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ALTERATION OF MICE L-TRYPTOPHAN METABOLISM BY THE ORGANOPHOSPHOROUS ACID TRIESTER DIAZINON

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Abstract—Diazinon [*O*,*O*-diethyl *O*-(2-isopropyl-6-methyl-4-pyrimidinyl) phosphorothioate] altered the formation of several L-tryptophan metabolites associated with the L-kynurenine pathway in mice. Liver kynurenine formamidase was inhibited almost completely by diazinon (10 mg/kg). The enzyme inhibition resulted in reduced L-kynurenine biosynthesis in livers with a concomitant accumulation of *N*-formyl-L-kynurenine. In contrast to the liver, plasma L-kynurenine increased up to 5-fold in diazinon-treated mice. Consequently, the urinary excretion of xanthurenic acid and kynurenic acid was raised 5- to 15-fold. The revelation of this novel mechanism of diazinon action is an important piece of information needed for a better understanding of the noncholinergic toxicity of organophosphorous acid triesters and methylcarbamates.

Organophosphorous acid triesters (OP‡) are inhibitors of numerous serine-hydrolases [1]. The major principle of their toxicity is acetylcholinesterase phosphorylation resulting in enzyme inhibition, acetylcholine accumulation and altered cholinergic neurotransmission [2]. However, some toxic effects of OPs are unrelated to this mechanism, e.g. various central nervous system-related symptoms [1, 3], and hyperglycemia [4].

Our previous investigation of NAD-related avian teratogenesis [5] found that liver kynurenine formamidase (KFase), the enzyme which catalyzes *N*-formyl-L-kynurenine (FK) hydrolysis in the L-kynurenine (KYN) pathway of L-tryptophan metabolism, is susceptible to OP inhibition [6]. L-Tryptophan metabolism is a unique process since it generates a variety of metabolites with diverse physiological activities, such as neuroactive kynurenic (KA) and quinolinic (QA) acids, 5-hydroxytryptamine (serotonin), vitamin nicotinates, the cofactor of oxidoreduction reactions NAD(P), or chelatogenic picolinic acid [7]. Therefore, compounds which are capable of altering L-tryptophan metabolism have a high potential of being toxic to organisms dependent on this system. In the present study, we focused on the effects of OP on the KYN pathway in mice. Diazinon [*O*,*O*-diethyl *O*-(2-isopropyl-6-methyl-4-pyrimidinyl) phosphorothioate], a commonly used insecticide, was used as a model OP since it is one of the most potent liver KFase inhibitors *in vivo* [6, 8]. Our study revealed a novel mechanism of diazinon action in mice, based on the altered formation of several L-tryptophan metabolites.

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‡ Abbreviations: FK, *N*-formyl-L-kynurenine; IDO, indoleamine 2,3-dioxygenase (EC 1.13.11.17); KA, kynurenic acid; KFase, kynurenine formamidase (aryl formylamine hydrolase) (EC 3.5.1.9); KYN, L-kynurenine; OP, organophosphorous acid triester(s); PDA, 2,4-pyridine dicarboxylic acid; QA, quinolinic acid; TDO, tryptophan 2,3-dioxygenase (EC 1.13.11.11); and XA, xanthurenic acid.

MATERIALS AND METHODS

Chemicals. Diazinon was obtained from Ciba-Geigy (U.S.A.). Its purity (>99%) was confirmed by GS–MS. Silica plates (Fisherbrand Gel GF, 0.25 and 1 mm thick layers) and Acrodisc LC13 PVD filters (0.2 μm) were purchased from Fischer Scientific (Santa Clara, CA). L-[side chain-2,3-^{3}H]Tryptophan (55 Ci/mmol) was obtained from Amersham (U.K.), and FK from the Chemical Dynamics Corp. (South Plainfield, NJ). All other chemicals of the highest available purity were purchased from the Sigma Chemical Co. (St. Louis, MO).

Animal treatment and sampling. Swiss–Webster male mice (Simonsen, Gilroy, CA; 22–35 g, 8–12 weeks old) were kept in stainless steel metabolic cages with free access to food (Purina Chow) and water on a 12-hr light/dark cycle. Diazinon in methoxytriglycol (20 μL) was administered i.p. to the mice (the 40 mg/kg dose used routinely throughout this study was about one-sixth of the LD_{50}; the dose caused only mild toxic symptoms which disappeared within 3 hr after diazinon administration). The control group of mice received an equal volume of the solvent carrier. Mice treatments and specimen collections were carried out at the same day/night times in order to avoid possible variations due to diurnal changes in L-tryptophan metabolism. Animals were killed by cervical dislocation. Organs if not used immediately were stored in isotonic sucrose solution at −80°. Blood was collected after cardiac puncture using a heparinized syringe. Plasma was separated by

centrifugation at 3000 *g* for 15 min and stored at −5°. Urine collected for a given period was preserved with the addition of hydrochloric acid (0.1 mL of 10 N HCl for 3 mL of urine) and stored at −5°.

