Bull. Environm. Contam. Toxicol. 25, 697-701 (1980)

In Vitro Metabolism of Carbofuran by Liver Microsomes of the Padifield Fish *Trichogaster pectoralis*

Sarjeet S. Gill
Division of Toxicology and Physiology, Department of Entomology, University of California, Riverside, CA 92521

Insecticides are increasingly being used in the padifields of West Malaysia. A result of such usage has been a rapid decline in padifield fish production (YUNUS and LIM 1971, TAN et al. 1972). Padifield fish forms an important protein source for the rural people in West Malaysia, in particular the poorer farmers. Of the padifield fishes, sepat siam, Trichogaster pectoralis (Regan) is probably the most important food fish.

Of the insecticides being increasingly used in West Malaysian padifields is carbofuran, an N-methylcarbamate. The fate of carbofuran is relatively well understood in insects, plants, mammals and soil (DOROUGH 1968, METCALF et al. 1968, VENKATESWARLU et al. 1977, DEUEL et al. 1979). However, the metabolic route of carbofuran in fish is poorly understood. A knowledge of this metabolic route along with an assessment of bioaccumulation would indicate the degree of potential threat of carbofuran residues to people consuming padifield fishes as do the rural population of West Malaysia. The present investigation is to determine the extent of metabolism carbofuran undergoes in vitro in fish utilizing T. pectoralis liver microsomal fraction.

MATERIALS AND METHODS

Chemicals. Carbofuran and ring-^{14}C-carbofuran (specific activity, 990 MBq/mmol) was kindly provided by FMC Corporation (Agricultural Chemicals Division, Middleport, N.Y., U.S.A.). Carbonyl-^{14}C-carbofuran (specific activity, 189 MBq/mmol) was obtained by reaction of methyl isocyanate-^{14}C (New England Nuclear, Boston, MA, U.S.A.) with carbofuran phenol (1.5 molar excess) in dry diethyl ether with triethylamine as catalyst. Carbofuran-^{14}C of both labels was purified by one dimensional thin-layer chromatography (tlc) on silica-gel GF_{254} plates (E. Merck, Darmstadt, W. Germany) to greater than 99% as determined in a number of solvent systems.

Unlabelled standards of possible carbofuran metabolites were 3-keto-carbofuran (3-keto-CF), 3-hydroxy-carbofuran (3-OH-CF), 3-keto-carbofuran phenol (3-keto-phenol), 3-hydroxy-carbofuran phenol (3-OH-phenol), carbofuran phenol,

0007-4861/80/0025-0697 $01.00

N-hydroxymethyl carbofuran (N-CH_2OH-CF), 3-keto-N-hydroxymethyl carbofuran (3-keto-N-CH_2OH-CF), 3-hydroxy-N-hydroxymethyl carbofuran (3-OH-N-CH_2OH-CF), N-desmethyl carbofuran (NH_2-CF), 3-keto-N-desmethyl carbofuran (3-keto-NH_2-CF).

Preparation of microsomes and incubation conditions. Padifield fish, T. pectoralis were collected from padifields in the Kuala Muda area, Kedah, Malaysia and maintained in the aerated well water at 28°C for one month prior to use. The fish of mixed sexes (40-70 g) were killed by decapitation. The livers were carefully taken to ensure that all non-hepatic tissue was removed. The livers were immediately chilled, washed and subsequently homogenized in 0.25M sucrose using a Potter-Elvehjem teflon homogenizer to give a 20% w/v homogenate. The homogenate was centrifuged at 8,500 g for 20 min and the supernatant futher centrifuged at 150,000 g for 70 min. The microsomal pellet was resuspended in 0.1M sodium phosphate buffer, pH 7.4 and used as soon as possible the same day. Protein concentration was measured by the method of Lowry et al. (1951) using bovine serum albumin, fraction V as the standard.

The incubation mixture, 2 mL in a 25 mL erlenmeyer flask, consisted of the following unless otherwise indicated: microsomal preparation (equiv. to 1 g wet tissue equivalent, ca. 7 mg protein), NADPH (2 μmol), $MgCl_2$ (15 μmol) and carbofuran (0.1 μmol in 20 μL ethanol). Radioactivity (840 Bq per flask) was added last in 10 μL of ethanol. Incubation flasks with no NADPH, and with NADPH but with boiled microsomes (15 min, 100°C) were used as controls.

