The Metabolism of the Insecticide Carbaryl (1-Naphthyl *N*-Methylcarbamate) by Fat Body of the Blowfly Larva *Calliphora erythrocephala*

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1. Carbaryl is metabolized more rapidly by fat body of the blowfly larva than by gut, muscle, cuticle or haemolymph. 2. Metabolism of carbaryl by the fat body is affected by the age of the larva, the pH of the incubation medium, and the concentration of magnesium chloride in the incubation medium. 3. Chloramphenicol, 2,4-dinitrophenol and 5-dimethylamino-6-nitro-1,3-benzodioxole (a carbaryl synergist) inhibit carbaryl metabolism by the fat body. 4. Subcellular fractionation of the fat body indicates that the pellet sedimenting at 30 000g is the most reactive with carbaryl. 5. Probable metabolites of carbaryl formed by the fat body include the 4- and 5-hydroxy derivatives, and, possibly, the *N*-hydroxymethyl and 5,6-dihydrodihydroxy derivatives.

The metabolic fate of carbaryl (1-naphthyl *N*-methylcarbamate), formerly known as Sevin, has been investigated in several insect species. Hydrolysis of the ester bond to 1-naphthol is an important degradation route in houseflies, *Musca domestica*, milkweed bugs, *Oncopeltus fasciatus*, and German cockroaches, *Blattella germanica* (Eldefrawi & Hoskins, 1961; Ku & Bishop, 1967). Non-hydrolytic degradation of carbaryl occurs after its injection into American cockroaches, *Periplaneta americana* (Dorough & Casida, 1964), after its topical application to houseflies, American cockroaches (Dorough & Casida, 1964), and cotton leaf worms, *Prodenia litura* (Zayed, Hassan & Hussein, 1966), and after its incubation with housefly abdomen homogenates (Tsukamoto & Casida, 1967) or housefly microsomes (Tsukamoto & Casida, 1967; Kuhr, 1969). In these instances, carbaryl formed some or all of the following: 4-hydroxy-, 5-hydroxy-, *N*-hydroxymethyl-, and 5,6-dihydrodihydroxy-carbaryl.

In the present study, the metabolism of carbaryl by various tissues of the blowfly larva, *Calliphora erythrocephala*, and by subcellular fractions of larval fat bodies, was investigated. Some of the results have been briefly reported (Price & Kuhr, 1968).

MATERIALS AND METHODS

Chemicals. [carbonyl-¹⁴C]Carbaryl (1-naphthyl *N*-methylcarbamate), specific radioactivity 26.7mc/m-mole, was

purchased from The Radiochemical Centre, Amersham, Bucks. A sample of the stock solution was evaporated to dryness and the residue was dissolved in ethanol. To this was added unlabelled carbaryl to give a final specific radioactivity of either 0.4mc/m-mole (20 µg. of carbaryl in 50 µl. of ethanol) or 0.08mc/m-mole (100 µg. of carbaryl in 50 µl. of ethanol). The 4-hydroxy and 5-hydroxy derivatives of carbaryl were a gift from Union Carbide Corp., South Charleston, W. Va., U.S.A. A carbaryl synergist (5-dimethylamino-6-nitro-1,3-benzodioxole) was synthesized by Wilkinson (1967). NADP, glucose 6-phosphate and glucose 6-phosphate dehydrogenase were purchased from Boehringer Corp. (London) Ltd., London W. 5. Cytochrome c was obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks., and was reduced by the method of Yonetani & Elliott (1966). MethylCellosolve (methylcellosolve) was purchased from Shell Chemical Co. Ltd., London S.E. 1, and silica gel H was obtained from Anderman and Co. Ltd., London S.E. 1. For other chemicals used see Price (1969).

Breeding of blowflies, *Calliphora erythrocephala*. Blowfly larvae were cultured as described by Price (1969).

