Possible Role of Tyrosinase and Cytochrome P-450 in the Metabolism of 1-Naphthyl Methylcarbamate (Carbaryl) and Phenyl Methylcarbamate by Houseflies

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Housefly microsomes plus $NADPH_2$ metabolize carbaryl to its 4-hydroxy, 5-hydroxy, and, probably, *N*-hydroxymethyl and 5,6-dihydrodihydroxy derivatives, while phenyl methylcarbamate forms one major organosoluble metabolite. The metabolism of both carbamates by housefly microsomes is inhibited by carbon monoxide, and this inhibition is partially reversed by light. Tyrosinase present in housefly microsome preparations does not appear to be involved in the metabolism of either of these carbamates. A soluble tyrosinase prepared from adult houseflies does not degrade carbaryl, Baygon, or phenyl methylcarbamate.

Housefly microsome preparations contain cytochrome P-450 (Ray, 1967) and tyrosinase (Wilkinson, 1965). Homogenates of adult houseflies contain a soluble tyrosinase which can be partially purified (Abd El-Aziz, 1967; Metcalf *et al.*, 1966). The purpose of this research was to acquire some information on the role tyrosinase and/or cytochrome P-450 might play in the metabolism of methylcarbamate insecticides by houseflies.

MATERIALS AND APPARATUS

Carbaryl carbonyl-C^{14}, Baygon isoprop-1,3-C^{14}-oxy, and phenol (U-C^{14}) were obtained from The Radiochemical Center, Amersham, England. Phenyl-C^{14}-labeled phenyl methylcarbamate (PMC) and unlabeled PMC were synthesized according to Kolbezen *et al.* (1954). Each radioactive carbamate had a radiochemical purity of 98% or better, and each was adjusted to a specific activity of 0.4 mc. per mmole. The 4-hydroxy and 5-hydroxy derivatives of carbaryl were supplied by Union Carbide Corp., South Charleston, W. Va. Nicotinamide adenine dinucleotide phosphate (NADP), glucose-6-phosphate (G-6-P), and G-6-P dehydrogenase were purchased from Boehringer, London, England.

Radioactive measurements were made with a Beckman liquid scintillation spectrometer, using vials containing 6 ml. of a mixture of 0.55% 2,5-diphenyloxazole in toluene and 2-methoxyethanol (2 to 1). Glass plates coated with 0.25 mm. of silica gel H (Anderman & Co., Ltd., London, England) were employed for thin-layer chromatography (TLC). Plates were developed in 3-to-1 ether-hexane. Radioautography was accomplished with x-ray film purchased from Kodak Limited, London, England. Ferric chloride–potassium ferricyanide and *p*-nitrobenzenediazonium fluoroborate were used to detect unlabeled compounds on TLC plates (Krishna *et al.*, 1962). Oxygen uptake was measured with a Clark electrode. Light from a 1000-W tungsten-iodine vapor lamp (Sylvania, Type DXN), passed through a 6-inch condenser and a copper sulfate solution as a heat filter, served to illuminate reaction vessels which were held in a constant temperature bath.

Unsexed, 2- to 4-day-old adult houseflies of a dieldrin-resistant strain (*Musca domestica vicina*) were used in all experiments. After emergence from puparia, flies were fed on sugar and water only. Centrifugation of fly homogenates was performed with a MSE high speed centrifuge.

METHODS

Preparation of Microsomes and Soluble Tyrosinase. Flies were immobilized with cold and 100 to 200 grams of flies, along with twice their weight in volume of 1.15% KCl, were homogenized in a Waring blender for 30 seconds. The homogenate was strained through muslin cloth, centrifuged at 21,000 G for 15 minutes, and the supernatant was filtered through glass wool before being centrifuged for 1 hour at 105,000 G. The microsome pellet was suspended in 1.15% KCl with a hand-driven PTFE pestle to give approximately 25 mg. of protein per ml., as measured by the Biuret method (Cleland and Slater, 1953). The supernatant remaining after the 105,000 G spin was considered the soluble fraction. In some cases, the microsomes were washed by resuspension in 1.15% KCl and resedimentation at 105,000 G for 1 hour. All operations were carried out at 0–5° C. with precooled apparatus. Microsomes and soluble fractions were held at 0° C. and used the same day they were prepared, unless indicated otherwise.

