Metabolism *in Vitro* of Diazinon and Diazoxon in Rat Liver

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The metabolism of ethoxy-1-^{14}C-labeled diazinon and diazoxon was studied *in vitro* using rat liver enzyme preparations. The degradation of diazinon is catalyzed by a microsomal enzyme system requiring NADPH and oxygen which is inhibited by carbon monoxide, presumably the NADPH-cytochrome P_{450} mixed function oxidase system. Diazoxon is degraded by enzymes located in the nuclear, mitochondrial, microsomal, and soluble fractions of rat liver. The diazoxon-degrading enzyme located in the microsomes is the most active and is not dependent on the addition of NADPH. Reduced glutathione has little or no effect on diazinon degradation by either microsomal or soluble enzymes. Diazinon is degraded to diethylphosphorothioic acid and diethylphosphoric acid, whereas diazoxon is degraded to diethylphosphoric acid. No direct evidence for desethylation was obtained with either diazinon or diazoxon in the microsomal and soluble fractions.

The hydrolases which are responsible for the hydrolysis of many organophosphate insecticides in mammals are well-documented (Aldridge, 1953; Augustinsson and Heimburger, 1954; Erdös and Boggs, 1961; Main, 1960a,b; Mounter, 1954; O'Brien, 1967).

In an early study of the degradation of diazinon [*O,O*-diethyl-*O*-(2-isopropyl-4-methyl-6-pyrimidinyl)phosphorothioate], no enzymatic activity was detected in either rabbit plasma or human serum (Augustinsson and Heimburger, 1954). In a later study the level of hydrolysis of diazinon was shown to be very low in enzyme preparations from the houseflies (Matsumura and Hogendijk, 1964).

Until recently little was known concerning the oxidative degradation of organophosphate insecticides. Oxidative degradation of parathion [*O,O*-diethyl-*O*-(4-nitrophenyl)phosphorothioate] and its analogs by mammalian liver microsomes was described by two groups of investigators (Nakatsugawa and Dahm, 1967; Nakatsugawa *et al.*, 1968, 1969b; Neal, 1967a,b). Subsequent studies on diazinon revealed that its degradation was catalyzed by a rat liver microsomal enzyme system which is dependent on NADPH and oxygen (Menzer and Dauterman, 1970; Nakatsugawa *et al.*, 1969a; Yang *et al.*, 1969).

More recently, Mücke *et al.* (1970) studied the fate of pyrimidine ring labeled ^{14}C-diazinon in the intact rat, and elucidated the metabolic pathway of the pyrimidine moiety. The metabolite, 2-isopropyl-4-methyl-6-hydroxypyrimidine, is first enzymatically cleaved from diazinon and subsequently gives rise to two hydroxylated metabolites and an unknown water soluble compound.

A recent abstract (Fukunaga *et al.*, 1967) indicated that diazinon was oxidized and diazoxon [*O,O*-diethyl-*O*-(2-isopropyl-4-methyl-6-pyrimidinyl)phosphate] hydrolyzed by microsomal enzymes from rat liver. No details were presented.

This study was undertaken to investigate the oxidative and nonoxidative metabolism of diazinon and diazoxon in subcellular fractions from rat liver.

MATERIALS AND METHODS

Synthesis of Radiochemicals. Ethoxy-1-^{14}C-labeled diazinon was prepared by reacting 2 mmoles of ethanol-1-^{14}C (specific activity 500 μCi per mmole) with 0.5/mmole of phosphorus pentasulfide under reflux for 12 hr. The resulting ^{14}C-diethyl phosphorodithioic acid was then chlorinated with sulfuryl chloride (SO_2Cl_2) to yield the intermediate, *O,O*-diethyl-^{14}C-phosphorochloridothionate. The intermediate was coupled with 2-isopropyl-4-methyl-6-hydroxypyrimidine according to the method of Krueger *et al.* (1960). The crude diazinon was purified by chromatography on a Celite column (Bowman and Casida, 1957). The chemical purity was confirmed by infrared spectrophotometry (Perkin-Elmer 237B) and paper chromatography (Mitchell, 1960). ^{14}C-Diazinon was obtained with 22.3% yield, and the specific activity was 1.98 μCi per mg.