Isolation of larval tissues. The fat body and gut were removed as described by Price (1969), the main structures that remained being the cuticle and its attached muscle. The cutaneous muscle was removed from the cuticle under Ringer solution by holding the cuticle at its posterior end and scraping firmly forward with the end of a spatula. To isolate haemolymph, the larva was held by its posterior end and a small cut with scissors was made at the anterior tip. The haemolymph that escaped was collected in a small centrifuge tube giving 15–20 µl./larva. When sufficient haemolymph had been collected, it was centrifuged at 1200g for 10 min. to sediment the blood cells.

Ringer solution and incubation medium. The composition of these solutions is given by Price (1969).

Incubation of larval tissues. In preliminary experiments, the tissues from ten larvae were incubated in 1 ml. of the

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above incubation medium in a wide-necked flask. In other experiments, the haemolymph alone (1 ml.) was used. The flasks were equilibrated on a Warburg bath for 5 min. at 30°. Then the [^{14}C]carbaryl (20 or 100 $\mu\text{g.}$) was added and the flasks were incubated with gentle shaking for the required period. In later experiments, fat bodies from five larvae were used.

Extraction of [^{14}C]carbaryl and metabolites. After incubation the flask contents were emptied into an all-glass Potter-Elvehjem homogenizing tube and the flask was rinsed with 2 ml. of water, which was also added to the tube. The tissue was homogenized at room temperature and the homogenate centrifuged at 1200g for 5 min. The supernatant was decanted and the residue, which was further homogenized with 2 ml. of water, was centrifuged as before. The combined supernatants were shaken with 10 ml. of ether for 4 min. in stoppered tubes. The ether phase was removed with a Pasteur pipette and the water phase was shaken with a further 10 ml. of ether. After this extraction the phases were separated by centrifugation at 600g for 3 min. and the ether extracts were combined.

Measurement of ^{14}C radioactivity. A sample of the ether extract (0.5 ml.) or of the water extract (0.2 ml.) was put into a glass scintillation vial together with 6 ml. of scintillation fluid (6.6 g. of 2,5-diphenyloxazole-800 ml. of toluene-400 ml. of methylCellosolve) and the ^{14}C radioactivity was measured in a Beckman liquid-scintillation counter, type LS200, over a 20 min. period. All counts were corrected for background radioactivity.

Thin-layer chromatography. Half of the remaining ether extract was evaporated to dryness at 40°, and the residue, after being dissolved in 0.2 ml. of acetone, was applied to a 20 cm. $\times$ 20 cm. glass plate coated with a film of silica gel H 0.25 mm. thick and chromatographed in ether-hexane (3:1, v/v). The plate was allowed to dry at room temperature and afterwards was placed in close contact with X-ray film (Kodak Ltd., London W.C. 2) for 3-5 days. By using the radioautograph as a guide, the radioactive zones on the thin-layer plate were located and the silica gel was scraped off into scintillation vials. The ^{14}C radioactivity was measured as described above. For co-chromatography experiments, 20 $\mu\text{g.}$ of the 4-hydroxy and 5-hydroxy derivatives of carbaryl were added to the 0.2 ml. of acetone before it was applied to the t.l.c. plate. After radioautography, the plate was sprayed with $\text{FeCl}_3\text{-K}_3\text{Fe}(\text{CN})_6$ solution, by the method of Krishna, Dorrough & Casida (1962), and the coloured spots were compared with the radioautograph.

Fractionation of fat body. The fat bodies from 250 larvae were dissected out in groups of 50 under ice-cold 0.25 M-sucrose or under 0.15 M-KCl. Each group of 50 fat bodies was transferred to an all-glass Potter-Elvehjem homogenizing tube surrounded by ice. The appropriate medium (3.5 ml.) was added and the fat bodies were homogenized by hand in the cold until the homogenate was of a creamy consistency and no large particles could be seen. The homogenates were combined and squeezed through two layers of muslin, previously washed by boiling in water. The filtered homogenate was centrifuged at 800g for 15 min. and the residue obtained was termed the nuclear fraction. The supernatant was centrifuged at 10000g for 15 min. to give a mitochondrial fraction. The supernatant from this was centrifuged at 30000g for 15 min. and the pellet obtained was termed the 'intermediate fraction'. The supernatant

from this was centrifuged at 105000g for 1 hr. to sediment a microsomal fraction, the final supernatant being termed the soluble fraction. Each of the fractions, apart from the soluble fraction, was resuspended in 3.5 ml. of the appropriate medium. All centrifugation was carried out in the cold in a MSE40 high-speed centrifuge.