Soluble tyrosinase was isolated and partially purified from adult houseflies according to Abd El-Aziz (1967), as reported by Metcalf *et al.* (1966).

Incubation, Extraction, and Chromatography Procedures. In the microsome studies, a typical incubation flask contained phosphate buffer, pH 7.7 ($5 \times 10^{-2}M$), KCl ($1.24 \times 10^{-2}M$), G-6-P ($2.8 \times 10^{-3}M$), G-6-P dehydrogenase (1 unit), NADP ($4.8 \times 10^{-4}M$), 0.5 ml. of microsome suspension, and water to total 5 ml. Radiolabeled carba-

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mates were added in 50 μl. of ethanol to give a final concentration of $2 \times 10^{-5}M$. Incubation, in air, was carried out at 30° C. for 20 minutes without shaking, in the case of carbaryl experiments, and 2 hours with shaking, in the case of PMC experiments. In some studies, incubation took place in an atmosphere of nitrogen or an 80% CO–20% O_2 mixture. For experiments on the effect of light, flasks containing buffer, KCl, G-6-P, and microsomes were preilluminated for 20 minutes before addition of NADP, G-6-P dehydrogenase, and substrate, and incubation in the usual manner. In certain experiments with carbaryl, flasks were illuminated during the 20-minute reaction period in air or in the CO–O_2 mixture.

Additions to incubation flasks included KCN ($10^{-3}M$), hydrogen peroxide ($10^{-3}M$), EDTA ($10^{-3}M$), and nicotinamide ($5 \times 10^{-3}M$). In control experiments, either the reaction mixture containing the microsomes was boiled for 10 minutes before the addition of G-6-P dehydrogenase, NADP, and substrate, or microsomes were omitted from the reaction flask. Slight adjustments to the volume of the incubation mixture were made to allow the addition of 2 ml. of soluble fraction. All experiments were performed at least three times with three different microsome preparations.

Soluble tyrosinase was incubated with 0.1 μmole of carbaryl, Baygon, or PMC for 2 hours at 30° C. in phosphate buffer, pH 7.0, according to Abd El-Aziz (1967). Either NADP alone or NADP, G-6-P, and G-6-P dehydrogenase were used as cofactors.

Reactions were stopped by the addition of 10 ml. of ether and vigorous shaking for 4 minutes. After the layers had separated, the ether was drawn off and another extraction with 10 ml. of ether followed. A portion of the combined ether extracts and of the remaining water fraction was counted. The ether extract was transferred to a test tube held in a 40° C. water bath and the ether was evaporated with a stream of air. The resulting residue consisted of a thin lipid-water coating on the inside of the lower portion of the tube. If the tube were left in the bath in the stream of air for a further 20 minutes (approximately), the residue would become completely dry. However, under these circumstances, PMC would volatilize from the tube. Thus, it was necessary to take care that the extracts containing PMC were removed from the bath after the ether was evaporated, but before the residue was completely dry, to avoid any loss of C^{14}-PMC. The residue containing parent carbamate and metabolites was taken up with 200 μl. of acetone and spotted on a TLC plate. After development, the plate was placed under x-ray film for 3 to 5 days before radioactive areas were scraped and counted. For cochromatography experiments, 20 μg. of each suspected unlabeled metabolite were added with the 200 μl. of acetone, and the TLC plate was sprayed, after radioautography, to match spots. Losses incurred during incubation and extraction were almost always less than 1%. Losses during evaporation of the ether extracts, TLC, and scraping of plates averaged 20% for all experiments.

Measurement of Tyrosinase Activity. The conditions used to measure the tyrosinase activity of the microsomes were, as nearly as possible, the same as those used in the microsome-metabolism studies. The electrode flask contained phosphate buffer, pH 7.7, G-6-P, and KCl in the same molar proportions used in incubation flasks. To this solution were added 50 μl. of microsome suspension or 200 μl. of soluble fraction, to give a final volume of 4 ml. After equilibration, *p*-cresol ($1.25 \times 10^{-3}M$) or 4-methyl catechol ($2.5 \times 10^{-4}M$) was added. Changes in the rate of oxygen uptake were noted in the presence of KCN ($10^{-3}M$, $10^{-4}M$), EDTA ($10^{-3}M$), nicotinamide ($5 \times 10^{-3}M$), or after preincubation of the flask contents plus microsomes in the presence of 80% CO–20% O_2 mixture, or in the presence of light.

