_5 is not known to combine with CO and no oxidized hemoglobin–CO difference spectrum is discernible in housefly preparations.

Morello *et al.* (1971) have reported on housefly preparations relatively free from cytochrome P-420. This may be, however, a consequence of cytochrome P-420 degradation caused by the addition of dithionite to the assay suspension prior to the establishment of a baseline and subsequent CO addition to the same cuvette (Omura and Sato, 1964a,b). This represents a modification of the cytochrome P-450 assay procedure published by Omura and Sato (1964a,b), who demonstrated that cytochrome P-450 is degraded when subjected to dithionite under aerobic conditions. This change in procedure, perhaps inadvertent, is a common one, particularly among workers using insects (Matthews and Casida, 1970; Morello *et al.*, 1971; Perry and Bucknor, 1970; Perry *et al.*, 1971). Figure 3 illustrates the effect on P-420 contaminations of adding dithionite before or after CO treatment.

Measurement of the amount of cytochrome P-420 present also represents a problem, since cytochrome P-420 is seen as an increase in absorbance relative to the cytochrome P-450 peak and should be measured relative to a hypothetical baseline, the cytochrome P-450 spectrum alone. Measurement relative to the initial baseline (*e.g.*, at 490 nm) is inappropriate. The principle is illustrated in Figure 4 in which the amount of cytochrome P-420 is the same, although the three spectra appear radically different.

Additional problems are encountered when whole flies are used in place of abdomens as a source of microsomes. The CO difference spectrum obtained from one of the whole fly preparations of Morello *et al.* (1971), the "homogenate preparation," shows a trough at 445 nm and a peak in the 430-nm region. It appears significant that this CO spectrum from whole fly preparations closely resem-

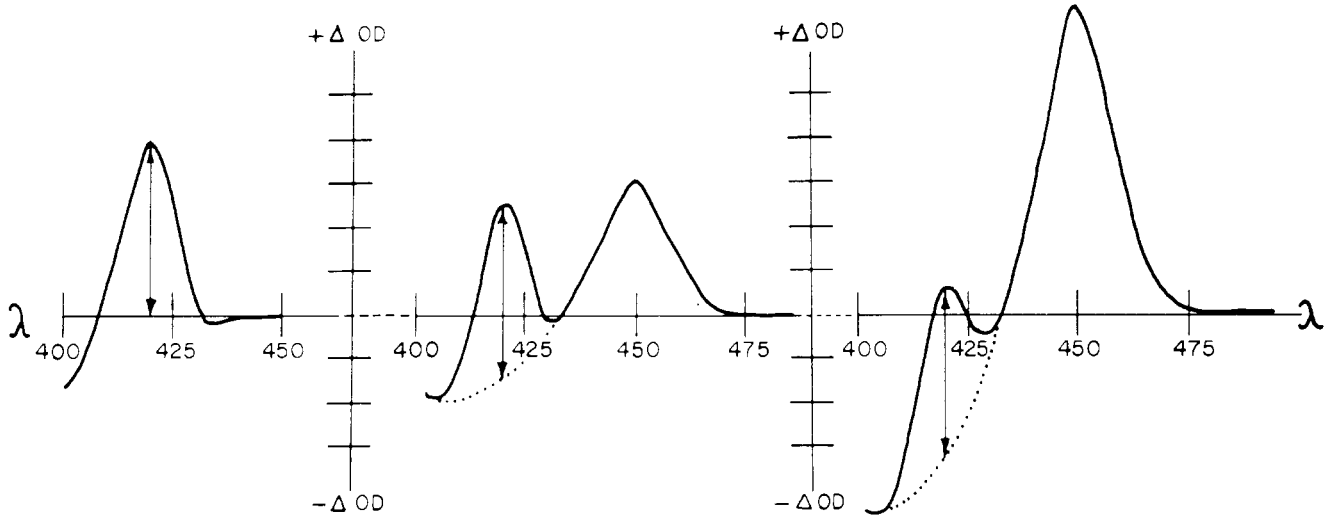


Figure 4. Estimation of cytochrome P-420 contamination in microsomal preparations. Although three different spectra, it can be seen that the amount of cytochrome P-420 is approximately the same in each case, an effect which would not be noted if the 420-nm peak was measured from the original baseline.

