Cytochrome *P*-450 and Hydroxylating Activity of Microsomal Preparations from Whole Houseflies

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1. A new microsomal preparation, obtained from whole houseflies is described in terms of its cytochrome *P*-450 content and its hydroxylating activity. 2. Microsomes prepared from whole-fly brei, obtained with the aid of a mortar (a procedure that avoids the destruction of sarcosomes), contain 0.265 nmol of cytochrome *P*-450 and hydroxylate naphthalene at a rate of 28.5 nmol/mg of microsomal protein in 30 min at 30°C. This corresponds to 104 nmol of naphthalene hydroxylated/nmol of cytochrome *P*-450. This is the highest rate ever reported for housefly and rat liver microsomal preparations. 3. Microsomal fractions prepared by procedures that do not retain the integrity of sarcosomes show the presence in the CO-difference spectrum of a 428 nm peak. This cytochrome is associated with sarcosomal microsomes and it may be involved in the inhibition of insect microsomal mixed-function oxidases, although other factors cannot be discarded at present. 4. The inability to show cytochrome *P*-450 in microsomal fractions isolated from whole houseflies by other procedures may be at least partially due to a masking effect brought about by contamination with the sarcosomal cytochrome.

Cytochrome *P*-450 has been shown to participate as a terminal oxidase in mixed-function oxidations in mammalian liver (Mason, Yamano, North, Hashimoto & Sakagishi, 1963). Although its presence in insects has been only recently reported (Ray, 1967), available evidence suggests that it also participates in insect microsomal hydroxylations (Perry, 1970; Mathews & Casida, 1970). The latter are important in resistance to several types of insecticides, especially since the enzyme(s) involved is induced by the same insecticides metabolized (Morello, 1964; Agosin, Scaramelli, Gil & Letelier, 1968).

Insect microsomal fractions have been usually prepared from housefly abdomens by the procedure outlined by Agosin, Michaeli, Miskus, Nagasawa & Hoskins (1961) or its modifications, since microsomal preparations from whole insects are much less active. Although the presence of cytochrome *P*-450 has been demonstrated in microsomal fractions from whole insects in one instance (Ray, 1967), it can be consistently shown only in microsomal fractions from abdomens, whereas an 'apparent' peak of cytochrome *P*-420 can be observed in microsomal fractions from whole flies (Perry, 1970).

These observations have led various authors to postulate the presence of microsomal inhibitors,

preferentially localized in the thoraxes and heads of the insects. These inhibitors would even act on microsomal oxidations by vertebrate preparations (Lewis, 1969; Mathews & Hodgson, 1966). The fact that microsomal fractions isolated from whole flies are still active in hydroxylation (Gil *et al.* 1968), although cytochrome *P*-450 cannot be usually detected in these microsomes (Perry, 1970), prompted us to characterize in more detail this type of preparation with the hope of obtaining more active microsomal fractions and the possibility of using them as a source of cytochrome *P*-450. This would avoid the more laborious procedure of isolating microsomal fractions from abdomens alone. In addition, cytochrome *P*-450 concentrations determined in microsomal fractions from whole insects could explain better the differences observed between insecticide-susceptible and -resistant housefly strains (Perry, 1970; Mathews & Casida, 1970). The present paper describes a microsomal preparation from whole houseflies that hydroxylates naphthalene at rates greater than previously described, and that contains appreciable amounts of cytochrome *P*-450. Naphthalene hydroxylation was used as the model reaction since naphthalene is a simple molecule with a metabolism extensively studied in insects and mammalian tissues (Schafer & Terriere, 1970; Booth & Boyland, 1958). It is

expected that this new preparation will be a tool for a better understanding of the metabolism of xenobiotics by insects.

MATERIALS AND METHODS

Chemicals. Naphthalene and 1-naphthol were obtained from Amersham-Searle, Des Plaines, Ill., U.S.A., and Baker Chemical Co., Phillipsburg, N.J., U.S.A., respectively. Glucose 6-phosphate, glucose 6-phosphate dehydrogenase and NADP were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. 2,6-Dichloroquinonechloroimide was purchased from Aldrich Chemical Co., Cedar Knolls, N.J., U.S.A. Highly polymerized RNA and bovine crystalline albumin were obtained from Sigma Chemical Co. The rest of the reagents were of the highest purity commercially available. Carbon monoxide was 99.9% pure.

Insect material. Unless otherwise indicated, non-sexed flies of the Fc strain 6–9 days old were used throughout. This strain is fairly resistant to naphthalene vapours and highly resistant to DDT [1,1,1-trichloro-2,2-bis-(*p*-chlorophenyl)ethane]. Other characteristics have been previously reported (Gil *et al.* 1968).