Measurement of carbaryl metabolism. Samples (0.5 ml.) of the subcellular fractions were incubated in 4.5 ml. of the following medium (final concentrations): $\text{KH}_2\text{PO}_4\text{-NaOH}$ buffer, pH 7.7, 50 mM; KCl, 12.4 mM; glucose 6-phosphate, 2.8 mM; glucose 6-phosphate dehydrogenase, 1 unit; NADP, 0.48 mM; [^{14}C]carbaryl, 20 $\mu\text{g.}$ in 50 $\mu\text{l.}$ of ethanol. Incubation was carried out at 30° for 30 min. At the end of the incubation 10 ml. of ether was added to the tube contents and the [^{14}C]carbaryl and its metabolites were extracted as described above.

Determination of protein. Samples (1 ml.) of each subcellular fraction were taken and their protein content was determined as described by Price (1969).

Determination of cytochrome oxidase activity. The cytochrome oxidase activity present in 50 $\mu\text{l.}$ of each subcellular fraction was measured by the method of Yonetani & Elliott (1966).

Determination of cytochrome P-450. The cytochrome P-450 content of each subcellular fraction was determined essentially as described by Ray (1967). Approx. 2 ml. of each fraction was mixed with 3 ml. of 0.1 M-tris-HCl buffer, pH 7.0, and reduced with approx. 1 mg. of $\text{Na}_2\text{S}_2\text{O}_4$. This solution was divided equally between two cuvettes. One cuvette was then saturated with CO and the difference spectrum was measured on a Unicam SP800 spectrophotometer.

RESULTS

Metabolism of carbaryl by various tissues. [^{14}C]Carbaryl was incubated with various tissues of the blowfly larva, and afterwards the flask contents were

Fig. 1. Metabolism of carbaryl by various tissues of the blowfly larva, *Calliphora erythrocephala*. Tissues from ten larvae were incubated at 30° in medium containing 20 $\mu\text{g.}$ of carbaryl. For details, see the Materials and Methods section. ○, Fat body; □, gut; △, cuticle; ●, muscle.

extracted, chromatographed and assayed as described in the Materials and Methods section. Carbaryl was metabolized much more rapidly by fat body than by cutaneous muscle or gut (Fig. 1); there was no detectable metabolism of carbaryl either by cuticle or by haemolymph.

Various factors affecting the metabolism of carbaryl by the fat body were then investigated.

Effect of age. Studies of protein synthesis by the fat body had shown that the rate of protein synthesis decreased rapidly as the larva aged (Price, 1966). The effect of age on carbaryl metabolism was also studied and the results indicated that the rate of carbaryl metabolism also decreased as the larva aged (Fig. 2). Over the age range investigated the rate was highest in 4–5-day-old larvae, so larvae of this age were used in subsequent experiments. The effect of age on carbaryl metabolism by fat body was also investigated in larvae that had been cultured at 10°, as described by Price (1969). In this case carbaryl was metabolized at a maximum rate by fat body from 9–11-day-old larvae (Fig. 2). At this age the larvae were about half-way through the larval period.

Effect of concentration of carbaryl. The concentration of carbaryl was varied from 20 µg./flask to 200 µg./flask. The rate of metabolism by the fat body over a 10 min. period was maximal when 100 µg. or more of carbaryl was added to the medium. In all subsequent experiments, 100 µg. of carbaryl was used.

Effect of time of incubation. The metabolism of carbaryl (100 µg.) by fat body over various periods was investigated and the results demonstrated that it was metabolized at a linear rate for at least 80 min.

Effect of pH. The effect of pH of the incubation

medium over the range 5.4–9.4 was studied and it was found that carbaryl metabolism by the fat body was maximal at pH 8.0 (Fig. 3). As the metabolism at pH 7.4 was nearly 90% of that at pH 8.0, and since previous experiments had been carried out in a medium buffered at pH 7.4, it was decided to continue investigations at pH 7.4.