Following incubation of the flasks at 37°C for 1 h in a reciprocal shaker (60 cycles/min) and in the presence of air, the incubation mixture was extracted once with 6 mL peroxide free diethyl ether. The aqueous phase was acidified with 1N HCl and further extracted twice with 6 mL ether. Extraction efficiency was between 87-92% using this procedure. Less than 2% of the radioactivity was recovered in the third ether extract. The ether fractions were combined, dried with anhydrous Na_2SO_4, the ether evaporated under a gentle stream of oxygen free nitrogen and the residue dissolved in 0.1 mL methanol. To identify metabolic products containing an intact N-methylcarbamate moiety, ether extractable products were treated with 2 mL of 1N NaOH for 2 h. Subsequently, the reaction mixture was acidified, extracted with ether, the ether phase dried, and the residue dissolved in methanol as explained above.

Chromatography. The metabolic products in methanol were separated by silica gel chromatoplates using ether-hexane (3:1, EH) and toluene-2-propanol (10:1, TP) as the solvent systems in two dimensional tlc. The radioactive spots detected by autoradiography were scraped and quantified by liquid scintillation counting (Packard Tricarb 3255) utilizing a 0.55% w/v 2,5-diphenyloxazole solution in toluene-2-methoxyethanol (2:1)

and quench corrected. Identification of the metabolites was based on cochromatography with authentic standards. In addition to solvents used above, hexane-acetonetoluene (3:1:1, HAT) and chloroform-dichloromethane-ethyl acetate-2propanol (10:10:1:1, CDEP) solvent systems were also used in the identification process.

RESULTS AND DISCUSSION

Carbofuran is metabolized by the microsomal-NADPH system of T. pectoralis liver to give two major metabolic products, N-CH_2OH-CF and 3-OH-CF, and two minor ones, 3-keto-CF and 3-keto-phenol (Table). Identification of N-CH_2OH-CF, 3-OH-CF, and 3-keto-CF as metabolic products was confirmed by cochromatography of the metabolites and their base hydrolysis products with authentic standards in a number of solvent systems. Carbofuran phenol and 3-keto-phenol were identified by cochromatography.

Formation of N-CH_2OH-CF, 3-OH-CF, 3-keto-CF, and 3-keto-phenol is dependent on the presence of NADPH since these metabolites are not formed or formed only in negligible amounts in the absence of NADPH. Formation of these metabolites occurs only in the presence of the microsomal fraction, and are not formed with boiled microsomes with or without NADPH. However, carbofuran phenol formation is not dependent on microsomes and/or NADPH, since similar amounts are also formed in the presence of boiled microsomes indicating a slow cleavage of the carbamoyl moiety at the slightly alkaline conditions used during incubation. Carbofuran phenol and 3-keto-phenol were not detected when carbonyl-^{14}C-carbofuran was used in the incubation reactions. However, the extent of oxidative hydroxylation occurring was similar. The only unidentified products isolated were at the origin and accounted for less than 0.4% of total radioactivity recovered.

The results show that oxidative hydroxylation is the major route of metabolism of carbofuran in T. pectoralis giving rise to the two hydroxylated products, N-CH_2OH-CF and 3-OH-CF. The occurence of oxidative hydroxylation as an important pathway in T. pectoralis was further confirmed by using p-nitroanisole as substrate (Gill, unpublished). Hydrolysis of the carbamoyl moiety is a minor pathway, if at all, in the in vitro microsomal-NADPH system used. Oxidative metabolism of carbofuran is similarly an important metabolic route in the rat microsomal-NADPH system (DOROUGH, 1968). However, the major metabolic product formed is 3-OH-CF with N-CH_2OH-CF formed in lesser amounts in contrast to the present study. Formation of other metabolites was also minor in the rat microsomal system. In earthworms in vivo, 3-OH-CF is also the major metabolic product formed (STENERSEN et al. 1973). In intact insect, mammalian and plant systems N-CH_2OH-CF is rarely isolated (IVIE and DOROUGH 1968, METCALF et al. 1968, KNAAK et al. 1970).

However, 3-OH-CF and its hydrolysis product 3-OH-phenol are often observed as significant metabolic products.