KFase assay. Tissues were homogenized in an Elvehjem–Potter homogenizer with 0.1 M sodium phosphate buffer (pH 7.4) [1:8 (w/v) for livers; 1:3 (w/v) for other tissues]. KFase was in the cytosolic fraction prepared by sequential centrifugation at 10,000 and 150,000 *g* [6]. For the assay [6], 5 μL of the 150,000 *g* supernatant (KFase) was added to 985 μL of 0.1 M sodium phosphate buffer (pH 7.4) and 10 μL of 0.15 M FK. FK hydrolysis was followed spectrophotometrically at 360 nm. The hydrolysis proceeded according to zero-order kinetics up to at least 4 min. Protein was assayed by the Lowry method using bovine serum albumin as a protein standard.

FK and KYN formation by liver slices.* Liver slices (about 20 slices per assay, 1 to 1.5 mm-side cubes, each of approximately 5 mg of wet weight) were prepared by sectioning livers of control and diazinon-treated mice with a scalpel. The slices were preincubated with 3 mL of Krebs solution at 37° for 30 min. The solution was then replaced with 1 mL of fresh Krebs solution containing 15 μmol of L-tryptophan. The reaction mixture was incubated in an ambient atmosphere and continuous shaking (50 strokes/min) for 120 min at 37°. The reaction proceeded according to zero-order kinetics under these conditions. The reaction was stopped by immersing the vials into ice. The medium was separated immediately from slices, 1 mL of precooled (−5°) methanol was added, and the mixture was spun at 5000 *g* for 15 min. The supernatant was filtered through Acrodisc LC13 PVD filters prior to HPLC analysis for KYN and FK with detection at 254 nm. Samples with L-[side chain-2,3-^{3}H]-tryptophan (10 μCi added to 1 mL of 15 mM L-tryptophan) were processed in a similar way for KYN and FK identification. Fractions (0.3 mL) were collected after HPLC and their radioactivity was counted by liquid scintillation spectrometry. The identity of FK and KYN was examined further by thin-layer chromatography (*n*-propanol–*n*-butanol–NH_4OH–water, 4:2:0.01:4/silica gel/detection under UV and by ninhydrin), spectrophotometrically (220–400 nm), and enzymatically (FK hydrolysis by liver KFase). The presence of KYN and FK in the media was confirmed by all the explored methods.

Liver tryptophan 2,3-dioxygenase (TDO). TDO activity in liver slices was expressed as the sum of FK and KYN formed by 1 mg of liver slices (wet weight) in 1 min at 37°.

Indoleamine 2,3-dioxygenase (IDO). Lung and colon IDO activities were determined by HPLC using L-tryptophan as a substrate [9, 10]. Tissues of diazinon-treated and untreated mice were homogenized with 0.1 M potassium phosphate buffer (pH 7.0) (1:3, w/v) in an Ultra-Turrax homogenizer. The tissue homogenate was fractionated by centrifugation as described above for KFase. The reaction mixture (1 mL) of methylene blue (25 μM), ascorbic acid (25 mM), L-tryptophan (0.2 mM), catalase (~500 IU) and IDO (500 μL of the soluble fraction corresponding to ~150 mg of wet tissue) was incubated for 2 hr at 37°. The enzymatic reaction was terminated by adding 0.3 mL of 10% trichloroacetic acid. After cooling to 0° the mixture was centrifuged at 5000 *g* for 15 min. The supernatant (2.5 to 5 μL) was analyzed by HPLC for KYN with detection at 360 nm.

Plasma KYN. Plasma (200–400 μL) was deproteinized by adding 3 vol. of 10% trichloroacetic acid at 0°. The precipitate was removed by centrifuging for 15 min at 5000 *g*. Deproteinized plasma was applied on Sep-Pak columns (C_{18} cartridges, Waters, MA) and washed with 1.5 mL of ice-cold water; KYN was eluted with 2.5 mL of 80% methanol. Methanolic eluates were taken to dryness in a vacuum rotary evaporator at 40°. The residue was dissolved in 100 μL of distilled water and analyzed by HPLC for KYN with detection at 360 nm.

