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Toxic Metabolites of Diazinon in Sheep

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Diazinon is oxidized in sheep to several cholinesterase-inhibiting metabolites. The structures of three of them were determined by direct spectroscopic measurement. Two are monohydroxy diazinons, and the third is a dehydration product of one of these; the three structures had already been proposed for diazinon metabolites produced by mice, when the establishment of structure was based on cochromatography with synthetic samples. Some quantitative aspects of the distribution of the compounds in sheep are reported.

Because diazinon is widely used as an agricultural insecticide in conditions where it may be ingested by mammals, its metabolism has been studied for many years. It is only recently, however, that specific structures have been put forward for some of the metabolites formed. In all these structures the pyrimidine moiety has been modified, either before or after cleavage from the diethyl phosphorothionyl group. Mucke *et al.* (1970) showed that in rats the isopropyl group of the pyrimidinol derived from diazinon was hydroxylated to give two isomeric hydroxypyrimidinones. These compounds are much less toxic than diazinon because they have lost the phosphorus-containing group. Miyazaki *et al.* (1970), in an extensive study of the metabolism of diazinon by mice, suggested structures for several urinary metabolites in which all the phosphate bonds were still intact. Identifications were based on a comparison of the chromatographic properties of the metabolites with those of synthetic compounds.

The present work is concerned with those metabolites of diazinon in sheep that are still indirect inhibitors of cholinesterase. Three of these were isolated in sufficient quantity to establish their structures by direct spectroscopic measurements. We have reported the occurrence of two of them (referred to below as I and II) in brief preliminary accounts (Machin *et al.*, 1971b, 1972).

EXPERIMENTAL SECTION

Materials. Solvents were analytical reagent grade and were distilled immediately before use. Chloroform was washed free from ethanol and dried before distillation.

Diazinon and diazoxon were gifts from Fisons, Ltd., Agrochemicals Division. Hydroxydiazinon (*O,O*-diethyl *O*-[(2-hydroxyprop-2-yl)-6-methylpyrimidin-4-yl] phosphorothionate) was prepared by irradiation of diazinon (Machin *et al.*, 1971a).

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Thin-layer chromatography (tlc) was on 8-in. square plates coated with silica gel G or GF/UV254 (Machery, Nagel & Co.); final separations were carried out on the specially purified grade N-HR/UV254. Column chromatography was on Florisil, Johns-Manville Celite 545, and Woelm silica gel and neutral alumina.

Gas Chromatography. Residues were determined and preparative separations of the metabolites monitored on a Varian Aerograph Model 204 chromatograph with a thermionic detector or on a chromatograph (Machin and Morris, 1972) which was also used to purify samples. Columns were 3 ft or 5 ft × ⅛ in. o.d. glass, packed with 1.5–2% XE-60 or 25% SE-30 on Aeropak 30, 100–120 mesh.

Isolation of Metabolites. Two sheep were dosed by stomach tube with diazinon (1 g/kg) which produced moderate symptoms of poisoning. One was killed after 48 hr. Urine was collected from the other for 3 days and the sheep was allowed to recover. Metabolites I and II were isolated from urine collected during this 3-day period. The urine (120 ml) was diluted to 500 ml with water and extracted for 48 hr with chloroform (800 ml) in a liquid–liquid extractor. The chloroform extract was divided into portions (4 × 200 ml), each of which was concentrated to 10 ml and applied to a column of silica gel (Brockman grade II, 25 g, ¾ in. diameter). The metabolites were eluted with 1:40 methanol–chloroform (150 ml) and the eluate was concentrated, first in a rotary evaporator and then in a stream of dry nitrogen, to 0.3 ml. After tlc with 1:4 acetone–hexane as mobile solvent, the bands containing I and II were eluted separately and chromatographed twice more, first with the same solvent and then with 2:3 ethyl acetate–hexane to remove an unknown contaminant detected by its fluorescence at 360 nm. I and II were again eluted with acetone and the solvent was evaporated. Metabolite I was also separated from the tissues, as described previously (Machin *et al.*, 1971b).