RESULTS

Metabolism by Microsomes. The pH of the incubation mixture was varied from 6.0 to 9.0, and pH 7.7 gave the most metabolism of carbaryl and PMC with the minimum amount of nonenzymic hydrolysis. In an average experiment, 14% of carbaryl was converted to metabolites in 20 minutes by the microsome-$NADPH_2$ system (11 mg. of microsomal protein). Of these metabolites, 78% were ether-soluble and 22% were water-soluble. With PMC, a 2-hour incubation with the microsome-$NADPH_2$ system (13 mg. of microsomal protein) converted 7.3% of the compound to metabolites, of which 50% were ether-soluble and 50% were water-soluble. Washing the microsomes usually improved the activity of the preparation toward carbaryl or PMC, based on micrograms of carbamate metabolized per milligram of microsomal protein.

At least five ether-soluble metabolites of carbaryl were detected after incubation in the microsome-$NADPH_2$ system. Two were tentatively identified, by cochromatography, as the 4-hydroxy and 5-hydroxy derivatives, which accounted for 44 and 29%, respectively, of the total ether-soluble metabolites. A metabolite which chromatographed in the position of the *N*-hydroxymethyl analog of carbaryl (Kuhr and Casida, 1967) accounted for 11% of the total metabolites. A small amount of residual material at the origin, and a compound just above the origin (perhaps the 5,6-dihydrodihydroxy carbaryl), together accounted for 14% of the ether-soluble metabolites. Only one major metabolite (R_f 0.25) of PMC was detected, representing about 94% of the total ether-soluble products. This may have been the 4-hydroxy derivative of PMC (Abd El-Aziz, 1967; Metcalf *et al.*, 1966).

The effect of various additions or omissions to the standard incubation flask is shown in Table I. In control flasks where microsomes were omitted or where the microsomes were boiled, less than 1% of the carbaryl or PMC was degraded, mostly to water-soluble products. This nonenzymic degradation was not affected by the addition or omission of any of the various materials listed in Table I. All experimental results, including the relative rates shown in Table I, were corrected for this nonenzymic degradation. Omitting NADP or oxygen gave 75 to 95% inhibition of the over-all degradation of both carbaryl and PMC, with over 90% inhibition of the production of ether-soluble derivatives. Soluble fraction alone did not produce metabolites of either carbamate, and inhibited the production of microsomal metabolites when added to the microsomes. Nicotinamide, EDTA, CO, and preincubation of unwashed microsomes in light all inhibited the microsomal metabolism of carbaryl and PMC, while per-

Table I. Effect of the Addition or Omission of Various Materials on the Metabolism of Carbaryl and Phenyl Methylcarbamate by Housefly Microsomes

Additions or Omissions to Microsome System[a]	Relative Rates of Metabolism	
	Carbaryl	PMC
None	100.0[b]	100.0[c]
Minus NADP	6.4	16.4
Minus oxygen	9.9	24.8
Plus soluble fraction	51.4	85.8
Plus nicotinamide $5 \times 10^{-3}M$	86.2	70.0
Plus KCN $10^{-3}M$	124.2	...
Plus EDTA $10^{-3}M$	93.6	84.1
Plus hydrogen peroxide $10^{-3}M$	119.5	109.7
Incubation in 80% CO–20% O_2	29.7	45.8
Preillumination	58.7	51.3

[a] The microsome system consisted of phosphate buffer, KCl, G-6-P, G-6-P dehydrogenase, NADP, microsomes, and substrate as described in the Methods section.
[b] The rate 100.0 is equal to 1.27% metabolism per mg. microsomal protein per 20 min.
[c] The rate 100.0 is equal to 0.56% metabolism per mg. microsomal protein per 2 hr.

oxide stimulated metabolism. In carbaryl experiments, illumination during the reaction gave approximately the same percentage inhibition of metabolism as preillumination. Washing the microsomes reduced this light inhibition from about 40 to 9%. However, washing the microsomes did not affect the amount of CO inhibition. By incubating the flasks in CO–O_2 during illumination, inhibition of carbaryl metabolism by CO was reversed from 70 to 63% inhibition with unwashed microsomes, and 70 to 33% with washed microsomes. KCN stimulated the formation of carbaryl metabolites, but had little effect on PMC metabolism, giving slight stimulation in some experiments and slight inhibition in others.