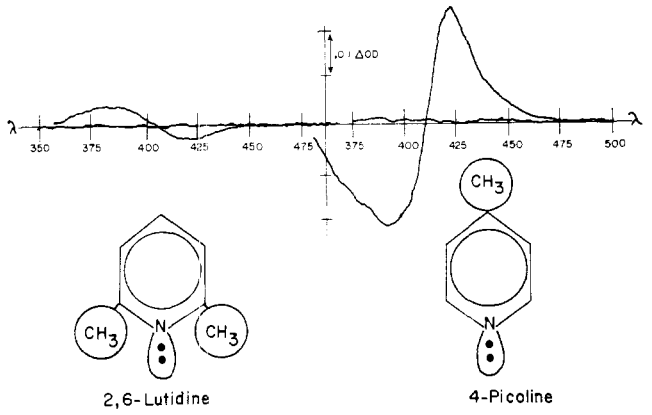


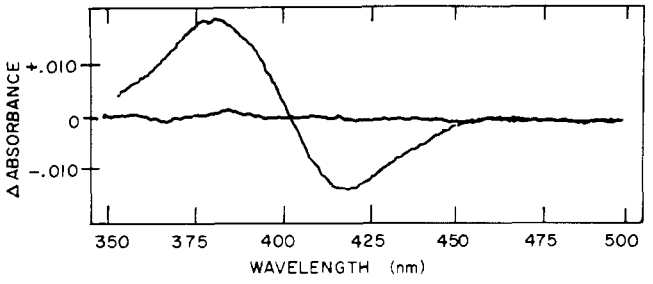
Figure 5. Nature of the type II spectral change. Spectra obtained using microsomes from the Fc strain of houseflies.

bles the difference spectrum of the CO complex of cytochromes oxidase and P-450 from adrenal mitochondria described by Kowal *et al.* (1970). This same spectrum has also been produced from mixtures of bovine heart electron-transport particles and liver microsomes. The CO difference spectrum observed from whole fly preparations is probably produced by the same two pigments, cytochrome oxidase and P-450. That this apparent mitochondrial contamination of whole fly preparations can be avoided by the use of mild homogenization techniques is demonstrated by Morello *et al.* (1971) using a different preparation, the "mortar preparation." However, preparations of this type from males and females of the resistant Fc housefly strain yielded less cytochrome P-450 (about 1.1×10^{-3} ΔA/fly) (see Figure 2, Morello *et al.*, 1971) than was reported by Philpot and Hodgson (1971a) for the abdomen of CSMA-susceptible flies (2.3×10^{-3} ΔA/abdomen). The use of preparations from housefly abdomens also avoids the endogenous mixed-function oxidase inhibitor described by Matthews and Hodgson (1966) and later characterized as xanthommatin by Schonbrod and Terriere (1971) and Wilson and Hodgson (1972).

The exact form of cytochrome P-450 CO difference spectrum and its variations will be considered, along with the possible role of this cytochrome in resistance.

Type I and Type II Substrate Difference Spectra. These spectra (Schenkman *et al.*, 1967) have been frequently used in investigations of mammalian cytochrome P-450, but few reports are available (Hodgson and Plapp, 1970; Philpot and Hodgson, 1971a; Tate *et al.*, 1973a,b) on insects, in which their form is similar to that in mammals, although there are striking differences between their occurrence in different genetic strains which are not seen in mammals. The type I spectrum has a peak at 385 nm and a trough at 420 nm, while the type II has a peak at about 430 nm and a trough at about 393 nm. There are several variations in the form of the type II spectrum and we have seen many of them in preparations of housefly cytochrome P-450.

