Metabolism of Isoxathion, O,O-Diethyl O-(5-Phenyl-3-isoxazolyl)phosphorothionate in Plants

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The absorption and metabolism of the insecticide, Isoxathion, on bean, cabbage and chinese cabbage plants were examined using carbon-14 labeled compound. Isoxathion penetrated into plant tissues was hydrolyzed to produce 3-hydroxy-5-phenylisoxazole, which was then rapidly converted to water soluble compounds. Among them, 3-(β-D-glucopyranosyl)-5-phenylisoxazole, 2-(β-D-glucopyranosyl)-5-phenyl-4-isoxazolin-3-one and 2-(β-D-glucopyranosyl)-5-p-hydroxy-phenyl-4-isoxazolin-3-one were unequivocally identified as the major metabolites. Another metabolic pathway of 3-hydroxy-5-phenylisoxazole via a reductive cleavage of the isoxazole ring to form benzoic acid was negligible.

Isoxathion, Karphos®️, O, O-diethyl O-(5-phenyl-3-isoxazolyl)-phosphorothionate, is a registered insecticide of a broad spectrum. The toxicity of the compound is as follows: acute (oral) LD₅₀ to rats is 112 mg/kg and non-effect level to rats in 90 days consecutive feeding test is approximately 1 mg/kg/day. Regarding the agricultural chemicals having isoxazole nucleus, fate of hymexazol (3-hydroxy-5-methylisoxazole) in rats, higher plants, soils, photolysis, and of isoxathion in rats, soils has been reported. Hymexazol treated in plants was rapidly absorbed and transformed to 2-(β-D-glucopyranosyl)-5-methyl-4-isoxazolin-3-one and 3-(β-D-glucopyranosyl)-5-methylisoxazole.

In the present paper, isoxathion labeled with carbon-14 was treated in plants to determine the absorption and metabolic fate of the compound.

MATERIALS AND METHODS

Chemicals. Radioactive O,O-diethyl O-(5-phenyl-3-isoxazolyl)-phosphorothionate-5⁻¹⁴C and 3-hydroxy-5-phenylisoxazole-5⁻¹⁴C were synthesized and purified as described elsewhere. The specific activity of each compound was 1.00 mCi/mM, and no radioactive impurity was detected by thin layer chromatography.

3-(2',3',4',6'-Tetra-O-acetyl-β-D-glucopyranosyloxy)-5-phenylisoxazole (I), 2-(2',3',4',6'-tetra-O-acetyl-β-D-glucopyranosyl)-5-phenyl-4-isoxazolin-3-one (II), 3-(β-D-glucopyranosyl)-5-phenyl-4-isoxazolin-3-one (III) and 2-(β-D-glucopyranosyl)-5-phenyl-4-isoxazolin-3-one (IV) were synthesized as follows: a mixture of 60 g (ca. 0.15 M) of 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl bromide, 24.2 g (0.15 M) of 3-hydroxy-5-phenylisoxazole, 16 g (ca. 0.15 M) of sodium carbonate, 60 g of Drierite and 500 ml of anhyd. acetonitrile was refluxed with stirring for 8 hr with exclusion of moisture. The mixture was cooled and filtered to remove inorganic materials. Solvent was evaporated, and remaining syrup was dissolved in 500 ml of benzene. The solution was washed with aq. saturated sodium bicarbonate and then with water, and dried over anhyd. sodium sulfate. The solvent was removed under vacuum, and a syrupy residue was chromatographed on a column of silica gel with ethyl acetate-ν-hexane (2:3 and 4:1, v/v). Eluates were monitored by thin layer chromatography of silica gel, and spots visualized under UV light. Evaporation of an earlier eluate gave 15.0 g of (I). It was recrystallized from acetone to afford colorless needles melting at 158°C. [α]D° -10.5° (c=1.00, chloroform). Anal. Found: C, 56.36; H, 5.18; N, 2.57. Calcd. for C₁₅H₁₇NO₄: C, 56.21; H, 5.13; N, 2.85%. IR νmax cm⁻¹: 3150 (C=CH), 1750 (OCOCH₃), 1625 (C=C), 1510 (C=N). The latter eluate gave 3.1 g of (II). It was recrystallized from n-propanol to afford colorless needles melting at 75°C. [α]D° -48.1° (c=1.00, chloroform). Anal. Found: C, 56.50; H, 5.24; N, 2.76%. IR νmax cm⁻¹: 3120 (C=CH), 1750 (OCOCH₃), 1700 (C=O), 1625 (C=C). To a solution of 7.5 g of (I) in 60 ml of methanol was added 60 mg of sodium methoxide. The mixture was stirred at room temperature...
for 6 hr, and then treated with Dowex 50W-X8 (H+ form) to neutralize the solution. After removal of the resin by filtration, methanol was evaporated to dryness, giving 4.6 g of crude (III). Recrystallization from ethanol afforded 4.0 g of (III) as colorless needles melting at 185~186°C. [α]D20 −27.9° (c = 0.70, H2O). Anal. Found: C, 55.62; H, 5.30; N, 4.20%. IR ν max cm⁻¹: 3450 (OH), 1630 (C=O), 1520 (C=C), 1465 (C-O). NMR δ (60 MHz, d6-DMSO): 7.95~7.45 ppm (m, aromatic), 5.13 (1H, d, J = 9.0 Hz, anomeric). 