Metabolite III was mainly concentrated in the fat and was extracted by macerating 200-g portions of fat three times with acetone (200 ml). After each maceration the suspension was centrifuged at 3000 rpm for 30 min at −20° and the supernatant was decanted. The extracts

Table I. Chromatographic Properties of Diazinon, Metabolites, and Derivatives

Compound	R_f value[a]	Relative retention time (diazinon = 1.00) On XE-60[b]	On SE-30[c]
Diazinon	0.40	1.00	1.00
Metabolite I	0.24	2.25	1.40
I-TMS ether		1.50	
Metabolite II	0.12	5.50	2.06
II-TMS ether		2.15	1.61
Metabolite III	0.43	1.65	1.30
Diazoxon	0.11	1.45	0.93

[a]Tlc on silica gel, developed with 1:4 acetone–hexane. [b]Glc on 1.7% XE-60, 5 ft × ⅛ in. o.d. column, 202°, carrier flow 20 ml per min, retention time of diazinon 1.60 min. [c]Glc on 25% SE-30, 5 ft × ⅛ in. o.d. column, 250°, carrier flow 20 ml per min, retention time of diazinon 2.25 min.

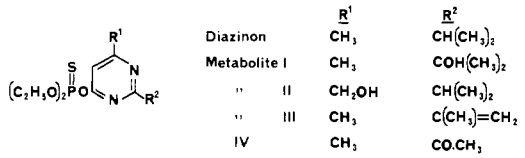

Figure 1. Structures of diazinon and degradation products.

from eight such aliquots were combined and concentrated in a rotary evaporator to a total volume of 60 ml, when the final solution separated into two layers with most of the diazinon and III in the upper layer. To remove fat from the upper layer (Wood, 1969), the solution (40 ml) was mixed with Celite 545 (40 g), the acetone was allowed to evaporate, and the mixture was packed into eight separate columns. Each was eluted with dimethyl sulfoxide (DMSO) (10 ml) onto a column containing Florisil (12 g) and alumina (12 g) which was eluted with hexane. The first eluate from each column (700 ml) contained most of the metabolite and about as much diazinon. The residues from these eluates were dissolved in DMSO (3 ml) and applied to eight Florisil–alumina columns (5 g of each). On elution with hexane the early fractions gave samples of III which were almost free from diazinon but still contained some fat; this was removed by the dimethylformamide extraction procedure of de Faubert Maunder *et al.* (1964).

Trimethylsilyl (TMS) ethers of metabolites I and II were prepared by evaporating solutions containing a few micrograms of the metabolites to dryness and treating with a solution (20% v/v) of *N,O*-bis(trimethylsilyl)acetamide (BSA) in chloroform or freshly distilled tetrahydrofuran.

Examination of Metabolites. The main characteristics of the compounds were established by their reactions with esterases (Bunyan, 1964; Mendoza *et al.*, 1968) and 2,6-dichloro-*p*-benzoquinone-4-chloroimine (Braithwaite, 1963) after separation by thin-layer chromatography.

Nuclear magnetic resonance (nmr) spectra were determined for samples dissolved in a deuteriochloroform containing tetramethylsilane as internal standard on a Perkin-Elmer R10 (60 MHz) spectrometer, linked to an NS-544 spectrum-accumulating computer. Up to 300 scans of each spectrum were accumulated. Mass spectra (ms) were determined for samples introduced at 50° on the direct insertion probe into a Hitachi Perkin-Elmer RMU6E spectrometer at 80 eV. Infrared (ir) spectra were recorded for samples in potassium bromide, chloroform, carbon tetrachloride, or carbon disulfide on a Perkin-Elmer 237 spectrophotometer fitted with microsampling equipment.

Determination of Residues. Diazinon and metabolite I were determined in the tissues, blood, and urine of the dosed sheep by the method of Machin and Quick (1969) but using larger volumes of eluant to ensure complete elution. Metabolite II was determined by the same method in the urine only. There was insufficient III available to calibrate the response of the glc detector and it was not determined quantitatively.

RESULTS AND DISCUSSION

Structure of Metabolites. All three metabolites were evidently fully esterified phosphorothionates because they inhibited cholinesterase after, but not before, oxidation with bromine and they were detected as red spots on tlc plates by 2,6-dichloro-*p*-benzoquinone-4-chloroimine. Thionates give red spots and thiolates give yellow ones with this reagent, as found by Stenersen (1971) with the analogous 2,6-dibromo compound.

The tlc R_f values and glc retention times of diazinon (see Figure 1), the three metabolites, and related compounds are given in Table I.

The identification of metabolite I as hydroxydiazinon was confirmed unequivocally by direct spectroscopic examination; its nmr, mass, and infrared spectra are identical with those published for hydroxydiazinon by Pardue *et al.* (1970). In addition, the TMS ether of the compound, prepared in tetrahydrofuran, gave a mass spectrum with a small molecular ion peak at m/e 392 and an intense M − 15 peak at m/e 377.