Ethoxy-1-^{14}C-labeled diazoxon was prepared by reacting 3 mmoles of ethanol-1-^{14}C (specific activity 166.7 μCi per mmole) with 1 mmole of phosphorus trichloride (PCl_3), followed by chorination to give *O,O*-diethyl phosphorochloridate (McCombie *et al.*, 1945). The intermediate was coupled with 2-isopropyl-4-methyl-6-hydroxypyrimidine by a method similar to that of Krueger *et al.* (1960). The crude compound was purified on a Celite column as mentioned previously. ^{14}C-Diazoxon was obtained with 29% yield, and the specific activity was 1.02 μCi per mg.

Chemicals and Supplies. The analytical standards of diazinon and diazoxon were kindly provided by Geigy Chemical Corp. Reduced forms of nicotinamide adenine dinucleotide phosphate (NADPH) and glutathione (GSH) were obtained from either Sigma Chemical Co. or Nutritional Biochemical Co. Dowex 1-X8 (50 to 100-mesh) was the product of J. T. Baker Chemical Co. All other chemicals used were of analytical reagent grade.

Enzyme Preparations. The subcellular fractions from rat liver were prepared as previously described (Yang *et al.*, 1969) with the exception that the 20% liver homogenate was made using 0.05*M* tris-HCl buffer, pH 8.0, instead of KCl-nicotinamide solution. In the case of diluted enzyme preparations, the dilution was made after the centrifugal fractionation of the 20% homogenate. All steps were carried out at 0° to 5° C and, unless otherwise started, fresh enzyme preparations were used in all experiments.

Enzyme Assay. A typical incubation system consists of 60 μmoles of tris-HCl buffer, pH 8.0; 1.1 μmoles of NADPH and/or 2.0 μmoles of GSH; 0.2 μmole of ^{14}C-diazinon, and enzyme preparation equivalent to 100 mg of liver (wet weight). Distilled water was added to bring the final volume to 2.0 ml.

The reactions were carried out in Kimax graduated glass tubes (16 × 125 mm) and incubated in a Water-Bath Shaker (Eberbach Corp., Ann Arbor, Mich.) at 37° C. The assay method was the same as that reported previously (Yang *et al.*,

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1969), the essential feature being a partitioning of diazinon and its metabolites between benzene and water, and the counting of the radioactivity of each phase.

Identification of Metabolites. Diazinon and diazoxon were separated and identified by the two-phase paper chromatographic system (mobile phase: 50% dimethylformamide in water; immobile phase: 10% mineral oil in ether) or Mitchell (1960). The radioactivity was detected by scanning the paper strips on a Packard Model 7201 Radiochromatogram Scanner. The diazinon standard was visualized with 0.5% 2,6-dibromoquinone-*N*-chloro-*p*-quinoneimine in cyclohexane (Menn *et al.*, 1957), and the diazoxon standard was detected by the phosphorus reagent reported by Hanes and Isherwood (1949) using 1% $SnCl_2$ in methanol as the reducing agent. The water soluble metabolites of diazinon and diazoxon were analyzed by the ion exchange chromatographic method of Plapp and Casida (1958a) using a 1.8 cm (i.d.) × 34 cm column of Dowex 1-X8 (50 to 100-mesh) anion exchange resin. The slightly modified solvent system was: (I) elution gradient (400 ml), pH 2 to pH 1 HCl; (II) elution gradient (400 ml), pH 1 HCl plus methanol (1 to 3) to 1*N* HCl plus methanol (1 to 3); (III) elution gradient (400 ml), 1*N* HCl plus methanol (1 to 3) to concentrated HCl, H_2O and methanol (1:1:6). The authentic standards were detected by a phosphorus method described by Allen (1940).