Urinary QA. In this assay which was modified after methods described in Refs. 11 and 12, urine (0.4 to 1.0 mL) was mixed with 1 vol. of 1 M perchloric acid and centrifuged at 5000 *g* for 15 min. The supernatant was neutralized by 3 M potassium hydroxide and potassium perchlorate was removed by centrifugation at 5000 *g* for 15 min. The internal standard 2,4-pyridine dicarboxylic acid (PDA) (1–10 nmol) was added to the neutralized supernatant and the mixture applied on a Dowex 1X8 column (5 × 15 mm column, 200–400 mesh Dowex 1X8 in a formate cycle). The column was eluted successively with 4.5 mL each of 1, 4 and 6 M formic acid. The 4 M formic acid fraction contained >97% of the quinolinic acid as determined by [^{14}C]quinolinic acid recovery. The fraction was evaporated to about 200 μL in a rotary vacuum evaporator at 40° [13] and taken to dryness at lowered pressure in a dessicator over calcium chloride. Hexafluoroisopropylalcohol (100 μL) and pentafluoroimidazole (100 μL) were added to the dried residue and the mixture was heated at 80° for 60 min. QA and PDA dihexafluoroisopropyl esters were then extracted into 250 μL *n*-heptane and the extract was concentrated at lower pressure to about 50 μL. One microliter was taken for GC–MS analysis.

Liver NAD. Livers were homogenized in an Elvehjem–Potter homogenizer in 1 M $HClO_4$ (1:3, w/v) and the homogenate was centrifuged for 15 min at 5000 *g*. The neutralized supernatant was used for NAD spectrophotometric assay with ethyl alcohol and alcohol dehydrogenase [14].

Urinary xanthurenic acid (XA) and KA. Urine (combined typically from two mice) was cleared by centrifugation at 5000 *g*. The supernatant was evaporated to dryness in a rotary vacuum evaporator at 40° and the residue dissolved in 10 mL of 0.01 M sodium hydroxide in 80% methanol. The precipitate formed after 5 hr at 0° was removed by centrifugation at 5000 *g* for 15 min. The supernatant was filtered through Acrodisc LC13 PVD filters prior to HPLC. XA and KA were detected at 254 nm; KA was also measured fluorimetrically at 300–400 nm after irradiation at 254 nm. The identity of XA was confirmed by thin-layer chromatography (the same solvent system as described above for MF/KYN with detection under UV and visible light), and

* Manuscript submitted for publication.

spectrophotometrically (200–400 nm) after HPLC fractionation. The identification of KA was based on cochromatography with standard in HPLC and the characteristic ratio of AUC absorbancy at 254 nm and fluorescence at 300–400 nm after irradiation at 254 nm. The major hydrolytic diazinon product 2-isopropyl-4-methyl-6-hydroxypyrimidine, prepared by controlled diazinon hydrolysis at pH 2.4 and 50° for 48 hr [15], did not interfere with XA and KA chromatography (it was eluted from the C_{18} column at 3.7 min compared to 13–15 min for XA and KA).

HPLC. A Spectra Physics IsoChrom LC Pump, a Rheodyne model 7125 Syringe Loading Sample Injector (10 μL sample loop) with a Spectra Chrom 100 UV, and Spectra Chrom 8410 fluorimetric detectors were the basic HPLC components (Spectra-Physics, San Jose, CA). Samples were separated on a reversed-phase C_{18} column (4.6 × 30 mm, 3 μm CR Pecosphere, Perkin–Elmer) with a mobile phase of 5% acetonitrile in 1 mM monosodium phosphate, pH 2.3, at a flow rate of 0.5 mL/min (the column was washed after each run successively with 25% acetonitrile in the phosphate buffer and methanol, respectively). Quantitation of metabolites was made either from the calibration curve or by addition of the standard compound.

GLC–MS. QA and PDA dihexafluoroisopropyl esters were analyzed using a Hewlett–Packard Gas Chromatograph 5890 with Mass Selective Detector 5970A. The samples were separated on a DB-1 fused silica capillary column (30 m × 0.25 mm i.d., J & W Scientific) with dimethyl polysiloxane phase and helium carrier gas (inlet pressure 70 kPa). The injector and detector temperature was 250°. The column was heated for the first min to 90°, then the temperature was increased to 120° at a rate of 2°/min, and subsequently to 250° at a rate of 30°/min. The temperature of the ion source of the Mass Selective Detector was kept at 200°. Ionization was achieved at 70 eV. Ion fragments (272 and 300 *m/e*) obtained by SIM were used for the analysis of QA and PDA derivatives. QA was calculated by linear regression from the plot of ratios of relative intensities of QA to PDA ion fragments against QA amount.