The spectra of metabolites II and III are shown in Figures 2–4. Their nmr spectra (Figure 2) show the similarities to the spectrum of diazinon (Keith *et al.*, 1968) to be expected if only ring substituents have been metabolized. The diethoxyphosphorus group, still present in both compounds, produces a 6 H triplet a at τ 8.6 (J = 7 Hz) and a 4 H multiplet b at 5.6 (J = 7, 10 Hz). In II (Figure 2, top) there is also a 6 H doublet e (τ 8.65, J = 7 Hz) together with a 1 H multiplet f centered on 6.8, showing that the isopropyl group is still present, whereas in III (Figure 2, bottom) these signals are absent. On the other hand, the

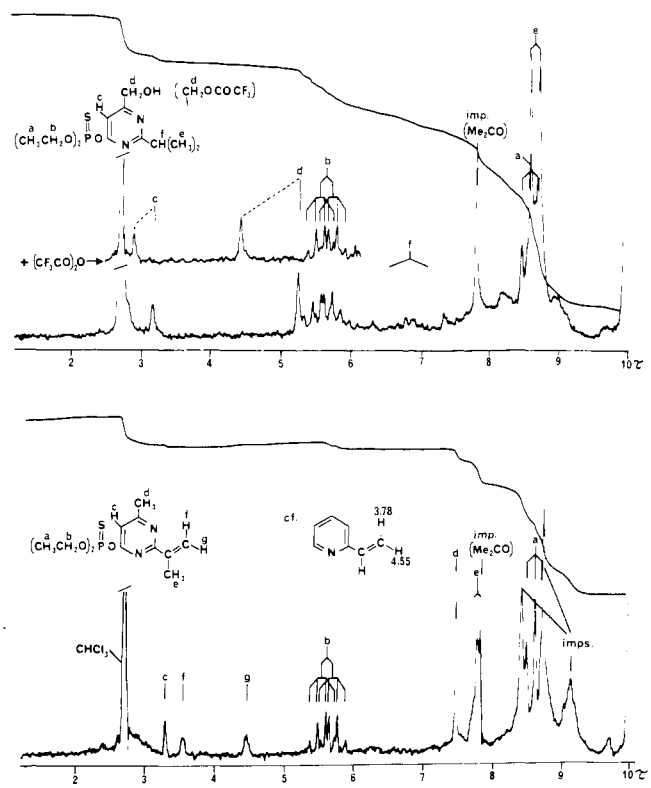

Figure 2. Nuclear magnetic resonance spectra. Metabolite II (272 runs accumulated) (top); Metabolite III (300 runs accumulated) (bottom).

ring methyl (at 7.57 in diazinon) is present in III (the 3 H singlet d at 7.49) but absent in II. These differences show that in II metabolic attack has occurred at the ring methyl and in III at the isopropyl group. The new peak in the spectrum of II, a 2 H singlet at τ 5.26, together with the shift of the pyrimidine ring proton c from 3.32 to 3.20, suggests that the ring methyl has been converted to hydroxymethyl. This interpretation was confirmed by the addition of trifluoroacetic anhydride. The 2 H signal was shifted downfield by 0.85 ppm; trifluoroacetylation of benzyl alcohol caused a corresponding shift of 0.75 ppm for the CH_2O signal.

In metabolite III there are new peaks, e, f, and g at τ 7.79 (3 H), 4.44 (1 H), and 3.56 (1 H), which are interpreted as arising from an isopropenyl group, $-C(CH_3)=CH_2$. The methyl group shows the expected chemical shift (the methyl peak in α-methyl styrene is at 7.88) and broadening due to allylic coupling with the olefinic protons. The unusually large difference in the chemical shifts of the two olefinic signals is similar to that observed in 2-vinylpyridine (Varian NMR Spectra Catalog, 1962; see inset, Figure 2, bottom) but is even more pronounced because of the second heterocyclic nitrogen atom.

The mass spectra of II and III (Figure 3) confirm the structures. In the spectrum of II all the significantly abundant ions above *m/e* 150 have counterparts 16 amu lower in diazinon [although only part of *m/e* 153 in II would correspond to 137 in diazinon, the remainder being $(C_2H_5O)_2PS^+$]. As expected, the compound formed a mono-TMS ether with a molecular ion at *m/e* 392, proving it to contain one hydroxyl group. This derivative formed readily in chloroform, whereas the ether of I formed only slowly, even in the more polar solvent tetrahydrofuran, presumably owing to the greater steric hindrance of its hydroxyl group. In III (Figure 3, bottom) peaks at *m/e* 302 (M^+), 274, 258, 246, 225, 214, 213, 197, 177, 150, and 153, corresponding to peaks 2 amu higher in diazinon, are all consistent with the proposed structure.