Metabolism by Soluble Tyrosinase. Two-hour incubations of Baygon or PMC with soluble tyrosinase plus NADP or the $NADPH_2$ generator failed to produce more than 1.5% degradation of either compound, of which almost all was as water-soluble products. About 4% of carbaryl was degraded in 2 hours, again mostly to water-soluble products. The only ether-soluble material detected was a compound that chromatographed in the position of 4-hydroxy carbaryl.

Microsomal Tyrosinase. The effect of various materials on the catecholase and cresolase activity of the microsomes is shown in Table II. The rate of oxygen uptake, after addition of *p*-cresol or 4-methyl catechol, was inhibited by about 60% in the presence of $10^{-4}M$ KCN or after preincubation of the microsomes in the CO–O_2 atmosphere. Nicotinamide, EDTA, or preincubation of unwashed microsomes in light did not affect the cresolase or catecholase activity of the microsomal tyrosinase. On storage at 0° C. for four days, the cresolase and catecholase activity of the microsomes remained high, while the ability of the microsomes to metabolize PMC and carbaryl fell (Figure 1). There was about six times as much tyrosinase in the soluble fraction as there was in the microsome pellet, based on gram weight of flies homogenized.

DISCUSSION

The results shown in Table I indicate that the metabolism of carbaryl and PMC by housefly microsomes requires

Table II. Effect of the Addition of Various Materials on the Activity of Housefly Microsomal Tyrosinase

Additions to Microsome System[a]	Relative Rates of Oxidation	
	4-Methyl catechol	*p*-Cresol
None	100.0	100.0
Plus nicotinamide $5 \times 10^{-3}M$	97.9	103.3
Plus KCN $10^{-3}M$	7.9	2.7
Plus KCN $10^{-4}M$	40.4	34.7
Plus EDTA $10^{-3}M$	100.0	102.6
Preincubation in 80% CO–20% O_2	40.4	42.7
Preillumination	104.7	103.4

[a] The microsome system consisted of phosphate buffer, KCl, G-6-P, microsomes, and substrate as described in the Methods section.

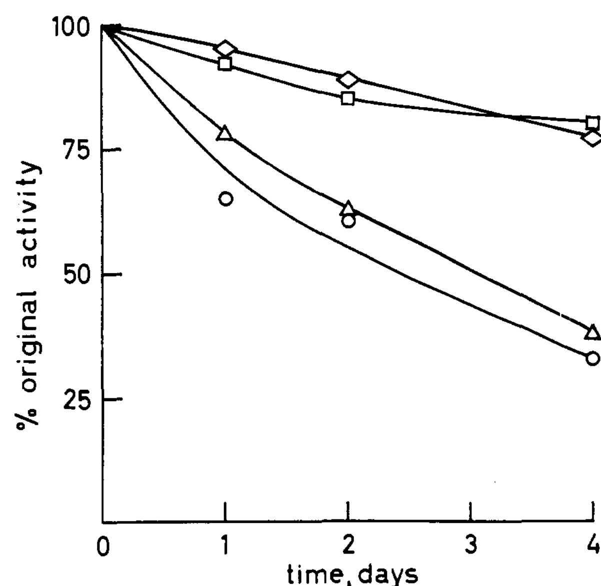

Figure 1. The effect of storage of housefly microsomes at 0° C. on the metabolic activity toward carbamates and on the oxidative activity toward *p*-cresol and 4-methyl catechol

◇ 4-Methyl catechol oxidation
□ *p*-Cresol oxidation
△ PMC metabolism
○ Carbaryl metabolism

$NADPH_2$ and molecular oxygen. The addition of soluble fraction or nicotinamide reduces the metabolic activity of the microsome-$NADPH_2$ system. The effect of KCN and EDTA on carbamate metabolism is similar to their effect on aldrin epoxidation by housefly microsomes (Ray, 1967). The inhibition of metabolism caused by light was thought to be due to the formation of hydrogen peroxide in the incubation flasks. However, addition of peroxide stimulated, rather than inhibited, carbaryl metabolism. The reason for this stimulation is not known.

