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Metabolism of Diazinon and Diazoxon in Fish Liver Preparations

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Although the metabolism of diazinon and diazoxon in mammals has been reported(NAKATSUGAWA et al. 1969, YANG et al. 1971a,b, SHISHIDO & FUKAMI 1972), little is known about the in vitro metabolism of diazinon and diazoxon in fish liver preparations(HOGAN & KNOWLES 1972). SEGUCHI & ASAKA(1981) reported the intake and excretion of diazinon and its in vivo metabolism by fresh-water fishes, and they found some metabolites. The present study was undertaken to investigate the in vitro metabolism of diazinon and diazoxon in fish liver preparations, and to identify the metabolites.

MATERIALS AND METHODS

Fish: Carp, Cyprinus carprio L.(50-70g); rainbow trout, Salmo gairdneri(60-80g); channel catfish, Ictalurus punctatus(130-169g); dace, Tribolodon hakonensis(40-50g) and yellow tail, Seriola quinqueradiata T.(200-300g) were obtained from a fish farm in Saitama and in Kanagawa, prefecture, Japan.

Chemicals: Diazinon and its related compounds were synthesized in our laboratory by the methods of MIYAZAKI et al.(1969) and KATO et al.(1973). The chemical structure of all the compounds were identified by ir, nmr and GC-MS. The reduced form of nicotinamide adenine dinucleotide phosphate(NADPH) and reduced glutathione(GSH) were obtained from Sigma Chemical Co. All the other chemicals were of analytical reagent grade.

Tissue preparations: After decapitation of fish, liver was immediately dissected, and hepatic subcellular fractions were prepared by differencial centrifugation in 0.05M Tris-HCl buffer(pH 8.0) as described by YANG et al. (1971a). Each precipitate was diluted with the same buffer to 16.7% homogenate. The enzyme preparations were prepared below 5°C and used immediately.

Difference spectra were recorded on Hitachi Two-Wavelength Double Beam spectrophotometer 356 and P-450 levels were determined in each microsomal suspension by the method of OMURA and SATO(1964).

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Incubation system: The final volume of the mixture of 19.7nmoles diazinon(6μg) and/or 20.8nmoles diazoxon (6μg), 3μmoles NADPH and/or GSH, 2ml enzyme preparation(equivalent to 333mg liver) was adjusted to 2.2ml with 0.05M Tris-HCl buffer(pH 8.0). The mixture was incubated with shaking at 30°C for 1h.

Analysis: After incubation, diazinon and diazoxon, and their metabolites were extracted with chloroform/isopropanol(1/1) and quantitated by gas chromatography(glc). Metabolites were identified by comparing the glc retention time with authentic standards and by mass fragmentography. Pyrimidones formed by cleavage of ester linkage were determined by glc in the same manner as the one reported by SEGUCHI and ASAKA(1981) except for the cleaning up of the pyrimidones before the derivatization, and extraction was made by using ethyl acetate instead of chloroform/isopropanol. GC-MS and mass fragmentography were carried out by the condition as follows: Shimadzu LKB 9000, Shimadzu High-speed MID-PM 9060S, column; 3% OV-17 on chromosorb W AW-DMCS HP(80-100 mesh), 1m X 3mm, carrier gas; He, 35ml/min, column temp.; 190°C, electron energy; 40eV.

RESULTS AND DISCUSSION

Degradation of diazinon and diazoxon by subcellular fraction of fish liver preparations: Centrifugal studies on fish liver homogenate showed that all the fractions possessed some capability for diazinon metabolism(Fig.1). Diazinon was degraded markedly in the NADPH containing fractions of nuclei, mitochondria and microsomes, whereas GSH had little or no effect. The highest effect of NADPH on microsomal fraction indicates that diazinon might be degraded by the enzyme systems of P-450 and the only slight effect with exogeneous GSH indicates that GSH-dependent O-alkyl and/or O-aryl conjugation was not active as previously stated in the case of channel catfish(HOGAN & KNOWLES 1972). Degradation activity of fresh-water fishes was almost the same but it was higher than that of yellow tail(sea-water fish).

In the case of diazoxon degradation, the activity of all the hepatic fractions of yellow tail was lower than that of fresh-water fishes. Nuclei, mitochondria and microsomes of carp possessed high activity in themselves, and neither NADPH nor GSH had increasing effect, whereas NADPH markedly increased the activity of microsomes of rainbow trout and mitochondria of dace.

It is therefore suggested that in subcellular fractions of carp except soluble fraction, there might be a hydrolase system(YANG et al. 1971a, SHISHIDO & FUKAMI 1972) which is indipendent of NADPH, and the hydrolase system hydrolyzes diazoxon and not diazinon.