We have examined (Kulkarni *et al.*, 1973) the difference spectra of some 206 organic compounds with cytochrome P-450 from the susceptible CSMA strain of houseflies and the resistant Fc strain. These include pesticides, insecticide synergists, and other compounds. Of these 122, a little over 60%, gave type II spectra and it is apparent, from work done on mammals (Mailman *et al.*, 1973) as well as houseflies, that the proper structure to cause a type II spectrum must include: spatially accessible sp^2 or sp^3 nitrogen or spatially accessible oxygen in certain configurations not yet fully defined but which seem to involve frequently an acidic character, *e.g.*, phenols or alcohols. Figure 5 shows a compound (4-picoline) with the nitrogen both in the correct configuration and spatially accessible compared to another (2,6-lutidine) in which the nitrogen,



PHOSPHATES	$\Delta od/\Delta od_{P450}$
trichlorfon	.142
azodrin	.117
CARBAMATES	
baygon	.122
carbaryl	.108
ORGANOCHLORINES	
aldrin	.114
DDT	.098

Figure 6. Type I difference spectra obtained using microsomes from the Fc strain of houseflies.

although appropriate, is spatially inaccessible because of the presence of two adjacent methyl groups.

Fifty-three compounds, almost all of those which do not form a type II spectrum, form a type I spectrum with the oxidized form of cytochrome P-450 from Fc flies. They include many insecticides, some of which are shown in Figure 6. A very small number of compounds appear to give a mixed type I/type II spectrum.

With susceptible CSMA flies the type I spectrum is essentially missing. In directly comparable experiments, 50 of the 53 compounds which gave type I spectra with the cytochrome P-450 from Fc flies failed to do so with the cytochrome from CSMA flies, including such well known type I compounds as benzphetamine. The remaining three, sulfoxide and *cis*- and *trans*-methylenedioxycyclohexane, gave something resembling a type I. However, in view of the complex interactions of methylenedioxy compounds with cytochrome P-450 (Hodgson *et al.*, 1973), it is apparent that these are not typical type I compounds. It is clear that type I spectral perturbations of the type seen in mammalian preparations cannot be demonstrated in susceptible flies.

***n*-Octylamine Spectra.** This is a special type of type II spectrum. It can be demonstrated in two forms, one with a double trough at 410 and 394 nm, characteristic of insecticide-susceptible strains of houseflies, and another with a single trough at 390 nm, characteristic of some resistant strains (Philpot and Hodgson, 1971a; Tate *et al.*, 1973a,b).

Ethyl Isocyanide Spectrum. This spectrum has been observed several times using housefly microsomal preparations (Capdevilla *et al.*, 1973; Matthews and Casida, 1970; Philpot and Hodgson, 1971a; Tate *et al.*, 1973a,b). It represents an interaction between EtNC and the reduced form of cytochrome P-450 and characteristically has two peaks in the Soret region, one at 455 nm and the other at 430 nm. These peaks are pH-dependent and if peak height is plotted against pH, the EtNC pH equilibrium point can be determined. This value has been used, in induction studies in mammals, to indicate whether or not a qualitative change has occurred in the cytochrome since, if the effect was simply a quantitative increase, the peak heights would change but not the equilibrium point (Sladek and Mannering, 1966).

Cytochrome P-420 also exhibits an ethyl isocyanide difference spectrum with a peak at 433 nm (Omura and Sato, 1964a,b) which may obscure the 430 peak and make calculation of this peak difficult.

Type III Spectrum. It has been demonstrated that *in vivo* administration of piperonyl butoxide to houseflies inhibits the formation of the CO–cytochrome P-450 difference spectrum (Matthews and Casida, 1970; Perry and Bucknor, 1970; Perry *et al.*, 1971). Perry and Bucknor (1970) found a good correlation between this inhibition and the toxic levels of several carbamate insecticides in resistant houseflies. We have recently related piperonyl butoxide inhibition of the CO–cytochrome P-450 difference spectrum in mice to a piperonyl butoxide–cytochrome P-450 interaction that prevents CO binding to the cytochrome (Hodgson *et al.*, 1973; Philpot and Hodgson, 1971b,c, 1972). The demonstration of this interaction from housefly preparations (Philpot and Hodgson, 1971a) suggests that the effect of piperonyl butoxide in the housefly is similar to that observed in mice. Piperonyl butoxide forms a typical type I (Hodgson and Plapp, 1970; Matthews *et al.*, 1970; Philpot and Hodgson, 1971b) spectrum with oxidized P-450 and the addition of dithionite eliminates this. After incubation with NADPH, a spectrum with peaks at 455 and 427 nm appears and dithionite simply serves to increase the size of this spectrum. If dithionite is not added but the preparation is allowed to use up all of the available NADPH, the spectrum assumes an oxidized type III form with a single peak at 437 nm; it does not return to the type I form but can be transformed from oxidized to reduced by addition of NADPH. This spectrum cannot be displaced by CO or any other ligand.