Preparation of microsomal fractions. Two procedures were used to prepare microsomes from whole flies. (a) Homogenizer microsomes. This is the commonly used method to prepare microsomal fractions and is as follows. About 500 flies (10 g), previously immobilized by cold, were homogenized in 40 ml of 0.1 M-potassium phosphate buffer, pH 7.5, in a Sorvall homogenizer at full speed for 60 s. The homogenate was filtered through two layers of cheesecloth and the filtrate was centrifuged at 10 000g for 10 min. The centrifugation was repeated three times, and the final supernatant was centrifuged at 214 000g in the 60 Ti rotor of a L2-65B Spinco centrifuge for 45 min. The microsomal pellet thus obtained was suspended in 5 ml of 0.1 M-potassium phosphate buffer, pH 7.5, and used as such. All these and the following operations were done at 2–4°C.

(b) Mortar microsomes. A 10 g sample of flies in 40 ml of 0.1 M-potassium phosphate buffer, pH 7.5, was placed in a previously chilled mortar, and the mash was gently pounded, without any grinding action, about 120 times, until a smooth brei was obtained. The brei was filtered through two layers of cheesecloth and the filtrate centrifuged at 750g for 10 min. The supernatant was then centrifuged at 3000g for 10 min to obtain intact sarcosomes (Watanabe & Williams, 1951). The supernatant was again centrifuged at 3000g for 10 min and the small pellet discarded. The supernatant was centrifuged at 10 000g for 10 min and the resulting pellet was suspended in 5 ml of 0.1 M-potassium phosphate buffer, pH 7.5. This preparation was termed 'heavy microsomes'. The supernatant was twice centrifuged at 10 000g for 10 min, the small pellets obtained were discarded, and centrifugation was carried out again at 214 000g for 45 min as above. The microsomal pellet ('mortar microsomes') was suspended in 5 ml of 0.1 M-potassium phosphate buffer, pH 7.5.

(c) Sarcosomal microsomes. The sarcosomal pellet obtained as described above was washed once with the phosphate buffer and then suspended in 40 ml of the same buffer. Homogenization was then carried out in a

Sorvall homogenizer at full speed for 120 s. The homogenate was centrifuged twice at 10 000g for 10 min and microsomes were obtained from the supernatant by centrifugation at 214 000g for 45 min as described above. The pellet was resuspended in 5 ml of the phosphate buffer.

(d) Thorax and head microsomes. Thoraxes and heads from 10 g of female flies were used to prepare microsomal fractions by the mortar procedure described above.

Difference spectra of microsomal fractions. The spectral characteristics of the various microsomal preparations were recorded in a Unicam SP.1800 spectrophotometer, by using cells of 1 cm light-path. To part of the microsomal suspension derived from 10 g of flies, a few milligrams of sodium dithionite were added and 1 ml portions of the suspension were added to the reference and sample cuvettes respectively. The base line was recorded, and then the sample cuvette was bubbled with CO for 30 s. The differential spectrum was then recorded. Cytochrome P-450 concentration was determined by using an extinction coefficient of $91 \text{ cm}^{-1} \cdot \text{mm}^{-1}$ for the ΔE between 450 and 490 nm (Omura & Sato, 1964).

Hydroxylation of naphthalene. Naphthalene hydroxylation was followed by measuring the sum of 1,2-dihydro-1,2-dihydroxynaphthalene and 1-naphthol produced. The incubation mixture in a final volume of 12 ml contained the following: microsomal fraction equivalent to 10 g of flies; 500 μmol of potassium phosphate buffer, pH 7.5; 80 μmol of MgCl_2 ; 10 μmol of NADP; 180 μmol of glucose 6-phosphate; 10 units of glucose 6-phosphate dehydrogenase; 6 μmol of naphthalene in 100 μl of ethanol. The reaction was started by the addition of naphthalene and incubation was carried out at 30°C. At timed intervals (see Fig. 6) samples of 0.5–1.0 ml were pipetted into centrifuge tubes containing 0.1 ml of conc. HCl. The tubes were then heated in a boiling-water bath for 10 min to convert the diol into 1-naphthol. The samples were extracted with 3 ml of ethyl acetate, and the 1-naphthol was determined in the ethyl acetate layer with 2,6-dichloroquinonechloroimide (Booth & Nayland, 1958). Zero-time values were obtained by using either boiled microsomal fractions or by pipetting a sample into the HCl immediately after addition of naphthalene.

Analytical procedures. RNA was measured by the orcinol procedure (Mejbaum, 1939) with highly polymerized commercial RNA as standard and protein by the method of Lowry, Rosebrough, Farr & Randall (1951).

All results presented in this paper are the average of four or more experiments. Variation between experiments was not more than 7%.