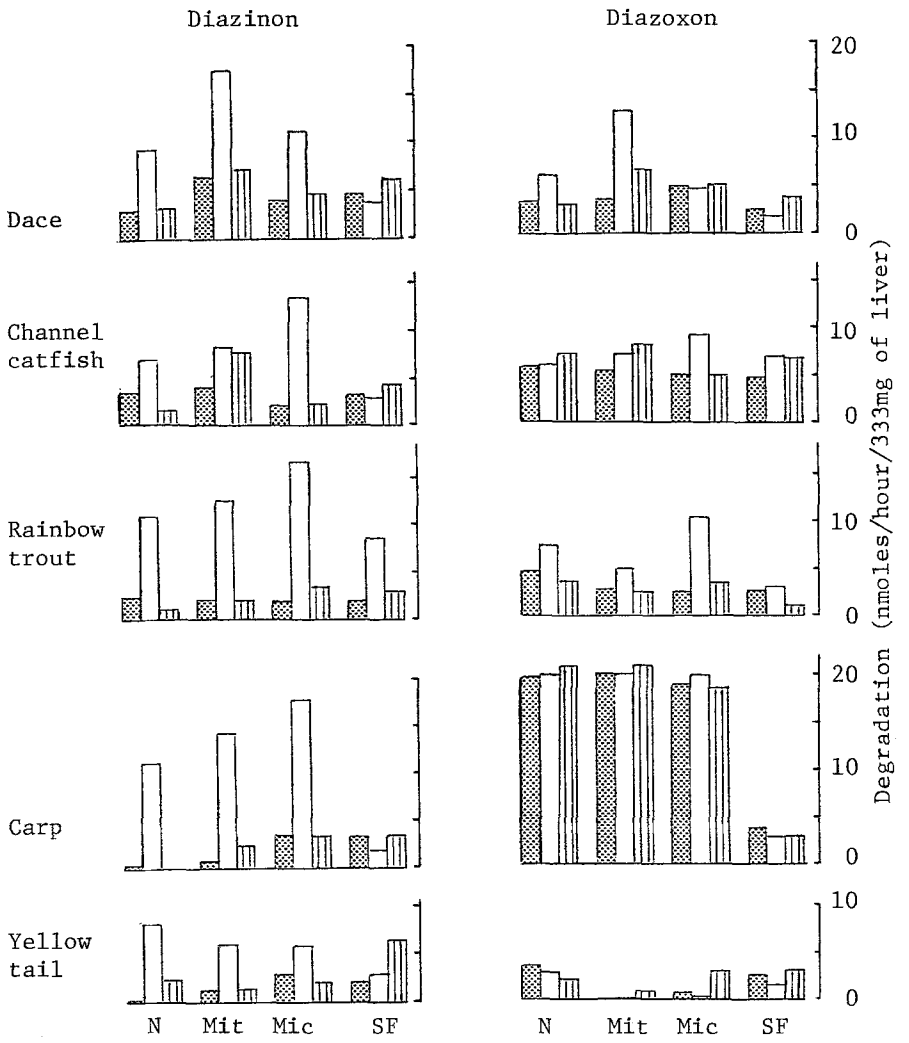

Fig. 1. Degradation of diazinon and diazoxon in subcellular fractions of fish liver homogenate.
▨ without cofactor, ▭ NADPH, ▤ GSH, N:nuclei, Mit:mitochondria, Mic:microsome, SF:soluble fraction

Identification of metabolites: Among the diazinon metabolites identified by the glc analysis from hepatic microsomal reaction mixture of fresh-water fishes, 2-isopropyl-4-methyl-6-pyrimidinol(hydroxy pyrimidine) was a predominant metabolite in vitro as well as in vivo (SEGUCHI & ASAKA 1981), whereas almost no hydroxy pyrimidine was formed in yellow tail as accounted from the Table 1. Diazoxon was found in large quantities in all the fishes tested, although it had not been detected in the previous in vivo study(SEGUCHI & ASAKA 1981) and O,O-diethyl-O-(2-isopropenyl-4-methyl-6-pyrimidinyl)

Table 1. Metabolite formation in microsomal preparation with NADPH.