Other Difference Spectra of Housefly Microsomes. Preliminary investigations show that pyrethroids such as allethrin form a spectrum with a peak at 418 nm but, unlike the type II spectrum, it has no trough at 390 nm and has a small trough at 443 nm. Little more can be said at this point, except that the spectrum is not caused by all pyrethroids and the structural reason for its occurrence is not yet apparent. It does, however, appear to represent a clear difference between houseflies and mammals since, in the latter, pyrethroids are type I substrates.

Wilkinson and coworkers (Wilkinson and Brattsen, 1973) have shown that the 1,2,3-benzothiodiazole synergists inhibit mixed-function oxidases and exhibit a different spectrum with reduced cytochrome P-450 with a peak at 446 nm, which shifts to 450 nm on the addition of CO.

CYTOCHROME P-450 IN INSECTICIDE RESISTANCE

Work in our laboratory as well as several others had indicated that an increased level of mixed-function oxidase activity is associated with the resistance of houseflies to several insecticides (see Wilkinson and Brattsen, 1972, for references). Since this is clearly related to cytochrome P-450, we decided to compare this cytochrome from flies of the susceptible CSMA and from the Rutgers diazinon-resistant strain (Philpot and Hodgson, 1971a). A comparison of these two strains based on the size and peak position of the CO, types I, II, III, *n*-octylamine, and the EtNC difference spectra, expressed on a per fly basis, show several points which bear particular attention. All spectra show an increase in resistance as opposed to susceptible flies, thus confirming the fact that P-450 is higher in this resistant strain. There is no detectable type I spectrum in the susceptible strain. This has been confirmed with a number of substrates and it is now quite clear that there is no type I binding site, of the type seen in mammals, in susceptible flies. Type II spectra are large as compared to mammals, particularly in the resistant strain. The *n*-octylamine spectrum shows a distinct difference between two strains, having a double trough at 394 and 410 nm in the susceptible strain and a single trough at 390 nm in the resistant strain. Probably of most significance is the observation that the resistant to susceptible ratios for the different parameters are not the same, as would be expected if the difference between the two strains was simply a quantitative increase in the amount of a single cytochrome P-450.

It is, in fact, strong evidence for a qualitative difference. This is borne out further when the data are calculated on the basis of OD units of the cytochrome P-450 CO difference spectrum. If there is only a quantitative difference, this should be eliminated and all ratios should be 1. In fact, they vary from 0.6 to 3.3.

It seems appropriate to mention that several of these parameters were used to support the contention that the cytochrome P-450 from 3-methylcholanthrene-induced mammals was a different cytochrome than that found in phenobarbital-induced mammals (Alvares *et al.*, 1967; Jefcoate *et al.*, 1969; Shoeman *et al.*, 1969; Sladek and Mannering, 1966). Another criterion used is that of the peak position, the wavelength of maximum absorption. Variations were between the two strains in this regard also, usually in the direction of 2–3 nm higher in the susceptible strain (*e.g.*, 451 *vs.* 449 in the CO spectrum, 426 *vs.* 424 in the pyridine type II spectrum).

We do not mean to imply that there is any direct relationship between induced cytochromes in mammals and resistance in insects. What the data do show, in each case, is that the differences cannot be explained on the basis of a quantitative change in a single cytochrome.