Some indirect evidence for a previously postulated fragmentation mechanism in diazinon (Damico, 1966) is provided by the spectra of its metabolites and of one of its irradiation products in which the isopropyl group has been oxidized to acetyl (IV, Machin *et al.*, 1971b). The relative abundances of *m/e* 179 in diazinon and IV, and the corresponding ions in the three metabolites (*m/e* 195 in I and II; *m/e* 177 in III) support Damico's explanation of the ion's formation in diazinon by migration of an ethyl group to the pyrimidinyl oxygen and expulsion of the methine hydrogen from the isopropyl group. Thus, in diazinon and II, which both have an intact isopropyl group, *m/e* 179 or 195 is the base peak; in I, III, and IV, where the methine hydrogen has been lost, the corresponding peaks are much smaller.

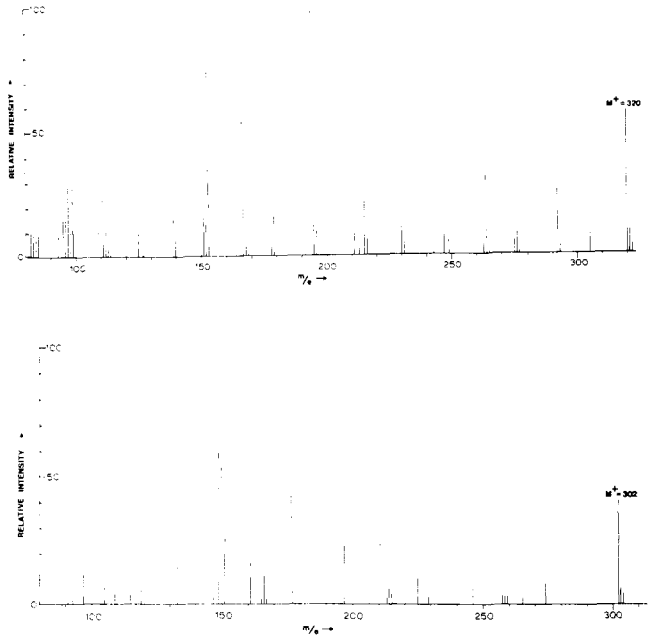

Figure 3. Mass spectra. Metabolite II (top); Metabolite III (bottom).

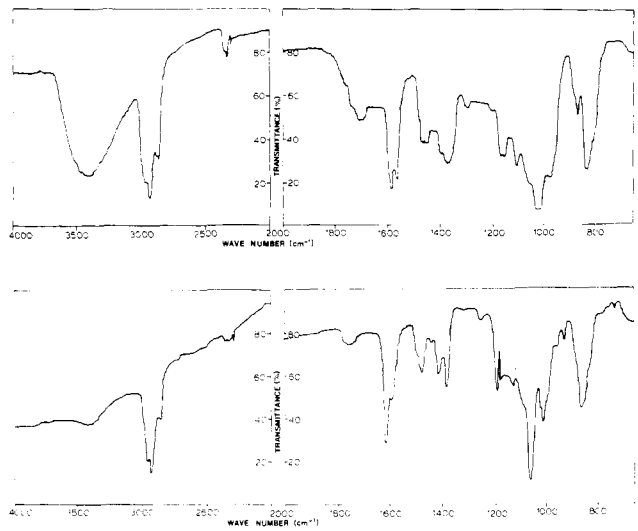

Figure 4. Infrared spectra. Metabolite II (top); Metabolite III (bottom).

The infrared spectra of II and III (Figure 4) show only small changes from the spectrum of diazinon. The most obvious difference is the decrease in the intensity of the methyl stretching bands at *ca.* 2970 and 2860 cm^{-1} compared with the methylene bands at *ca.* 2920 and 2850 cm^{-1}, owing to the absence of the ring methyl group in II and an isopropyl methyl in III.