RESULTS

KFase inhibition. KFase of the liver was affected the most by diazinon (Fig. 1A). The enzyme was inhibited almost completely by a single dose of diazinon (>10 mg/kg). Administration of multiple doses of diazinon maintained reduced KFase activity. Spontaneous recovery of diazinon-inhibited KFase was slow; 50% of the enzyme activity was attained in 3–5 days after administration of 1 and 10 mg diazinon/kg. Neither 2-PAM (2 × 15 mg/kg) nor hydrocortisone (50 mg/kg) alleviated the inhibition of KFase by diazinon. KFase activity in the lung and colon was affected to a lesser extent by diazinon (i.e. 70–80% inhibition by 10 mg diazinon/kg) (Fig. 1B). Enzyme activity returned to normal at a rate similar to that of the liver.

FK and KYN formation in liver slices. KYN formation in slices prepared from the livers of diazinon-treated mice was reduced by about 50%

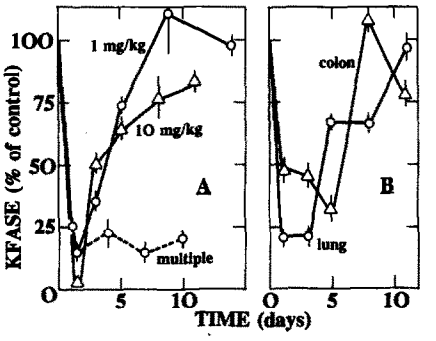

Fig. 1. Kynurenine formamidase inhibition by diazinon. Diazinon was administered i.p. to mice. KFase was measured in the cytosolic fraction of organ homogenate fractionated by centrifugation at 150,000 *g*. Enzyme activity was determined spectrophotometrically with FK (1.5 mM). Values are the means of two assays with the range indicated by bars. (A) Effects of a single (1 or 10 mg/kg) and multiple doses of diazinon (1 mg/kg on days 0, 3, 6 and 9). A dose of 40 mg/kg maintained >99% inhibition for at least 24 hr. (B) Nonhepatic KFase after diazinon administration (10 mg/kg). Control values ± SD: liver, 424 ± 75 (N = 14); colon, 3.5 ± 1.3 (N = 11); and lung, 4.4 ± 0.5 (N = 13) nmol·mg protein^{-1}·min^{-1}.

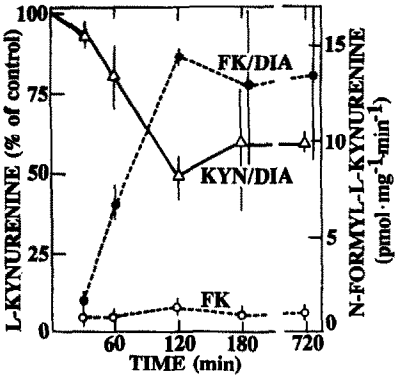

Fig. 2. Effects of diazinon on *N*-formyl-L-kynurenine and L-kynurenine formation by liver slices. Liver slices prepared at the indicated times after diazinon i.p. administration (40 mg/kg) were incubated with 15 mM L-tryptophan were assayed in Krebs solution at 37° for 120 min. FK and KYN were assayed in the media by HPLC. Values are the means of two assays with the range indicated by bars. Key: KYN: control, 21.4 ± 8.0 pmol·mg^{-1}·min^{-1} (SD, N = 5); and FK/DIA, KYN/DIA; diazinon-treated.

120 min after drug administration (Figs. 2 and 3). Diazinon doses over 40 mg/kg did not restrict KYN production any further (Table 1). The reduced KYN formation was accompanied by FK accumulation (Figs. 2 and 3, Table 1). Neither 2-PAM (2 × 15 mg/kg) nor hydrocortisone (50 mg/kg) alleviated the effects of diazinon on KYN/FN production.