The liver microsomal-NADPH system of T. pectoralis similarly metabolizes carbaryl by hydroxylation of the N-CH_3 moiety (GILL 1976) indicating that N-hydroxylation is the major route of metabolism of N-methylcarbamates in T.

TABLE

Rf values of possible standard metabolites of carbofuran and metabolism of ring-^{14}C-carbofuran by the liver microsomal fraction of T. pectoralis.

Compound	Solvent System				% of recovered radioactivity[a]	
	EH	HAT	CDEP	TP	with NADPH	without NADPH
Carbofuran phenol[b]	0.62	0.49	0.68	0.57	1.2	1.1
3-Keto-phenol[b]	0.42	0.22	0.35	0.33	0.2	-
3-OH-phenol[b]	0.49	0.35	0.56	0.44	-	-
Carbofuran	0.39	0.36	0.57	0.45	81.3	98.3
3-Keto-CF	0.35	0.32	0.54	0.42	0.2	-
3-OH-CF	0.22	0.16	0.32	0.34	4.9	0.2
N-CH_2OH-CF	0.21	0.14	0.31	0.30	11.9	0.2
NH_2-CF	0.32	0.24	0.42	0.36	-	-
3-Keto-N-CH_2OH-CF	0.17	0.11	0.27	0.26	-	-
3-Keto-NH_2-CF	0.29	0.20	0.41	0.33	-	-
3-OH-N-CH_2OH-CF	0.10	0.04	0.10	0.18	-	-
Origin	-	-	-	-	0.4	0.3

[a] Results are average values of experiments run in triplicate. The experiment was repeated once. Each enzyme preparation was from an average of 5 - 6 fish.

[b] Compounds not detected when carbonyl-^{14}C-carbofuran is used in metabolism studies.

pectoralis. The extent of metabolism by the liver microsomal-NADPH system of carbofuran and as reported earlier for carbaryl indicates that oxidative hydroxylation activity in T. pectoralis liver is comparatively lower than in laboratory experimental animals. Thus care has to be taken in extrapolating degradation rates obtained from laboratory animals to that of padifield fish.

ACKNOWLEDGEMENTS

R. Robinson, FMC Corporation, Middleport, N.Y., U.S.A. and M.A.H. Fahmy, formerly of the University of California, Riverside, CA., U.S.A., kindly provided the ring-labelled carbofuran and standard metabolites respectively. Financial assistance from the International Atomic Energy Agency (SD/2087), Universiti Sains Malaysia short-term Research Grant and the Ford Foundation is gratefully acknowledged. This work was completed while the author was a faculty member at Universiti Sains Malaysia, Penang, Malaysia.

REFERENCES

DEUEL, JR., L.E., PRICE, J.D., TURNER, F.T. and BROWN, K.W.: J. Environ. Qual. 8, 23 (1979).

DOROUGH, H.W.: J. Agric. Food Chem. 16, 319 (1968).

GILL, S.S.: Malaysian Agric. J. 50, 545 (1976).

IVIE, G.W. and DOROUGH, H.W.: J. Agric. Food Chem. 16, 849 (1968).

KNAAK, J.B., MUNGER, D.M., McCARTHY, J.F. and SATTER, L.D.: J. Agric. Food Chem. 18, 832 (1970).

METCALF, R.L., FUKUTO, T.R., COLLINS, C., BORCK, K., EL-AZIZ, S.A., MUNOZ, R. and CASSIL, C.C.: J. Agric. Food Chem. 16, 300 (1968).

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. and RANDALL, R.J.: J. Biol. Chem. 193, 265 (1951).

STENERSEN, J., GILMAN, A. and VARDANIS, A.: J. Agric. Food Chem. 21, 166 (1973).

TAN, C.E., CHONG, B.J., SIER, H.K. and MOULTON, T.: A report on paddy and paddy-field fish production in Krian, Perak. Bull. No. 128, Ministry of Agriculture and Fisheries, Kuala Lumpur, Malaysia.

VENKATESWARLU, K., SIDDARAME GOWDA, T.K. and SETHUNATHAN, N.: J. Agric. Food Chem. 25, 533 (1977).

YUNUS, A. and LIM, G.S.:Malaysian Agric. J. 48, 167 (1971).