RESULTS AND DISCUSSION

Degradation of Diazinon and Diazoxon by Subcellular Fractions of Rat Liver Homogenate. The degradation of diazinon by microsomal enzymes from rat liver has been reported previously (Nakatsugawa *et al.*, 1969a; Yang *et al.*, 1969). This reaction requires NADPH and O_2, and is inhibited by carbon monoxide (Yang *et al.*, 1969). It was indicated in previous reports (Nakatsugawa *et al.*, 1969a; Yang *et al.*, 1969) that approximately one-third of the total radioactivity in the aqueous phase after enzymatic degradation of diazinon was diethyl phosphoric acid. A possible route for the formation of this metabolite is the oxidation of diazinon to diazoxon, and subsequent hydrolysis at the pyrimidinyl phosphate bond. Therefore, the *in vitro* study of the degradation of diazoxon by rat liver enzymes was undertaken. The results, as shown in Table I, suggest that a hydrolase system, which is independent from NADPH, hydrolyzes diazoxon and not diazinon. Diazinon is completely immune from the hydrolytic attack by the enzyme system.

The data also indicate that although the microsomal fraction has the highest enzymatic activity, the nuclear, mitochondrial, and soluble fractions also possess considerable activity. Since the subcellular fractions were all resuspended and recentrifuged during preparation, cross contamination from other fractions should be minimized. In view of the fact that there is more than one paraoxon-degrading enzyme in the rat liver homogenate (Kojima and O'Brien, 1968), it is therefore possible that in different subcellular fractions of the rat liver cell there might be more than one hydrolase which degrades certain organophosphate nonspecifically (Hodgson and Casida, 1962). In the case of diazoxon degradation, the microsomal hydrolase(s) is the most active.

In the study of diazoxon degradation, the substrate level was elevated 50-fold, and the incubation time and the enzyme level were reduced to $1/2$ and $1/10$, respectively, as compared to the diazinon study (Yang *et al.*, 1969). A comparison of initial rates per unit wet weight of rat liver shows the microsomal diazoxon-degrading enzyme to be approximately 1000 times more active than the mixed function oxidase system which degrades diazinon.

Table I. Degradation of Diazoxon in Subcellular Fractions of Rat Liver Homogenate[a]

Fraction	Degradation (μmoles/30 min/10 mg of liver) with NADPH	without NADPH
Nuclei	2.2	2.5
Mitochondria	1.4	1.6
Microsomes	7.0	7.8
Soluble fraction	0.3	0.2

[a] Incubation mixture consists of 60 μmoles tris-HCl buffer, pH 8.0; 10 μmoles ^{14}C-diazoxon; 1.1 μmole NADPH; enzymes equivalent to 10 mg liver and distilled H_2O to a final volume of 2 ml.

Table II. Effect of Reduced Glutathione on the Degradation of Diazinon and Diazoxon by Rat Liver Enzymes

Enzyme Source	Cofactors	Degradation (mμmoles/hr/100 mg of liver)
	Diazinon	
Microsomes	None	0.4
	GSH	1.8
	NADPH	100.9
	NADPH + GSH	98.4
Soluble fraction	None	14.3
	GSH	19.0
	NADPH	15.5
	NADPH + GSH	20.2
	Diazoxon	**(μmoles/30 min/10 mg of liver)**
Microsomes	None	6.1
	GSH	5.7
	NADPH	5.2
	NADPH + GSH	4.9
Soluble fraction	None	0.4
	GSH	0.3
	NADPH	0.2
	NADPH + GSH	0.3

Effect of Cofactors on the Degradation of Diazinon and Diazoxon by Rat Liver Enzymes. The requirement of NADPH for the oxidative degradation of diazinon by rat liver microsomal enzymes has been shown by Yang *et al.* (1969). Since GSH dependent desalkylation of organophosphates by soluble enzymes has been shown to be a common detoxication route in many species of animals (Fukami and Shishido, 1963, 1966; Hollingworth *et al.*, 1967; Kojima and O'Brien, 1968; Miyamoto *et al.*, 1968; Plapp and Casida, 1958b,c; Shishido and Fukami, 1963), the effect of GSH on the degradation of diazinon and diazoxon by microsomal and soluble fractions of rat liver was compared to that of NADPH. The results are shown in Table II.