Liver TDO. TDO assayed in liver slices was

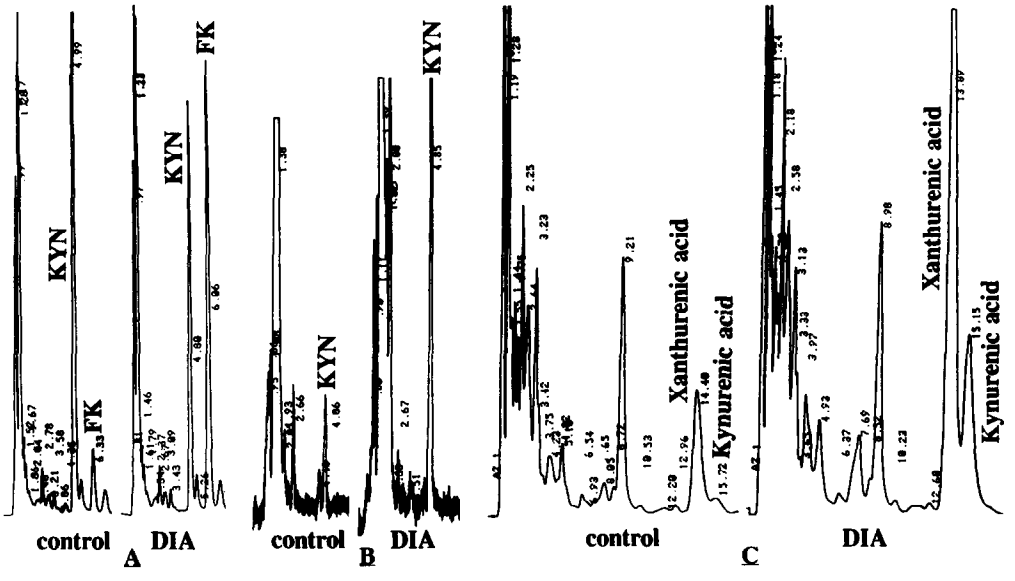

Fig. 3. Alteration by diazinon of metabolite formation in the L-kynurenine pathway. Diazinon (40 mg/kg) was administered i.p. to mice. HPLC of liver slice media, plasma and urine was performed on a reversed-phase C_{18} column with 5% CH_3CN in 0.001 M sodium phosphate buffer, pH 2.3. FK and KYN (liver slices), XA and KA were detected at 254 nm, and KYN (plasma) at 360 nm. (A) KYN and FK formation in liver slices 12 hr after diazinon (DIA) administration. (B) Plasma KYN 12 hr after diazinon administration. (C) XA and KA in urine collected for 11 hr following diazinon administration.

enhanced moderately in mice treated with high diazinon doses (Table 1).

Plasma KYN. Plasma KYN increased 3- to 5-fold between 6 and 24 hr after diazinon administration (40 mg/kg) (Figs. 3B and 4). It peaked at 12 hr and returned to basal level by 72 hr. Cycloheximide did not alleviate diazinon-induced plasma KYN.

Liver NAD, urinary and plasma QA, and IDO. Diazinon did not affect the liver NAD pool 24 hr after the mice were treated (Table 2). Urinary QA excretion was increased moderately during the 16-hr period following administration of diazinon while the plasma QA concentration remained steady (Table 2). Colon IDO activity in the mice was reduced by diazinon 2 hr after treatment (Table 2).

Urinary XA and KA. Diazinon markedly induced urinary XA and KA excretion in mice (Figs. 3C, 5 and 6). Higher XA was detected in urine as early as

Table 1. Effect of varying diazinon doses on *N*-formyl-L-kynurenine/L-kynurenine formation and tryptophan 2,3-dioxygenase in liver slices

Diazinon ($mg \cdot kg^{-1}$)	KYN	FK ($pmol \cdot mg^{-1} \cdot min^{-1}$)	TDO
0 (3)	26.7 ± 3.6	0.2 ± 0.1	26.9 ± 3.6
1 (2)	31.3 ± 8.9	1.2 ± 0.7	32.5 ± 9.4
10 (2)	23.8 ± 3.8	2.8 ± 0.9	26.6 ± 4.6
40 (2)	16.4 ± 1.9	13.6 ± 6.7	30.0 ± 7.0
80 (1)	15.3	17.1	32.4
160 (1)	16.3	27.3	43.6
240 (1)	17.1	26.2	43.3

Liver slices were prepared 36–39 hr after i.p. diazinon administration. The number of independent experiments of duplicate assays is shown in parentheses in the first column. Diazinon and diazoxon (10^{-4} M) preincubated with liver slices for 60 min at 37° reduced KYN formation by 34 and 28%, respectively. Control values are means ± SD; where only two experiments were performed, values are means ± range.

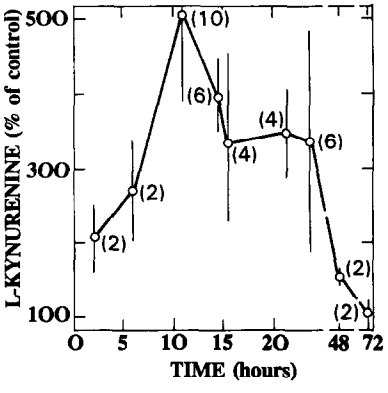

Fig. 4. Plasma L-kynurenine in diazinon-treated mice. Diazinon (40 mg/kg) was administered i.p. to mice. Deproteinized plasma was fractionated on a reversed-phase C_{18} column (Sep-Pak) prior to HPLC. The number of independent determinations is given in parentheses; the standard deviations (or a range for two assays) are indicated by bars. KYN at 11–24 hr differed significantly from the control value at $P < 0.001$ (*t*-test for differences between two means). The basal level was 1460 ± 582 nmol KYN · mL plasma^{-1} (SD, N = 32). The KYN increase in mice treated with diazinon and cycloheximide (5 mg/mouse) was 448% at 14 hr.