Effect of magnesium chloride. The concentration of magnesium chloride in the incubation medium has a marked effect on the rate of protein synthesis by the fat body (Price, 1967). The effect of various concentrations of magnesium chloride on the rate of carbaryl metabolism was also studied, and it was found that the rate of metabolism was maximal at the lowest concentration of magnesium chloride used (10 mM). At concentrations above 30 mM, inhibition of carbaryl metabolism was observed. Thus this result is somewhat different from that obtained for protein synthesis, where a maximum effect was obtained at a concentration of magnesium chloride of 30 mM in the medium (Price, 1967).

Effect of inhibitors of protein synthesis. The effect of three inhibitors of protein synthesis [the L-(–)-erythro isomer of chloramphenicol, cycloheximide and 2,4-dinitrophenol] on carbaryl metabolism by fat body was investigated. Chloramphenicol (7.7 mM) inhibited carbaryl metabolism by 75%, but at lower concentrations (7.7 µM–0.77 mM) there was virtually no inhibition (Fig. 4). Dinitrophenol (10 mM) completely inhibited carbaryl metabolism, but at 10 µM there was no inhibition (Fig. 4). Thus the metabolism of carbaryl is inhibited by concentrations of chloramphenicol and dinitrophenol similar to those which inhibit protein synthesis (Price, 1969). However, cycloheximide over the range 0.1 µM–0.1 mM did not inhibit carbaryl metabolism at all, although protein synthesis is

Fig. 2. Metabolism of carbaryl by fat bodies from larvae of different ages reared at 25° (●) or 10° (○). For the larvae reared at 25°, five fat bodies were incubated at 30° for 15 min. in a medium containing 20 µg. of carbaryl, and for the larvae reared at 10°, five fat bodies were incubated for 30 min. with 100 µg. of carbaryl. For details of the culturing of the larvae see Price (1969).

Fig. 3. Effect of pH on the incubation medium on the metabolism of carbaryl by fat body. Five fat bodies from 5-day-old larvae, reared at 25°, were incubated at 30° for 15 min. in a medium containing 100 µg. of carbaryl.

Fig. 4. Effect of various materials on the metabolism of carbaryl by fat body. Fat bodies from five 4-day-old larvae, reared at 25°, were incubated at 30° for 30 min. in a medium containing 100 μ g. of carbaryl. $\circ$, Chloramphenicol; Δ , 2,4-dinitrophenol; $\square$, 5-dimethylamino-6-nitro-1,3-benzodioxole.

completely inhibited by 10 μ M-cycloheximide (Price, 1969).

Effect of a synergist. The synergist used in the present study was 5-dimethylamino-6-nitro-1,3-benzodioxole, a compound that has been shown to enhance the toxicity of carbaryl to the housefly (Wilkinson, 1967). The effect of various concentrations of this synergist on carbaryl metabolism was studied and it was found that at a concentration of 0.1 mm, carbaryl metabolism was inhibited by 70% (Fig. 4).

Carbaryl metabolism by subcellular fractions. The rate of carbaryl metabolism by various subcellular fractions of the fat body was investigated as described in the Materials and Methods section. The specific activity (μ g. of carbaryl metabolized/30 min./mg. of protein) was highest in the 'intermediate fraction' when either sucrose or potassium chloride was used as the isolation medium (Table 1). With potassium chloride the next highest activity was found in the mitochondrial fraction, but with sucrose the next highest activity was found in the microsomal fraction. With both potassium chloride and sucrose, there was very little activity in the soluble fraction.

Distribution of cytochrome P-450. Definite evidence for the presence of cytochrome P-450 was obtained only for the 'intermediate fraction'. Thus this pigment was found in the fraction that possessed the highest rate of carbaryl metabolism.

Distribution of cytochrome oxidase activity. The cytochrome oxidase activity of the various subcellular fractions was measured as described in the Materials and Methods section. When the fractions

Table 1. Metabolism of carbaryl by subcellular fractions from fat bodies of 4-day-old blowfly larvae isolated either in 0.15M-potassium chloride or in 0.25M-sucrose

The subcellular fractions were isolated and carbaryl metabolism was measured as described in the Materials and Methods section. The values given are from two experiments with each isolation medium.