Comparing the effects of GSH and NADPH on the degradation of diazinon and diazoxon indicated that in the case of diazinon NADPH markedly increased the activity of the microsomes but not the solution fraction, whereas GSH had little or no effect. With diazoxon, neither cofactors had an appreciable effect on the degradation by microsomes on the supernatant.

Effect of Sodium Fluoride on the Activity of the Microsomal Diazoxon-Degrading Enzymes in Rat Liver. It has been reported that sodium fluoride (NaF) inhibits the *in vitro*

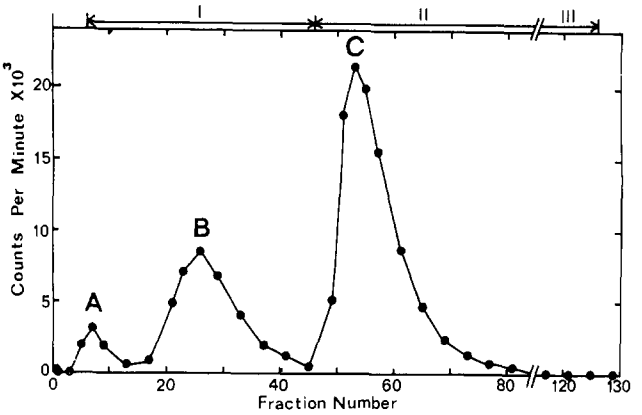

Figure 1. Ion exchange chromatogram of water soluble metabolites of diazinon degradation. Incubation mixture: Tris buffer, 425 μmoles; ^{14}C-diazinon, 1 μmole; NADPH, 11 μmoles; GSH, 20 μmoles; microsomes equivalent to 800 mg liver and distilled water to a final volume of 10.5 ml. After extraction twice with toluene, 5.0 ml of the aqueous phase was chromatographed. The Roman numerals shown on the top of the Figure are in accordance with those of the elution gradient mentioned in Materials and Methods

degradation of the oxygen analogs of certain organophosphate compounds (Dahm *et al.*, 1962; Murphy and DuBois, 1957). An attempt was made, therefore, to study the effect of NaF on diazoxon degradation by rat liver microsomes.

An initial experiment was performed by incubating diazoxon with microsomal enzymes in the presence of 0.1 mmole of NaF. The result was compared to a parallel test without the addition of NaF. It was found that diazoxon was degraded

Table III. Effect of Sodium Fluoride on the Degradation of Diazoxon

Addition	NaF added (mmole)	% Degradation of diazoxon
Tris buffer	None	0.9
	0.8	37.1
	1.6	44.2
Microsomes	None	48.6
	0.8	36.2
	1.6	44.3

to the same extent in both cases. Thus NaF at a concentration of 0.1 mmole seemed to have no inhibitory effect on the microsomal degradation of diazoxon. A subsequent experiment was then conducted using higher concentrations of NaF, and the results are shown in Table III.

It is interesting to note that NaF apparently served as a chemical catalyst in the hydrolysis of diazoxon. These data can be explained by assuming that the enzymatic degradation is inhibited extensively by NaF, while at the same time NaF catalyzes a nonenzymatic hydrolysis of diazoxon. Whether or not this chemical hydrolysis is common to all the oxygen analogs of the organophosphates remains to be investigated.