Table 2. Liver NAD, urinary and plasma quinolinic acid, and colon indoleamine 2,3-dioxygenase in control and diazinon-treated mice

	NAD ($nmol \cdot g^{-1}$)	QA		IDO† ($nmol \cdot hr^{-1} \cdot g^{-1}$)
		Plasma* ($pmol \cdot mL^{-1}$)	Urine ($nmol \cdot mL^{-1}$)	
Control	515 ± 0 (2)	161 ± 57 (2)	15 ± 6 (6)	43 ± 18 (3)
Diazinon	521 ± 8 (3)	177 ± 102 (7)	22 ± 7 (7)‡	20 ± 13 (2)

Diazinon (40 mg/kg) was administered i.p. to mice. Liver NAD was assayed 24 hr, plasma QA and colon IDO 2 hr, and urinary QA 2, 6 and 16 hr later. Values are means ± SD (ranges for two assays). The number of independent determinations of duplicate assays is indicated in parentheses.

* Plasma QA increased from 958 ± 332 (2) to 12,000 ± 498 (3) $pmol \cdot mL^{-1}$ following induction by i.p. administered L-tryptophan (rats).

† Low lung IDO activity was not increased by diazinon. Both lung and colon enzymes were induced by lipopolysaccharide (phenolic extract of *Salmonella abortus equi* purified chromatographically.

‡ Significantly different from the control at $P < 0.05$ (*t*-test for paired comparison, 3 d.f.).

2–6 hr after diazinon administration, reaching a maximum in 24- and 48-hr urine collections (Fig. 5). Urinary XA remained elevated for 4 days following a single diazinon dose (40 mg/kg). The dose-dependence study revealed that 5 mg of diazinon/kg was the lowest dose required for increased XA excretion (Table 3). Diazinon at doses higher than 10 mg/kg did not increase XA excretion further. KA excretion was enhanced more profoundly, compared to that of XA (Fig. 6). A more than 15-fold increase in KA was found in urine collected for the first 14 hr following diazinon administration (40 mg/kg). Higher urinary KA excretion continued up to 48 hr after diazinon treatment. Similar to the diazinon dose effect on XA excretion, 40 mg/kg was not more effective in inducing KA excretion than the 10 mg dose.

DISCUSSION

Diazinon readily inhibits liver KFase. The KFase active center has many features in common with the

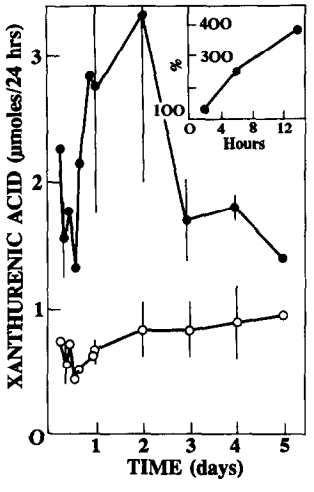

Fig. 5. Urinary xanthurenic acid excretion in control and diazinon-treated mice. Diazinon (40 mg/kg) was administered i.p. to mice. Urine was collected from two mice at the times indicated. Standard deviations indicated by bars were calculated from 3–4 independent experiments. Values at 0.5, 1, 2, 3 and 4 days were significantly greater than the control at $P < 0.02, 0.05, 0.02$, and 0.1, respectively (*t*-test for differences between two means, 5–6 d.f.). The inset shows XA excretion [percent of control, i.e. 1332, 654 and 927 $nmol \cdot (24\ hr)^{-1}$ at 2, 6 and 14 hr] for the initial period. Key: (○) control; and (●) diazinon-treated.