Perry *et al.* (1971) also suggested, based on the peak position of the CO spectrum, that qualitative differences exist between a susceptible strain and one of several resistant strains examined, Diazinon-R (this strain and Rutgers diazinon-resistant are both derived from the same strain). The differences in peak wavelength, 448 *vs.* 452 nm in their hands compared to 449 *vs.* 451 nm in ours (Philpot and Hodgson, 1971a), may well be due to the experimental problems discussed earlier. Based primarily on EtNC spectra, Matthews and Casida (1970) suggested qualitative differences might exist between susceptible and resistant strains and, more recently, Capdevilla *et al.* (1973) have suggested, again based on EtNC spectra, that qualitative differences exist between the cytochrome P-450 of normal and phenobarbitol- or naphthalene-induced flies of the Fc resistant strain.

In this latter regard it should be noted that induction does not necessarily result in a cytochrome P-450 with altered spectral properties, since it has been shown (Tate *et al.*, 1973a) that induction of the Orlando strain with DDT results in a higher level of cytochrome P-450, which has spectral properties identical to those of the uninduced insect.

It should also be noted that none of the investigations reported, from this or other laboratories, have demonstrated that changed spectral properties are due to changes in the cytochrome *per se*. It is entirely possible, although less likely, that the changes noted are due to changes in other constituents of the microsomal membrane. Solubilization and purification studies now in progress should be helpful in this regard.

GENETICS OF CYTOCHROME P-450 IN HOUSEFLIES

At this juncture it occurred to us that since much was known of the genetics of resistance, particularly that due to oxidative metabolism, and we knew that the cytochrome involved could be different in different strains, this was an unparalleled opportunity to at least approach the molecular biology of insecticide resistance. That is to say, we could try to relate specific genes to specific cytochromes and eventually to understand how the genetic makeup of the insect related to specific cytochromes P-450.

The genetics of the resistant strain we were working with, Rutgers diazinon-resistant, were unknown at that time so we needed to work with strains of known genetics. In the Fc strain, resistance was known to involve a gene

Table I. Comparison of Cytochrome P-450 Spectral Characteristics between Resistant and Susceptible Housefly Strains

Spectrum	Strain	
	Susceptible[a]	Resistant[b]
P-450–CO absorbance maxima, nm	451	449
P-450 titer/female abdomen (% susceptible level)	100	150–200
Formation of Type I difference spectrum	−	+
Forms a single trough Type II *n*-octylamine spectrum	−	+
Magnitude of Type III ethyl isocyanide 455 nm peak, % CO value	100	60

[a] CSMA- and sbo-susceptible strains. [b] R-Fc, Diazinon-R, and Dimethoate-R strains.

on the fifth chromosome, while in the Baygon-resistant strain the gene was known to be on the second chromosome. The use of visible mutant markers (Tsukamoto, 1964) is well known and will not be discussed further. In our investigations the following recessive markers were used: stubby wings (stw), the gene for which is located on chromosome II; brown body (bwb), chromosome III; ocra eyes (ocra), chromosome V. The cytochrome P-450 of the triply marked susceptible strain (sbo) showed no significant spectral differences to the CSMA-susceptible strain.

The initial study (Tate *et al.*, 1973a) was to examine the cytochrome P-450 in flies from as many strains as possible, including some marked strains. These initial studies involved no new crosses, since the strains were already in existence and had been inbred for a number of years. The cytochrome P-450 levels were determined for the following strains: CSMA; Fc_{NCSU}; Fc_{Texas}; Dimethoate-R; R-Baygon, bwb, ocra (R-chromosome II); stw, bwb, R-Fc (R-chromosome V); and stubby winged, bwb, ocra, (sbo) susceptible.

The data showed that: cytochrome P-450 levels are highest in the Fc_{NCSU} and Dimethoate-R strains but only slightly higher than susceptible in Fc_{Tex}; no differences from susceptible levels could be seen in the marked Fc or marked Baygon strains; the absorption maximum was shifted to 449 in the two strains with the highest level; a simultaneous increase in microsomal protein occurs with the increase in P-450, thus raising the possibility that one effect may be an increase in endoplasmic reticulum.