O-Desethyl-oxathion (1.6 μCi) in 0.03% Tween 20 was applied to the surface of leaves of bean, cabbage and chinese cabbage. After exposure, treated plants were washed thoroughly in running acetone to remove radioactivity not absorbed. Each tissue was homogenized with glass homogenizer in dry-ice acetone, then filtered through a glass filter. The filtrate was concentrated at 40~45°C in vacuo. The aqueous concentrate was transferred to a separatory funnel and chloroform was added. After shaking for 10 min, the layers were separated and made up to volume for radioassay. Sediments on the glass filter were dried, then a portion from each sample was burned in an Automatic Sample Oxidizer (Packard) and radioassayed. On water culture experiments, bean plants were transferred to jars containing Knop’s solution. One day was allowed for adaptation to the new conditions, then treatment was started by transfer of each plant to 5 ml of aqueous solution containing 1.6 μCi of isoxathion in 0.03% Tween 20. When the solution had been almost absorbed, the nutrient solution was added. The individual plant was sampled 1, 3, 5 and 7 days after the initiation of treatment. Radioactivity in shoots and roots was separately assayed after homogenization and extraction by the same manner as described above. For autoradiography of whole bean plants, the plants were sampled periodically, separately dried and then subjected to autoradiography with Sakura Type N X-ray film for constant 7 days.

**Separation of isoxathion and its metabolites.** Thin layer chromatography with silica gel GF254 (Merck, thickness 0.25 mm) was conducted to separate the radioactive metabolites. Solvent systems used were (A) benzene-acetic acid (8:1, v/v) (B) n-hexane-ethyl acetate-formic acid (70:30:1, v/v/v) (C) benzene-ethanol-water (6:5:1, v/v/v) (D) n-propanol-conc. ammonium hydroxide-acetone (60:20:3, v/v/v) (E) chloroform-methanol-conc. ammonium hydroxide (4:4:1, v/v/v) (F) n-butanol-acetic acid-water (8:1:1, v/v/v) (G) ethyl acetate-methylethylketone-formic acid-water (5:3:1:1, v/v/v/v). Locations of the radioactive metabolites were detected by autoradiography with Sakura Type N X-ray film. Radioactivity in each spot was determined by scraping the corresponding silica gel area from the plates.

**Hydrolyses of three metabolites (M1, M2 and M3).** Radiochemically isolated M1 (or M2, M3) was hydrolyzed for 1 hr on a steam bath with 1 n hydrochloric acid or 1 n sodium hydroxide. Enzymatic hydrolyses with β-glucosidase (Sigma Chemical Co.), in 0.1 m acetate buffer (pH 5.0), were also carried out at 37°C for 4 hr.

**Isolation of unknown metabolites.** Isolation and identification of the metabolites were conducted with the shoots of bean plants. The upper shoots were dipped in the nutrient solution containing 500 ppm of unlabeled 3-hydroxy-5-phenylisoxazole. After 7 days the shoots were collected and homogenized in dry ice acetone with glass homogenizer, then filtered...
through a glass filter. The filtrate was concentrated at 40-45°C in vacuo. The aqueous concentrate was extracted with chloroform to remove soluble materials. The aqueous layer was lyophilized and the residue was column chromatographed (3 x 40 cm) with silica gel using benzene, benzene-methanol (8: 2, 7: 3, 1: 1, v/v) and methanol as the solvent systems. A small portion from each fraction was chromatographed on thin-layer chromatography with standards for M1 and M2, and spots were visualized under UV light. For M3 and M4, radiochemically isolated ones were used for standards, and spots visualized under UV light were compared with those of radioactivity detected by autoradiography. An earlier eluate (M1 and M2) was evaporated to remove solvent. Silica gel column chromatography (2 x 30 cm) eluted with methylethylketone as the solvent was successively performed for separation of M1 and M2. A latter eluate (M3 and M4) was evaporated to remove solvent and the residue was again column chromatographed with silica gel using ethyl formate, and finally purified by thin-layer chromatography with solvent systems (C) and (G).

Acetylations of M1, M2 and M3. Twenty mg of M1 (or M2, M3) was acetylated in 2 ml of acetic anhydride-pyridine (1: 3, v/v) for 24 hr at room temperature. The reaction mixture was transferred to a separatory funnel and 20 ml each of water and ethyl acetate was added. After shaking for 5 min, the ethyl acetate layer was separated and dried up.