RESULTS

'Homogenizer microsomes.' The yield of microsomal fraction from 10 g of flies by this procedure was about 64 mg of protein, with a protein/RNA ratio of about 5.0 (Table 1). Fig. 1 shows the CO-differential spectrum of microsomal fractions prepared by this method. A large peak at 428 nm is evident, and only a very small one at 458 nm. The spectrum shows a large trough at 445 nm, which is similar to spectra reported for whole fly microsomal fractions by Perry (1970). The peak at 458 nm is clearly asymmetrical, suggesting that

material absorbing at 450nm is being masked by the trough at 445nm.

These preparations hydroxylate naphthalene at very low rates (Table 1) similar to the activity shown by Arias & Terriere (1962) for microsomal fractions of other housefly strains prepared by essentially the same procedure.

'Mortar microsomes.' The yield of microsomes from 10g of flies was much less than for the 'homogenizer microsomes', about 23mg of protein. The protein/RNA ratio was also smaller, about 2.0 (Table 1). The CO-differential spectrum of this preparation is shown in Fig. 2. A large and straight peak of cytochrome P-450 is clearly evident, without any 428 or 420nm peaks, except for a very small shoulder at about 428nm. In agreement with this high content of cytochrome P-450, microsomal fractions prepared by the mortar procedure hydroxylated naphthalene at rates 25 times higher than the 'homogenizer microsomes' (Table 1). It is possible that this difference may be higher, since it was difficult to measure naphthalene hydroxylation by 'homogenizer microsomes' at that rate, owing to the limitations of the colorimetric determination procedure used.

Thorax and head microsomes. Contrary to what was expected on the basis that microsomal inhibitors could be present in fly thoraxes and heads, microsomal fractions derived from these tissues, and prepared by the mortar procedure, hydroxylated naphthalene in terms of their cytochrome P-450 at rates similar to those for 'mortar microsomes' from whole flies (Table 1), far better than the rates shown by 'homogenizer microsomes'. The CO-differential spectrum of head and thorax microsomal fractions showed the presence of a clear peak of cytochrome P-450 (Fig. 3). The protein/RNA ratio of these microsomes was similar to that of 'mortar microsomes' from whole flies (Table 1).

Sarcosomal microsomes. These, prepared as

described in the Materials and Methods section, did not hydroxylate naphthalene at detectable rates (Table 1). Surprisingly, the CO-differential spectrum (Fig. 4) showed the presence of a large peak at 428nm with a deep trough at 445nm as described for microsomal fractions prepared by the homogenizer procedure.

'Heavy microsomes.' These contained, in addition to cytochrome P-450, considerable amounts of the 428nm peak, which suggests that this preparation contains sarcosomes and/or sarcosomal fragments (Fig. 5). This is also supported by the high protein/RNA ratio shown by these preparations (Table 1). The hydroxylating activity of these 'heavy microsomes' was not determined.

Effect of sarcosomal microsomes on the rate of hydroxylation by 'mortar microsomes.' In experiments not shown here, it was found that 'sarcosomal microsomes' oxidized NADPH at rates that could interfere with the hydroxylation reaction by competing for the availability of NADPH. These microsomal fractions also oxidized NADH at higher rates than NADPH. When increasing amounts of sarcosomal microsomes were added to the hydroxylation reaction mixture containing 'mortar microsomes,' it was found that hydroxylation decreased proportionately to the amount of sarcosomal protein added (Table 2).

DISCUSSION

The 'mortar microsomes' preparation from whole insects is more active than those reported for abdomen and whole insect microsomes prepared by the homogenizer procedure (Mathews & Casida, 1970; Arias & Terriere, 1962; Schonbrod & Terriere, 1966). Fig. 6 shows the kinetics of hydroxylations of naphthalene by microsomal fractions prepared by the 'mortar' and the 'homogenizer' procedure respectively. The concentration

Table 1. Characteristics of housefly microsomal fractions

Results are given for 10g of flies. For experimental details, see the Materials and Methods section. Cytochrome P-450 was measured by using the extinction coefficient $91 \text{ cm}^{-1} \cdot \text{mm}^{-1}$ and ΔE 450–490nm.

Source of microsomes	Procedure	Protein (mg)	RNA (mg)	Protein/RNA ratio	Cytochrome P-450 content		Naphthalene metabolized	
					Total (nmol)	(nmol/mg of protein)	(nmol/15 min)	(nmol/15 min per nmol of cytochrome P-450)
Total insect	Mortar	23.8	12.2	2.0	6.2	0.265	334	54
	Homogenizer	64.0	12.7	5.0	1.4*	0.046	14	10
Thorax and Head	Mortar	9.7	5.4	1.8	0.8	0.083	45	57
Sarcosomes	Homogenizer	12.4	0.6	20.7	0.0	0.00	0	0
Heavy microsomes	Mortar	13.6	3.4	4.0	1.6	0.118	—	—

* ΔE 458–490nm.