Analysis of type I (benzphetamine), type II (pyridine), type III (EtNC), and piperonyl butoxide and *n*-octylamine spectra revealed the following. Most obvious difference in these strains is presence or absence of detectable type I binding. The marked Fc strain, although resembling the susceptible in P-450 level and optimum, does resemble the other Fc strains in having a type I binding site. It could be noted at this point that supposedly identical strains come to vary from one another following isolated inbreeding over many generations in different laboratories. The Fc_{NCSU} and Fc_{Tex} strains are an excellent illustration of this. The results seem to indicate that the presence of type I binding is related to resistance associated with chromosome V, at least in the Fc strain. The presence of a double trough in the *n*-octylamine spectrum is a susceptible characteristic seen also in Baygon, Fc_{Tex}, and marked Fc but not in Fc_{NCSU}. Although Baygon has high oxidase activity (Plapp and Casida, 1969), its P-450 seems to be essentially the same as that in susceptible strains, indicating that the cytochrome is not rate limiting and that other factors must be examined. Cytochrome P-450 reductase is one obvious place to start. A small 455 ethyl isocyanide peak seems to be linked to the presence of a double trough in the *n*-octylamine trough.

Table I is a summary of those spectral characteristics which appear to be of particular importance in the different forms of P-450 which are associated with some resistant strains of the housefly, namely type I binding, P-450 titer and absorbance maximum, *n*-octylamine spectrum, and the ethyl isocyanide spectrum.

Recently (Tate *et al.*, 1973b) we have used crossing experiments with marked strains to provide new information on the cytochrome P-450 in resistant strains. The Rutgers diazinon-resistant strain was of unknown genetics. We crossed this with the marked susceptible (sbo) and then back-crossed the F_1 ♂ progeny to the sbo strain.

High P-450, presence of type I binding, single trough *n*-octylamine spectrum, and low ethyl isocyanide spectrum are all associated with the presence in the substrains of chromosome II from the resistant strain; *i.e.*, substrains +++, ++o, +b+, and +bo. In contrast, strains without resistant chromosomes II (s++, s+o, sb+, and sbo) show susceptible spectral characteristics. LD_{50} determinations confirm that resistance is associated primarily with chromosome II in this strain.

In a similar examination of the Fc strain, high P-450 levels are seen in phenotypes containing the second chromosome from the resistant strain, although much of the resistance in this strain is due to chromosome V (Oppenoorth and Houx, 1968; Plapp and Casida, 1969). However, the presence of the type I spectrum is associated with V, that is substrains +++, +b+, sb+, and s++. A single-trough *n*-octylamine spectrum could not be found in the progeny nor did the ethyl isocyanide spectrum show any striking differences between the different phenotypes, suggesting that anciliary factors may be involved in the expression of these last two characters.

It is interesting to speculate on the genetics of cytochrome P-450 in the housefly. Even at this early stage in the investigations, it is apparent that at least four genes (three on chromosome II and one on chromosome V) are required to explain the spectral variants alone. Moreover, the gene on V required for type I binding appears to be similar to the gene for the same characteristics on II in another strain, raising the further speculation that a translocation from II to V may have occurred during the development of the Fc strain.

I would not like to leave the impression that all of these results are simply interpreted. It is apparent that even though resistant flies may be brought about by selection for a small number of genes, the effects are modified in subtle ways by many other genes and by the genetic background of the strain with which experimental crosses are made. Some of these genes may be increasing formation of smooth endoplasmic reticulum and therefore by force of circumstance, cytochrome P-450, while others may cause specific changes in either the cytochrome P-450 molecule or in the way it is bound in the membrane. The answer to many of these problems lies in solubilizing the cytochrome from different strains and this is a task we have started on.

It is apparent, however, that the genetics of cytochrome P-450 and the genetics of some types of resistance appear to be inextricably linked and that this type of analysis represents a new tool with which to further explore the mechanism of resistance at the molecular level.