Fraction	Expt. no. ...	Carbaryl metabolism by fractions expressed as μ g. of carbaryl metabolized/30 min./mg. of protein			
		KCl		Sucrose	
		1	2	1	2
Nuclear	...	0.89	2.68	0.77	1.24
Mitochondrial	...	2.30	4.35	1.82	1.98
Intermediate	...	6.46	10.90	5.91	4.62
Microsomal	...	1.65	1.09	3.69	2.78
Soluble	...	0.13	0.19	0.12	0.22

were isolated either in sucrose or in potassium chloride, the cytochrome oxidase activity was highest in the mitochondrial fraction. With potassium chloride the activity in the mitochondrial fraction was 30 times that in the 'intermediate fraction' and approx. 3 times that in the nuclear fraction. With sucrose the activity in the mitochondrial fraction was 8 times that in the 'intermediate fraction' and slightly greater than that in the nuclear fraction.

Identity of carbaryl metabolites. At least four ether-soluble metabolites were separated on t.l.c. plates after carbaryl was incubated with gut, whole fat body, or the subcellular fractions of the fat body. Two of these were tentatively identified, by co-chromatography, as the 4-hydroxy (R_F 0.29) and 5-hydroxy (R_F 0.38) derivatives of carbaryl. Two other metabolites, with R_F 0.04 and 0.23, were probably the 5,6-dihydrodihydroxy and *N*-hydroxymethyl derivatives respectively, the identification being based on their relative R_F values (Kühr & Casida, 1967). In experiments with subcellular fractions, approx. 80% of the metabolites formed were ether-soluble, but with whole fat bodies, only 40–50% of the metabolites were ether-soluble, the remainder being water-soluble metabolites of unknown identity.

DISCUSSION

Of the various tissues of blowfly larvae, the fat body showed the greatest metabolic activity towards carbaryl. Further, carbaryl metabolism and protein synthesis by the fat body had several

features in common. For example, both functions are maximal in fat body from younger larvae and in an incubation medium buffered at or near pH 8.0. To discover if there is a relationship between protein synthesis and carbaryl metabolism, other conditions were varied. Thus, protein synthesis by the fat body is extremely sensitive to the magnesium chloride concentration of the medium (Price, 1967). A change in concentration from 10 to 30 mM produced a 14-fold increase in protein synthesis, but the amount of carbaryl metabolism over this range remained about the same. The effect of various inhibitors of protein synthesis on carbaryl metabolism was also investigated. Chloramphenicol [L-(—)-erythro isomer] at a concentration of 0.7 mM had no effect on protein synthesis by the fat body, but at 7 mM complete inhibition occurred (Price, 1969). These concentrations of chloramphenicol produced similar effects on carbaryl metabolism, that is, up to 0.7 mM there was only slight inhibition, whereas at 7 mM, 73% inhibition was obtained. The reason for this inhibition of carbaryl metabolism by chloramphenicol is not known. With dinitrophenol, protein synthesis was completely inhibited by 0.1 mM (Price, 1969) whereas a concentration of 10 mM was needed to inhibit carbaryl metabolism completely. Finally, cycloheximide completely inhibited protein synthesis by the fat body at 10 μ M whereas carbaryl metabolism was not affected at ten times this concentration.

All subcellular fractions derived from the fat body, with the exception of the soluble fraction, were able to metabolize carbaryl. However, the greatest activity was found in the pellet sedimenting at 30000g, whether the isolation of the fractions took place in sucrose or in potassium chloride. In preliminary experiments, carbaryl-metabolizing activity was greatest in a fraction obtained by centrifuging at 20000g. This result suggested that this mitochondrial fraction might have contained microsomes because it had been previously shown by Gemrich (1967) that the microsomal fraction of fat body from the cockroach *Blaberus giganteus* was the most active in metabolizing the carbamate Banol (2-chloro-4,5-dimethylphenyl *N*-methylcarbamate). Also, when homogenates of housefly abdomina were fractionated, the greatest metabolic activity toward Baygon (2-isopropoxyphenyl *N*-methylcarbamate) was found in the microsomal fraction (Tsukamoto & Casida, 1967). To decrease the likelihood of contamination of the mitochondria with microsomes, mitochondria were sedimented at 10000g. Likewise, to reduce the likelihood of contamination of the microsomes with mitochondria, the mitochondrial supernatant was centrifuged at 30000g before the final 105000g centrifugation.