Fig. 1. CO-differential spectrum of microsome fraction isolated from whole flies by the homogenizer procedure. The concentration of the microsome fraction was 2 g of flies/ml. See the Materials and Methods section for further details.

of cytochrome *P*-450 in the mortar preparation was 0.265 nmol/mg of microsome protein, a value similar to that reported by Mathews & Casida (1970) for abdomens of Fe strain flies, but higher than for Diazinon strain flies (Perry, 1970). The recovery of total cytochrome *P*-450 appears to be slightly better in the mortar preparation if the contents of 'mortar microsomes' and 'heavy microsomes' are added together. The concentration of cytochrome *P*-450 in our preparation is about one-third of rat liver and about one-fifth of guinea-pig liver (Gillette & Gram, 1969). The latter corresponds to the highest value reported in the

Fig. 2. CO-differential spectrum of microsome fraction isolated from whole flies by the mortar procedure. Experimental details are as for Fig. 1.

literature. When the preparations are compared in terms of nmol of substrate hydroxylated/nmol of cytochrome *P*-450 per 30 min our preparation is far better than those of Mathews & Casida (1970),

Fig. 3. CO-differential spectrum of microsomal fraction isolated from thoraxes and heads by the mortar procedure. Experimental details are as for Fig. 1.

and even better than rat liver microsomes (Booth & Boyland, 1958).

The main difference between the 'mortar microsomes' and microsomes prepared by other procedures, such as Schafer & Terriere's (1970), is that the integrity of sarcosomes is preserved by the mortar. As a result, sarcosomes can be easily eliminated by differential centrifugation, avoiding the contamination of microsomal fractions by sarcosomes and/or sarcosomal fragments. Sarcosomal contamination may be suspected when (a) there is an increase in the amount of microsomal protein above normal values ['clean' microsomes have about 23.8mg of protein/10g of flies, against 64mg of protein when homogenization is done by other procedures (Table 1)], (b) the CO-differential spectrum of 'clean' microsomal fractions shows only the presence of cytochrome *P*-450 whereas microsomal fractions prepared by more disruptive procedures have a spectrum closely resembling that of sarcosomes. Our results strongly indicate that 'clean' microsomal fractions show the presence of only cytochrome *P*-450, and that the 'apparent' cytochrome *P*-420 found in other preparations may correspond to sarcosomal contamination and not to conversion of cytochrome *P*-450 into cytochrome *P*-420. This is further supported by the fact that

Fig. 4. CO-differential spectrum of sarcosomal microsomes. Experimental details are as for Fig. 1.

conversion of cytochrome *P*-450 into cytochrome *P*-420 by mild detergent treatment does not result in a deep trough at 445nm (Perry, 1970). Microsomes prepared from whole flies by the homogenizer

Fig. 5. CO-differential spectrum of 'heavy microsomes' obtained by the mortar procedure. Experimental details are as for Fig. 1.

procedure show the presence of small amounts of cytochrome *P*-450, but the peak is shifted to higher wavelengths, about 458 nm. This is understandable if we realize that the sarcosomal cytochrome is masking the cytochrome *P*-450 peak (Fig. 1). Under these conditions, the absorption at 450 nm becomes relatively negative. When the flies are homogenized in a tight Potter-Elvehjem homogenizer or when they are frozen before homogenization, large amounts of the 428 nm peak material are apparent. This indicates that these procedures result in sarcosomal disruption.

It is not clear from our results whether true inhibitors of microsomal inhibitors are present in our preparations. If the oxidations are localized mainly in the head and thorax, one should expect no activity from these tissues, but cytochrome *P*-450 is present in thorax and head microsomes, which are also capable of hydroxylating naphthalene (Table 1, Fig. 3). However, inhibition of 'mortar microsomes' activity was observed when sarcosomal microsomes were present in the reaction mixture. It is possible that the sarcosomal cytochrome may be involved in the inhibition by competing for NADPH, but other factors cannot be discarded at present.

Fig. 6. Kinetics of hydroxylation of naphthalene by whole fly microsomal fraction (10g) prepared by the homogenizer and the mortar procedures. Incubation was for the indicated time-intervals at 30°C. ●, Microsomes isolated by homogenizer procedure, 62 mg of protein; ○, microsomes isolated by the mortar procedure, 23 mg of protein.

Table 2. Effect of sarcosomal microsomes on the rate of hydroxylation of naphthalene by microsomes prepared by the mortar procedure

Sarcosomal microsomes added (mg of protein)	Naphthalene metabolized (% of control)
0 (Control)	100
12.4	54
6.2	82
3.1	100

Although insect microsomal oxidations are considered to be important in the detoxication of several types of insecticides (Gil *et al.* 1968), one puzzling aspect was the generally low activity of these microsomal preparations. The present report of a microsomal system that is in the activity range of mammalian liver microsomes will enhance the role of mixed-function oxidases in insecticide resistance. It is also expected that it will result in a re-evaluation of the problem.

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