The reduced form of cytochrome P-450 forms a complex with CO which is reversed by light (Cooper *et al.*, 1965). Housefly microsomes contain P-450 and the metabolism of carbaryl and PMC by housefly microsomes is inhibited by CO (Table I). Reversal of this CO inhibition by light was only partially successful, one reason being that light alone caused inhibition of carbamate metabolism. Washing the microsomes reduced this light inhibition from 40 to

9%. Washing did not affect the amount of CO inhibition, however, which remained about 70%. Reversal of the CO inhibition improved with washed microsomes, going from 70% inhibition to 33% inhibition instead of 70 to 63%. Thus, the presence of P-450 in housefly microsomes which metabolize carbaryl, the inhibition of this metabolism by CO, and the partial reversal of this CO inhibition by light would seem to indicate that cytochrome P-450 might be involved in the metabolism of carbaryl, and perhaps other carbamates, by housefly microsomes.

However, it is not known whether housefly microsome preparations contain other enzymes which form a complex with CO that is reversible by light. Studies with microsomes prepared from rat livers indicated that these preparations were less active than fly microsomes at metabolizing carbaryl, but had a much higher proportion of P-450 expressed in terms of cytochrome P-450 per mg. of microsomal protein (Kuhr, 1968). Preliminary studies with other insect microsomes containing P-450 showed that these preparations were unable to metabolize carbaryl to any great extent (Heslop, 1968). Thus, there is not always a correlation between the amount of P-450 in microsomes and their ability to metabolize carbaryl. However, endogenous inhibitors in these microsome preparations may mask activity so that correlation with P-450 is not possible. Also, P-450 may be rate-limiting in microsomes from some species, but not in others. More evidence is needed before one can say that P-450 is or is not involved in carbamate metabolism.

Microsomal tyrosinase does not appear to be associated with the metabolism of carbaryl or PMC by housefly microsomes. This can be seen by comparing the results shown in Tables I and II. Cyanide is a very good inhibitor of microsomal tyrosinase, but stimulates the microsomal metabolism of carbaryl and has little effect on the metabolism of PMC. Nicotinamide and EDTA both inhibit carbaryl and PMC metabolism, but neither has any effect on tyrosinase activity. Preincubation of microsomes in light causes inhibition of carbamate metabolism, but does not affect tyrosinase activity. The catecholase and cresolase activity of the microsome preparation remains high after 4 days, but the metabolic activity toward carbaryl and PMC falls over this time period (Figure 1). Although the soluble fraction contained six times as much tyrosinase as the microsomes, it did not metabolize carbaryl or PMC. However, since the soluble fraction inhibited microsomal metabolism, its potential activity may have been masked by the presence of endogenous inhibitors.

Soluble tyrosinase prepared from whole flies by Abd El-Aziz (1967) hydroxylated certain phenols and phenyl methylcarbamates, including PMC. However, the activity of this preparation toward Baygon, Furadan, and carbaryl was very poor. The soluble tryosinase in this report was prepared as described by Abd El-Aziz, but a different strain of housefly was employed. This soluble preparation not only failed to metabolize carbaryl and Baygon to any extent, it also failed to hydroxylate PMC.

Thus, microsomal tyrosinase would seem to play a minor role, if any, in the metabolism of carbaryl and PMC by housefly microsomes. Whether this is true for other methylcarbamates, or for other insect microsomes, is not known. Soluble tyrosinase, prepared from houseflies, is almost inactive toward carbaryl and Baygon, but, in certain cases, metabolizes PMC (Abd El-Aziz, 1967; Metcalf *et al.*, 1966). If tyrosinase is involved in carbamate metabolism by houseflies, its role as a detoxication enzyme is restricted to certain carbamates and, perhaps, to certain strains of houseflies.

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