Nature of Metabolites. A total of 32 analyses, 16 by paper chromatography of the organic phase and 16 by ion-exchange column chromatography of the aqueous phase, which were the result of experiments on the metabolism of diazinon and diazoxon by either rat liver microsomal or soluble enzymes in the presence of various cofactors (none, GSH, NADPH,

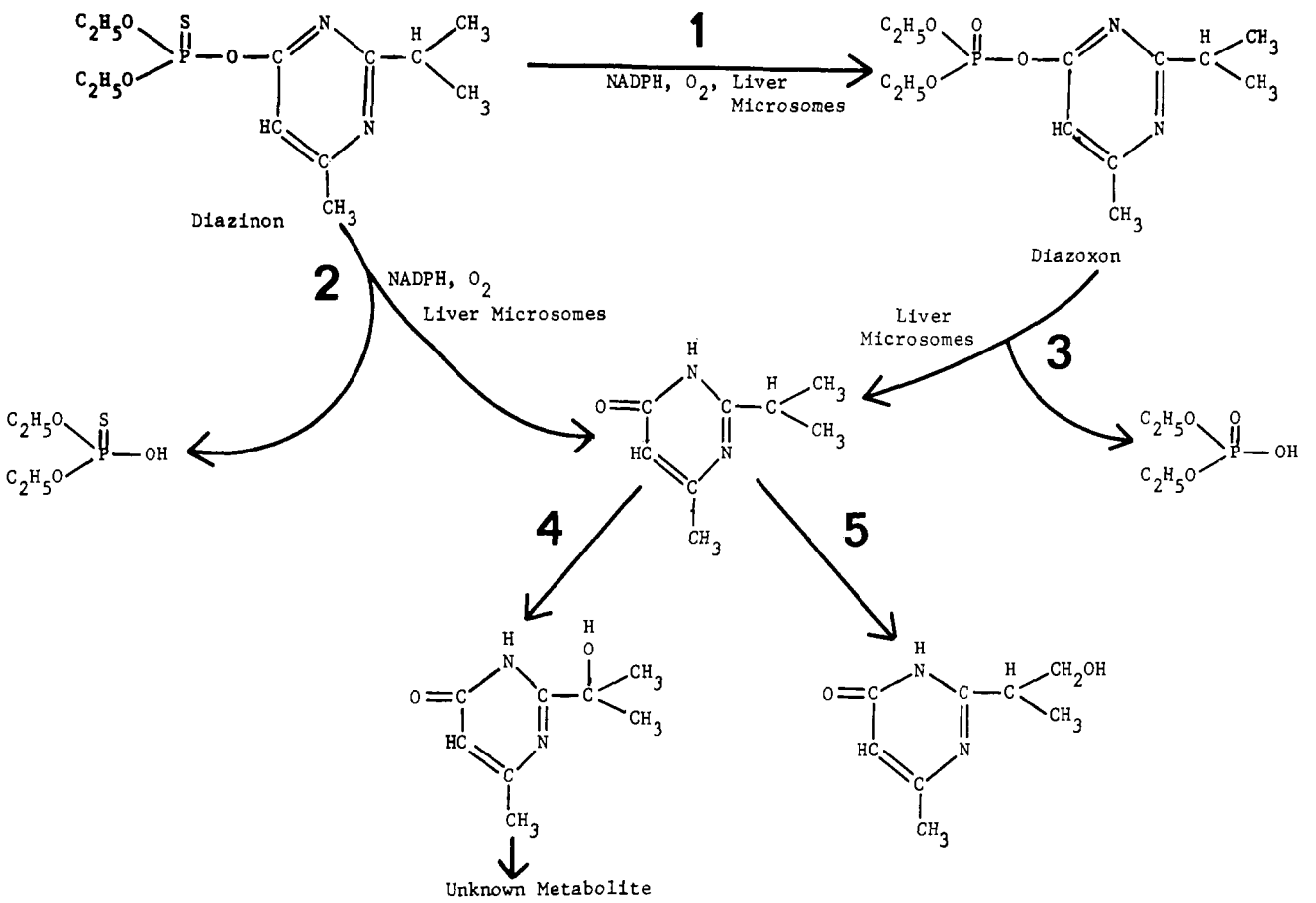

Figure 2. Proposed metabolic pathway of diazinon in the rat

NADPH + GSH), were carried out. Each analysis was duplicated.