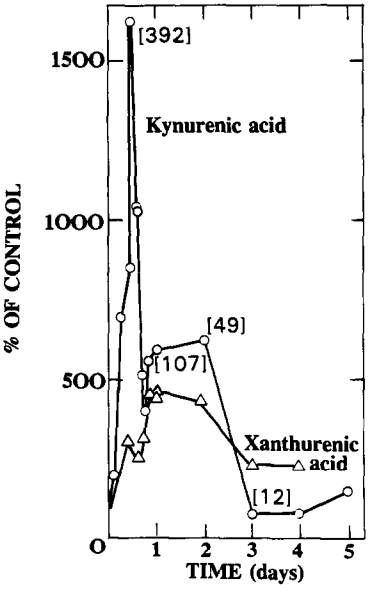

Fig. 6. Effect of diazinon on urinary xanthurenic and kynurenic excretion. Diazinon (40 mg/kg) was administered i.p. to mice. Urine was collected from two mice at the times indicated. XA percentages were calculated from the data in Fig. 5. KA standard deviations or range (3 or 2 independent determinations) are shown in square brackets. KA values from 0.5, 1 and 2 days were significantly greater than the controls at $P < 0.1$, 0.05 and 0.1 levels (*t*-test for differences between means, 2–4 d.f.), respectively. The basal values for XA and KA were 737 ± 256 (SD, N = 27) and 250 ± 52 (SD, N = 13) $nmol \cdot (24\ hr)^{-1}$, respectively.

Table 3. Effect of varying diazinon doses on xanthurenic and kynurenic acid urinary excretion

Diazinon (mg·kg^{-1})	Xanthurenic acid (nmol·(24 hr)$^{-1}$)	Kynurenic acid (nmol·(24 hr)$^{-1}$)
0 (27) (13)†	737 ± 256	250 ± 52
1 (3)	855 ± 199	235 ± 80
5 (1)	1754	ND
10 (2)	3913 ± 479‡	1038 ± 20§
20 (1)	2417	ND
40 (13) (6)†	3670 ± 752‖	1245 ± 255‖
80 (1)	3169	ND

Urine was collected for 24 hr after i.p. diazinon administration. The number of independent determinations is shown in parentheses in the first column. Where multiple experiments were done, values are means ± SD; where only two experiments were performed, values are presented as means ± range. ND = not determined.

† For KA.

‡–‖ Significantly different from the control (*t*-test for differences between two means) at: ‡ $P < 0.1$ (27 d.f.); § $P < 0.01$ (13 d.f.); and ‖ $P < 0.001$ (38 and 17 d.f., respectively).

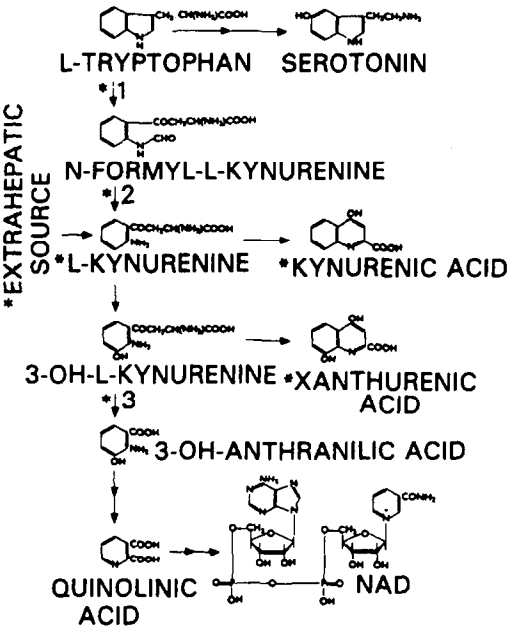

Fig. 7. L-Kynurenine pathway of L-tryptophan metabolism with sites of diazinon interference (*). Key: (1) TDO, (2) KFase, and (3) kynureninase.

cholinesterases and other serine-hydrolases, making it susceptible to phosphorylation by OP [6]. Reactivation of the phosphorylated enzyme, either spontaneously or by oxime treatment, is slow and incomplete [6]. Thus, *in vivo* recovery of inhibited liver KFase in mice is due primarily to *de novo* protein synthesis rather than spontaneous reactivation, following fast metabolic depletion of diazinon [16]. Administration of multiple doses of diazinon, simulating a subchronic exposure to the insecticide, maintained reduced liver KFase activity. This finding deserves special consideration in view of the possible toxic consequences due to prolonged KFase inhibition in humans or animals exposed to (sub)chronic doses of diazinon. KFase in the lung and colon was affected by diazinon to a lesser extent than liver KFase. Perhaps this reflects lower diazinon distribution and availability in the lungs and colon, or their limited ability to activate diazinon to the ultimate KFase inhibitor diazinon [6, 8]. Since KFase activity of these tissues is low compared to that of the liver, the residual lung and colon KFase seems unable to compensate for diminished liver enzyme.