The nature of the particles in the 30000g pellet is not known, but it is likely to consist of a mixture of 'light' mitochondria and 'heavy' microsomes. This intermediate fraction exhibited some cytochrome oxidase activity, although, as expected, most of this activity was associated with the mitochondrial fraction.

Examination of the subcellular fractions with the light-microscope revealed that the nuclear fraction was heavily contaminated with mitochondria. It was, however, virtually free of cell debris, which had presumably been retained when the whole homogenate was squeezed through muslin. The mitochondrial fraction was fairly homogeneous, very few nuclei being discernible. Mitochondria were present in the intermediate fraction but none was seen in the microsomal fraction. The intermediate fraction was the only fraction that consistently contained a measurable amount of cytochrome *P*-450, a cytochrome often associated with oxidative reactions in microsomes. This may indicate that cytochrome *P*-450 is involved in carbaryl metabolism by the fat body. The fact that a methylenedioxypheyl synergist inhibited carbaryl metabolism by the fat body suggests that a mixed-function oxidase may be involved.

The metabolites of carbaryl were tentatively identified as the 4-hydroxy, 5-hydroxy and, probably, *N*-hydroxymethyl derivatives. These metabolites have been shown to be produced by other insects (Dorough & Casida, 1964; Kuhr, 1969; Tsukamoto & Casida, 1967), by plants (Kuhr & Casida, 1967), and by mammals (Dorough & Casida, 1964; Leeling & Casida, 1966; Oonithan & Casida, 1966, 1968).

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REFERENCES

- Dorough, H. W. & Casida, J. E. (1964). *J. agric. Fd Chem.* **12**, 294.
Eldefrawi, M. E. & Hoskins, W. M. (1961). *J. econ. Ent.* **54**, 401.
Gemrich, E. G. (1967). *J. agric. Fd Chem.* **15**, 617.
Krishna, J. G., Dorough, H. W. & Casida, J. E. (1962). *J. agric. Fd Chem.* **10**, 462.
Ku, T. Y. & Bishop, J. L. (1967). *J. econ. Ent.* **60**, 1328.
Kuhr, R. J. (1969). *J. agric. Fd Chem.* (in the Press).
Kuhr, R. J. & Casida, J. E. (1967). *J. agric. Fd Chem.* **15**, 814.
Leeling, N. C. & Casida, J. E. (1966). *J. agric. Fd Chem.* **14**, 281.
Oonithan, E. S. & Casida, J. E. (1966). *Bull. environ. Contam. Toxicol.* **1**, 59.

- Oonithan, E. S. & Casida, J. E. (1968). *J. agric. Fd Chem.* **16**, 28.
- Price, G. M. (1966). *J. Insect Physiol.* **12**, 731.
- Price, G. M. (1967). *J. Insect Physiol.* **13**, 69.
- Price, G. M. (1969). *J. Insect Physiol.* (in the Press).
- Price, G. M. & Kuhr, R. J. (1968). *Biochem. J.* **108**, 20P.
- Ray, J. W. (1967). *Biochem. Pharmacol.* **16**, 99.
- Tsukamoto, M. & Casida, J. E. (1967). *Nature, Lond.*, **213**, 49.
- Wilkinson, C. F. (1967). *J. agric. Fd Chem.* **15**, 139.
- Yonetani, T. & Elliott, W. B. (1966). *Biochem. Prep.* **11**, 14.
- Zayed, S. M. A. D., Hassan, A. & Hussein, T. M. (1966). *Biochem. Pharmacol.* **15**, 2057.