Miyazaki *et al.* (1970), on the basis of chromatographic comparison with synthetic compounds, proposed the structures I–III for three of the metabolites they detected in mouse urine; they also suggested routes to account for the formation of all the metabolites they reported. The present work, which was completed before the findings of

Table II. Distribution of Diazinon, Hydroxydiazinon (Metabolite I), and Isohydroxydiazinon (Metabolite II) in Sheep Dosed at 1000 mg/kg

Residue in	Time after dosing, hr	Diazinon, ppm	Hydroxydiazinon, ppm	Isohydroxydiazinon, ppm
Blood	3	1.6	1.0	
Blood	6	2.5	1.4	
Blood	24	5.4	1.6	
Blood	30	5.3	1.4	
Blood	48	4.9	1.9	
Liver	48	18	2.1	
Kidney	48	12	0.6	
Brain	48	15	0.7	
Muscle	48	14	0.5	
Fat	48	624	9.2	
Urine	0–2	2.1	0.3	7.2
	2–4	1.1	4.3	38
	4–6	1.4	0.4	6.5
	6–24	2.6	2.6	15
	24–26	2.6	4.4	5.8
	26–30	3.3	6.7	3.2
	30–48	5.0	17	61

Miyazaki *et al.* were known, establishes the identity of the metabolites with certainty and suggests that the metabolic sequence proposed by Miyazaki *et al.* is a common one in mammals.

Residues in Blood, Urine, and Tissues. Table II shows the residues of diazinon and hydroxydiazinon found in the blood, urine, and tissues of the sheep killed after 48 hr.

The proportion of hydroxydiazinon found in the blood was surprisingly high, varying from about 25 to 70% of the diazinon content. It was much lower in the tissues, from about 1.5% in fat to about 12% in the liver, and the proportions of II and III were clearly lower still, although these compounds were not determined quantitatively. The compounds were distributed among the tissues according to their relative polarities. Thus, diazinon and metabolite III were largely concentrated in fat (the concentration of diazinon in the fat was over 100 times its concentration in the blood). The more polar hydroxydiazinon was more evenly distributed, with only about five times as much in the fat as in the blood, and the most polar of the compounds, metabolite II, was most prominent in the urine.

There is little information about the toxicity of these metabolites to mammals. Miyazaki *et al.* (1970) determined the oral toxicity to mice of several of the compounds they detected but mentioned only the value for hydroxydiazinon (120 mg/kg). This is close to the figure (82 mg/kg) quoted by Martin (1968) for the toxicity of diazinon. On the other hand, Sawicki (1971) found that hydroxydiazinon was considerably more toxic than diazinon to a susceptible strain of houseflies (the LD_{50}'s were, respectively, 0.12 and 0.30 μg per female fly when topically applied in acetone as 1-μl drops). Evidently the toxicity of these cholinesterase-inhibiting metabolites to other species cannot be inferred from the available data and the possible hazards of these residues cannot yet be assessed.

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Distribution of Picloram Residues in Sugarcane

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[^{14}C]Picloram absorbed through the roots of sugarcane plants (a hybrid of *Saccharum* spp.) accumulated in the leaves. Residues retranslocated from older to younger leaves without detectable radioactivity in either the apical meristem of the primary stalk or the stalk. With the depletion of the supply to the roots over a period of 13 weeks, the leaf residues reached a maximum and began to decline, with a portion of the radioactivity lost from the plant in the senescent abscised leaves. Picloram had a constant linear inhibiting effect on the elongation of leaf tissue from the primary spindle.

Two patterns of residue distribution in sugarcane from the root absorption of labeled herbicides have been described previously (Hilton *et al.*, 1970). The contact herbicide, pentachlorophenol (PCP), adsorbed strongly on the root surfaces with no ^{14}C appearing in the leaves and with only 5% of the recoverable activity in the stalk at the end of 8 weeks. In contrast, ^{14}C-containing residues from the ring-labeled *s*-triazine herbicides, atrazine and ametryne, moved freely upward through the stalk to accumulate in the leaves. The roots retained 25 to 35% of the recovered radioactivity; stalk residues were low, about 3 to 4% for atrazine and 6 to 7.5% for ametryne over a 13-week absorption period. Movement of radioactive products to the leaves appeared to take place through the xylem system with transpired water. The regular senescence and abscission from the stalk of the older lower leaves resulted in the removal of the major part of the residue deposits.

Plants treated with PCP and the ring-labeled *s*-triazine herbicides lost substantial amounts of total radioactivity with time, even when the triazine residues from the abscised leaves were added to those remaining in the growing plant. The loss of PCP was attributed to its volatility; sugarcane appears to be able to break the *s*-triazine ring quite readily with the production of volatile compounds from the roots and leaves (Goswami, 1972).

In this study we have used carboxyl-labeled picloram as an example of a herbicide known to be xylem- and

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