The liver and plasma KYN pool responded to diazinon treatment in a distinct way. In the livers, diazinon reduced KYN production by inhibiting KFase. However, blockade of KYN formation in liver slices was only partial, despite the almost complete KFase inhibition in cytosolic preparations. This partial blockade could be caused by a combination of several factors. First, diazinon concentration in the cytosolic enzyme preparation may have been increased by its release from storage in lipophilic hepatocyte membranes upon liver homogenization. Second, the ultimate KFase inhibitor diazoxon, which is formed from diazinon by hepatic mixed-function oxidases, may be deactivated [15, 16] at a higher rate in slices than in cytosolic preparations. The low diazoxon *in vitro* inhibitory potency in liver slices (Table 1) was probably due to its loss by enzymatic degradation. Third, TDO has been induced moderately by diazinon, the mechanism based probably on glucocorticoid release [17, 18]. The elevated intracellular FK may then increase the rate of FK hydrolysis by the residual KFase. FK accumulation in liver slices is another important phenomenon resulting from KFase inhibition by diazinon. The potential for cellular damage due to increased FK concentrations (elevation more than 100-fold) is at this time only speculative: its mechanism may include direct FK effects on some enzymes of L-tryptophan metabolism (e.g. on kynureninase, EC 3.7.1.3 [19]) (Fig. 7) or disruption of intracellular homeostasis by scavenging carbonyl compounds due to FK reactivity (our unpublished results).

In contrast to the liver, plasma KYN was increased up to 5-fold by diazinon treatment. Plasma KYN is formed in nonhepatic tissues from L-tryptophan with IDO involved in the initial L-tryptophan oxidative cleavage [20]. IDO can be induced under various pathological conditions with the most marked effects being observed in the epididymis, lungs and colon [20]. Diazinon did not increase IDO activity in either of the latter tissues. On the contrary, colon IDO was depressed, perhaps due to a regulatory response to increased plasma KYN. The epididymis was not considered for examination since the effects of diazinon on L-tryptophan metabolism were profound in both male and female mice (our unpublished results). We also examined whether KYN can be transported into plasma from the liver. Almost all of the hepatic KYN formed after *in vivo* TDO induction with L-tryptophan was processed immediately into QA and released into the systemic

circulation. There was a 12-fold increase in plasma QA compared to only a 1.3-fold increase in plasma KYN. This finding is in accord with the proposed scheme of KYN flow in mice [20], i.e. KYN is delivered unidirectionally from the plasma pool into the liver. KYN release from yet unidentified storage is another hypothetical mechanism for increased plasma KYN. The ineffectiveness of cycloheximide, a known inhibitor of IDO synthesis [21], to alleviate diazinon-induced plasma KYN may be an argument for this mechanism.

The liver content of NAD, the end-product of the KYN pathway of L-tryptophan metabolism, remained steady following diazinon administration in spite of the increased KYN plasma pool. Formation of QA, the metabolite closer to KYN in the metabolic route between KYN and NAD (Fig. 7), had increased only moderately as monitored by its appearance in plasma or excretion in urine. These findings suggest two important points. First, homeostasis of two physiologically important metabolites is maintained due to the increase in plasma KYN which compensates for the reduced liver KYN biosynthesis. Second, there must be an additional mechanism for regulating KYN plasma overproduction, located between KYN and QA in the KYN metabolic pathway.

The urinary excretion of XA and KA was induced markedly by diazinon, in support of point two above. The induction correlated with the increase of KYN in the plasma. The signal for this diversion in the KYN pathway may be given by FK accumulated in the liver. This mechanism was indicated to regulate flow through the KYN pathway in *Neurospora* [19]. Concordance of diazinon doses which cause FK accumulation in the liver with those that induce XA and KA urinary excretion supports a cause-and-effect hypothesis.

The increased plasma KYN pool and the induced XA urinary excretion (Fig. 7) have several implications in the assessment of diazinon noncholinergic toxicity. First, XA urinary elevation deserves special attention due to its possible involvement in bladder malignancies in mice [22, 23]. Second, an increase in XA formation may alter glucose metabolism. XA has been reported to form a complex with insulin [24, 25] and damage pancreatic beta-cells [26]. Third, elevated plasma KYN may alter KYN transport into the brain. Since over 40% of brain KYN originates from the systemic circulation [27], cerebral biosynthesis of neuroactive KYN metabolites such as QA and KA may change. Finally, increased consumption of L-tryptophan for building a high KYN pool may affect L-tryptophan availability for other L-tryptophan-dependent processes, e.g. for the 5-hydroxytryptamine biosynthesis route. The magnitude of a single diazinon dose for inducing changes in L-tryptophan metabolism implies the importance of this novel mechanism for a better understanding of the noncholinergic toxicity of OP and methylcarbamate [6, 8] KFase inhibitors in occupational and accidental poisonings.

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