LITERATURE CITED

Alvares, A. P., Schilling, G., Levin, W., Kuntzman, R., *Biochem. Biophys. Res. Commun.* **29**, 521 (1967).
Capdevilla, J., Morello, A., Perry, A. S., Agosin, M., *Biochemistry* **12**, 1445 (1973).
Folsom, M. D., Philpot, R. M., Hodgson, E., *Comp. Biochem. Physiol.* **39B**, 589 (1971).
Gillette, J. R., Conney, A. H., Cosmides, G. J., Estabrook, R. W., Fouts, J. R., Mannering, G. T., Ed., "Microsomes and Drug Oxidations," Academic Press, New York, N. Y., 1969.

Hodgson, E., Ed., "Enzymatic Oxidation of Toxicants," North Carolina State University, Raleigh, N. C., 1968.
Hodgson, E., Plapp, F. W., Jr., *J. Agr. Food Chem.* **18**, 1048 (1970).
Hodgson, E., Philpot, R. M., Baker, R. C., Mailman, R. B., *Drug Metab. Disposition* **1**, 391 (1973).
Jefcoate, C. R. E., Gaylor, J. C., Calbrese, R. L., *Biochemistry* **8**, 3455 (1969).
Kowal, J., Simpson, E. R., Estabrook, R. W., *J. Biol. Chem.* **245**, 2438 (1970).
Krieger, R. I., Wilkinson, C. F., *Biochem. Pharmacol.* **18**, 1403 (1969).
Kulkarni, A. P., Mailman, R. B., Baker, R. C., Hodgson, E., submitted for publication.
Mailman, R. B., Kulkarni, A. P., Baker, R. C., Hodgson, E., submitted for publication.
Matthews, H. B., Casida, J. E., *Life Sci.* **9**, 989 (1970).
Matthews, H. B., Hodgson, E., *J. Econ. Entomol.* **59**, 1286 (1966).
Matthews, H. B., Skrinjoric-Spoljar, M., Casida, J. E., *Life Sci.* **9**, 1039 (1970).
Morello, A., Bleecker, W., Agosin, M., *Biochem. J.* **124**, 199 (1971).
Nishibayashi, N., Sato, R., *J. Biochem. (Tokyo)* **63**, 766 (1968).
Omura, T., Sato, R., *J. Biol. Chem.* **239**, 2370 (1964a).
Omura, T., Sato, R., *J. Biol. Chem.* **239**, 2379 (1964b).
Omura, T., Sato, R., Cooper, D. Y., Rosenthal, O., Estabrook, R. W., *Fed. Proc.* **24**, 1181 (1965).
Oppenoorth, F. J., Houx, N. W. H., *Entomol. Exp. Appl.* **11**, 81 (1968).
Orrenius, S., Berg, A., Ernster, L., *Eur. J. Biochem.* **11**, 193 (1969).
Perry, A. S., Bucknor, A. J., *Life Sci.* **9**, 335 (1970).
Perry, A. S., Dale, W. E., Bucknor, A. J., *Pestic. Biochem. Physiol.* **1**, 131 (1971).
Philpot, R. M., Hodgson, E., *Chem. Biol. Int.* **4**, 399 (1971a).
Philpot, R. M., Hodgson, E., *Chem. Biol. Int.* **4**, 185 (1971b).
Philpot, R. M., Hodgson, E., *Life Sci.* **10**, 503 (1971c).
Philpot, R. M., Hodgson, E., *Mol. Pharmacol.* **8**, 204 (1972).
Plapp, F. W., Jr., Casida, J. E., *J. Econ. Entomol.* **62**, 1174 (1969).
Ray, J. R., in "Pest Infestation Research," HMSO, London, 1965.
Ray, J. R., *Biochem. Pharmacol.* **16**, 99 (1967).
Schenkman, J. B., Remmer, H., Estabrook, R. W., *Mol. Pharmacol.* **3**, 113 (1967).
Schonbrod, R. B., Terriere, L. C., *Pestic. Biochem. Physiol.* **1**, 409 (1971).
Shoeman, D. W., Chaplin, M. D., Mannering, G. J., *Mol. Pharmacol.* **5**, 412 (1969).
Sladek, N. E., Mannering, G. J., *Biochem. Biophys. Res. Commun.* **24**, 668 (1966).
Tate, L. G., Plapp, F. W., Jr., Hodgson, E., *Chem. Biol. Int.* **6**, 237 (1973a).
Tate, L. G., Plapp, F. W., Jr., Hodgson, E., *Biochem. Genet.* in press (1973b).
Tsukamoto, M., *Botyu-Kagaku* **29**, 51 (1964).
Wilkinson, C. F., Brattsen, L. B., *Drug Metab. Rev.* **1**, 153 (1972).
Wilson, T. G., Hodgson, E., *Pestic. Biochem. Physiol.* **2**, 64 (1972).