		nmole							
		Metabolites					Remaining		Recovery
		A	B	C	D	E	diazinon	Total	(%)
	min.								
Dace	15	0.5	0.8	2.7	ND	4.7*	11.0	15.0	76.1
	30	0.6	1.0	3.4	ND	5.7*	9.0	14.0	71.1
	60	0.7	1.0	4.0	ND	8.4*	5.6	11.3	57.4
	120	0.7	1.0	4.5	ND	9.5*	3.9	10.2	51.8
Channel	15	0.5	0.9	3.3	ND	4.3	10.5	19.5	99.0
catfish	30	0.6	1.0	3.5	ND	7.0	7.1	19.2	97.5
	60	0.8	1.1	4.9	ND	6.5	5.6	18.9	95.9
	120	0.9	1.3	3.9	ND	9.0	3.8	18.9	95.9
Rainbow	15	0.4	ND	1.6	ND	7.6	9.9	19.5	99.0
trout	30	0.4	ND	2.0	ND	8.9	6.8	18.1	91.9
	60	0.4	ND	2.5	ND	8.8	4.0	15.7	79.7
	120	0.3	ND	2.3	ND	10.0	1.9	14.5	73.6
Carp	15	0.1	0.3	0.8	ND	4.5	11.5	17.2	87.3
	30	0.3	0.4	1.4	ND	7.4	8.8	18.3	92.9
	60	0.3	0.5	2.0	ND	10.3	6.5	19.6	99.5
	120	0.3	0.5	1.9	ND	13.0	3.8	19.5	99.0
Yellow	5	ND	ND	0.2	1.2	0.0*	18.3	19.7	100.0
tail	15	ND	ND	0.6	3.9	0.0*	15.2	19.7	100.0
	30	ND	ND	1.2	3.9	0.9*	12.7	17.8	90.4
	60	ND	ND	1.5	2.6	5.1*	10.5	14.6	74.1

A:isopropenyl diazinon, B:hydroxy diazinon, C:diazoxon, D:hydroxymethyl diazinon, E:hydroxy pyrimidine, Total=Remaining diazinon + measured metabolites, *:E=(19.7-Total)

phosphorothioate(isopropenyl diazinon) was also detected in all the fresh-water fishes. Furthermore, O,O-diethyl-O-[2-(α-hydroxyisopropyl)-4-methyl-6-pyrimidinyl] phosphorothioate(hydroxy diazinon) was found in carp, dace and channel catfish.

With the yellow tail study, the major metabolite detected was O,O-diethyl-O-(2-isopropyl-4-hydroxymethyl-6-pyrimidinyl) phosphorothioate(hydroxymethyl diazinon).

When the diazoxon was examined as initial substrate in the microsome system of rainbow trout and channel catfish, detectable amount of a new metabolite was formed in the presence of NADPH. It was tentatively identified by glc and mass fragmentography as O,O-diethyl-O-(2-isopropenyl-4-methyl-6-pyrimidinyl) phosphate(isopropenyl diazoxon).

Among the identified 6 diazinon metabolites, hydroxy primidine and isopropenyl diazinon had already been detected in our previous in vivo study(SEGUCHI & ASAKA 1981), but hydroxydiazinon, hydroxymethyl diazinon and isopropenyl diazoxon were new metabolites detected in fish.

It is considered that susceptibility of sea-water fishes to organophosphate or carbamate inseticides is higher than that of fresh-water fishes(KANAZAWA 1976). In the case of diazinon, it was shown that the susceptibility of yellow tail was more than 84-fold higher than that of carp in term of Tlm 48(IWATA 1979). The relative susceptibility of fishes to poisoning by diazinon might be determined by the rates at which the oxgen analogues accumulate in the organisms as well as degradation of diazinon(SHISHIDO & FUKAMI 1972, YANG et al. 1971a,b). Acetylcholinesterase inhibitory activity of diazoxon and isopropenyl diazoxon is about 10^4 fold higher than diazinon but those of the phosphorothioate metabolites are almost the same as diazinon(MIYAZAKI et al. 1969). In fresh-water fishes, over 70% of diazinon was metabolized and nontoxic hydroxy pyrimidine was formed up to half amount of treated diazinon during 1h incubation, while in yellow tail more than 70% of treated diazinon remained as toxic phosphorous esters including diazoxon. Furthermore, carp and rainbow trout have higher P-450 level than yellow tail as shown in Table 2. It suggests that metabolic activity has close relation to P-450 levels as stated by KOBAYASHI(1979). Therefore, it is presumed that one of the causes of differential sensitivity to diazinon between fresh-water fishes and sea-water fishes may be attributable to the differential degradation activity due mainly to the P-450 levels.

Table 2. P-450 levels in fishes.

	P-450 nmole/mg protein
Carp	0.33
Rainbow trout	0.31
Yellow tail	0.15

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