Organic extracts of the experiments in Tables II and III were cochromatographed with the analytical standards. In all the diazinon studies, diazinon was found to be the major radioactive compound in every case. When the reaction mixture contained microsomes and NADPH, both diazinon and a trace of diazoxon were detected. This confirmed the previous finding that diazoxon is formed oxidatively (Dahm *et al.*, 1962). With the diazoxon studies, the only organic soluble compound detected was diazoxon.

A typical ion exchange chromatogram of the water soluble metabolites of diazinon is presented in Figure 1.

Peaks B and C (Figure 1) were identified by cochromatography with authentic standards on a Dowex-1 column as diethylphosphoric and diethylphosphorothioic acids, respectively. The identity of the minor metabolite (peak A) is unknown.

The analyses of the effluent from the ion-exchange columns of the degradation products of diazinon formed in the presence of other cofactors (none, GSH, NADPH) and other enzyme sources (microsomes, soluble fraction) showed similar results. Only traces of diethylphosphoric acid were detected in addition to diethylphosphorothioic acid when diazinon was incubated with the soluble fraction. In the case of diazoxon degradation, diethylphosphoric acid (peak B) was the sole metabolite obtained with either microsomal or soluble fraction and either cofactor.

Attempts to prepare desethyl diazinon utilizing ethanolic KOH (Plapp and Casida, 1958c), and benzenethiol (Miller, 1962) were unsuccessful. No desethyl diazoxon was obtained with the NaI method (Spencer *et al.*, 1958). Since standard desethyl diazinon and desethyl diazoxon were not available, these compounds were assumed to be eluted at the elution gradient III, as reported previously by Plapp and Casida (1958a).

If this assumption is correct, no desethyl diazinon or desethyl diazoxon was detected in all the experiments conducted with various cofactors (none, GSH, NADPH, GSH + NADPH) and enzyme sources (microsomes, soluble fraction).

These and previous findings are presented in Figure 2, a metabolic pathway for diazinon in the rat.

Once diazinon has entered the body of the rat, the insecticide is activated to a more potent cholinesterase inhibitor, diazoxon, to exert its toxic action. The activation of diazinon (reaction 1) is the result of the liver microsomal enzyme system which requires NADPH and O_2 (Dahm *et al.*, 1962). Meanwhile, diazinon is also degraded oxidatively (reaction 2) by the liver microsomal enzyme system (Nakatsugawa *et al.*, 1969a; Yang *et al.*, 1969). These two oxidative reactions are probably catalyzed nonspecifically by the same mixed function oxidases which are responsible for many other oxidations of xenobiotics (Hodgson, 1968). Due to the effective hydrolases in the microsomal and other subcellular fractions of the liver (Table I), diazoxon is degraded hydrolytically (reaction 3) once it is formed. In insects, diazoxon is degraded slowly by the microsomal mixed function oxidase system, while hydrolysis does not occur. It has been suggested that this is a possible explanation for diazinon selectivity (Yang *et al.*, 1971).

The two metabolites, diethyl phosphorothioic and diethyl phosphoric acids, being water soluble, are excreted rapidly (Robbins *et al.*, 1957). The further hydroxylation of the other metabolite, 2-isopropyl-4-methyl-6-hydroxypyrimidine (reactions 4 and 5), was demonstrated *in vivo* in the rat by Mücke *et al.* (1970). Whether or not these reactions involve the mixed function oxidase remains to be demonstrated.

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Received for review July 8, 1970. Accepted September 8, 1970. Paper number 3230 of the Journal Series of the North Carolina State University Agricultural Experiment Station, Raleigh, N.C. Work supported in part by Grant No. ES-00044 from the U.S. Public Health Service.