Received for review April 30, 1973. Accepted September 11, 1973. Paper number 4041 of the Journal Series of the North Carolina State University Agricultural Experiment Station, Raleigh, N. C. This work was supported, in part, by grant number ES-00044 from the U. S. Public Health Service. Presented at a symposium on "Biochemistry of Insect Resistance," 165th National Meeting of the American Chemical Society, Dallas, Tex., April 11, 1973.

The Induction of Detoxifying Enzymes in Insects

Leon C. Terriere* and Shyi J. Yu

Microsomal oxidases and DDT-dehydrochlorinase activity are increased in houseflies treated with insecticides such as aldrin, dieldrin, and DDT, the barbiturate phenobarbital, and the insect hormones or hormone analogs such as ecdysone and juvenile hormone. There is evidence that in housefly strains resistant to insecticides by virtue of increased detoxication activity, inducing chemicals cause greater increases in enzyme activity than in susceptible strains treated similarly. It is postulated that this is due to the presence, in the resistant strains, of multiple sets of genes coding for the induced enzymes. The possibility that induction is, or has been, a factor in insect control is discussed. It is concluded that because of the high doses required, at least in the cases examined so far, there is little likelihood that induction has been a factor.

The phenomenon of induction is an appropriate topic of discussion at this symposium on the biochemistry of resistance because in some respects it resembles resistance. Shortly after various species of animals are treated with inducing chemicals the activity of certain enzyme systems is increased, sometimes as much as 25-fold. In this respect such animals are similar to insecticide-resistant insects which are often resistant because they can metabolize the insecticide rapidly enough to escape its toxic effects. Since some of the very insecticides to which insects are now resistant have also been found to be inducers of the detoxication enzymes, it is not surprising that some investigators have wondered whether there is a connection between these two events, *i.e.*, whether induction by these chemicals has been a factor in their ability to select the resistant population.

Probably the first reports of induction in insects were those of Agosin and coworkers (Agosin and Dinamarca, 1963) who observed the phenomenon in *Triatoma infestans*, a blood-sucking insect, when this species was treated with DDT. They found that DDT increased the level of NADP, which is an important cofactor in microsomal oxidation. Later, they showed that the increased level of NADP following DDT treatment resulted from increased activity of NAD-kinase (Ilevicky *et al.*, 1964). These workers suggested that, since DDT was metabolized in some species by the microsomal oxidases (Agosin *et al.*, 1961), its induction of this system might be related to DDT resistance. In a series of papers that followed this discovery, these investigators established that the DDT-stimulated increase in enzymes involved the synthesis of new (*i.e.*, additional) protein (Agosin *et al.*, 1965, 1967), that RNA synthesis was involved (Balazs and Agosin, 1968; Litvak *et al.*, 1968), and that DDT metabolism was indeed more rapid in treated insects (Agosin *et al.*, 1969).

Turning next to a study of the phenomenon in the housefly (*Musca domestica*) Gil *et al.* (1968) obtained results similar to those with *T. infestans* but noted that only certain strains of resistant houseflies, *i.e.*, those with resistance which involved oxidative mechanisms, were inducible with DDT. In several of their reports, the Chilean workers suggested a connection between induction by

Departments of Agricultural Chemistry and Entomology, Oregon State University, Corvallis, Oregon 97331.