RESULTS AND DISCUSSION

Absorption and translocation

As indicated in Fig. 1, time course experiments showed that isoxathion applied on leaves was absorbed by all examined species. Radioactivity deposited on the surface gradually decreased with time, while radioactivity in chloroform soluble, water soluble fractions and unextractable residue material increased. These results showed that isoxathion deposited on leaf surface was absorbed gradually to plant tissues, then metabolized to water soluble compounds, and finally in part to bound materials.

The uptake of isoxathion via the roots of the intact bean seedlings appeared to be very slow as indicated by the autoradiograms (Fig. 2). Radioactivity absorbed from the roots did not localize to the apical meristem region (top leaves) in the plant, but distributed uniformly. Also as indicated in Fig. 3, radioactive materi-
chromatogram is reproduced in Fig. 4. The major metabolites of isoxathion in bean plants were the same as those in cabbage and Chinese cabbage plants. Relative contents of the metabolites of isoxathion in water soluble fraction of bean plants are shown in Table I. Water soluble extracts of bean plants treated with 3-hydroxy-5-phenylisoxazole in nutrient solution also yielded the same metabolites, al-
though the amounts varied. Therefore, the isolation of the metabolites was conducted with stem treatment of 3-hydroxy-5-phenylisoxazole. The major metabolites (M1, M2 and M3) were isolated and identified, whereas attempts to determine structures for others were unsuccessful mainly because they were obtained in small amounts.

M1 produced 3-hydroxy-5-phenylisoxazole readily upon hydrolysis in 1 N hydrochloric acid or 1 N sodium hydroxide and β-glucosidase, and ninhydrin reaction on this metabolite was negative. Co-chromatographic identity of M1 with an authentic sample supported that the structure of M1 is 3-(β-D-glucopyranosyl)-5-phenylisoxazole. IR spectrum of M1 and NMR and IR spectra of acetylated M1 were all identical with those of synthesized (III) and (I).

M2 produced only a trace of 3-hydroxy-5-phenylisoxazole upon hydrolysis in 1 N hydrochloric acid or 1 N sodium hydroxide, or β-glucosidase, and ninhydrin reaction on this metabolite was negative. Co-chromatographic identity of M2 with an authentic sample supported that the structure of M2 is 2-(β-D-glucopyranosyl)-5-phenyl-4-isoxazolin-3-one. NMR and IR spectra of the purified M2 and the acetylated M2 were identical with those of synthesized (IV) and (II).

M3 produced a trace of an unknown compound (presumably 3-hydroxy-5-(p-hydroxyphenyl) isoxazole) upon hydrolysis in 1 N hydrochloric acid or 1 N sodium hydroxide. It remained unaffected with β-glucosidase. Spot test with p-nitrobenzenediazonium fluoroborate of M3 was positive, but ninhydrin reaction was negative. IR spectrum of M3 was almost identical with that of an authentic 2-(β-D-glucopyranosyl)-5-phenyl-4-isoxazolin-3-one (Fig. 5), indicating that the former is a derivative of the latter. In NMR spectrum (60 MHz) of M3 (Fig. 6), an A₂B₂ signal at 7.48 ppm (2H, J=9 Hz) and 6.88 ppm (2H, J=9 Hz) was clear enough to show the presence of p-substituted phenyl group. Also the NMR spectrum showed a single at 6.03 ppm (1H, C=CH (4)) and a doublet at 5.26 ppm (1H, J=8.5 Hz, anomeric), indicating the presence of the isoxazole ring and a sugar conjugate with β-configuration. NMR spectrum

![FIG. 5. Infrared Spectra of N-G and M3.](image)

N-G: 2-(β-D-glucopyranosyl)-5-phenyl-4-isoxazolin-3-one.
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FIG. 6. Nuclear Magnetic Resonance Spectrum of M3 (solvent CD$_3$OD, 60 MHz).

(100 MHz) of acetylated M3 revealed a single acetyl group at 2.35 ppm, in addition to four equivalent ones at 2.00 ppm due to substitution on a sugar moiety. In view of the positive result of M3 on the spot test for phenolic derivatives, the former acetyl group was apparently a substituent on the phenyl group. From these results, M3 was assigned as 2-(β-D-glucopyranosyl)-5-p-hydroxyphenyl-4-isoxazolin-3-one.

M4 was readily hydrolyzed to afford 3-hydroxy-5-phenylisoxazole with 1 N hydrochloric acid or 1 N sodium hydroxide or β-glucosidase. M4 may be therefore a glycoside of 3-hydroxy-5-phenylisoxazole. Desethyl isoxathion, desethyl-oxon and oxon were not detected. 3-Hydroxy-5-phenylisoxazole, the hydrolytic compound of isoxathion, were barely detectable, presumably because of its rapid glucosidation.

Previous studies on the metabolism in rats, and in soils have shown that 3-hydroxy-5-phenylisoxazole partially undergoes a reductive cleavage of the isoxazole ring to benzoic acid via the formation of benzyolacetamide. In the present study, when the water soluble materials of cabbage and chinese cabbage plants were hydrolyzed with 6 N hydrochloric acid followed by extraction with ether, small amounts of radioactive benzoic acid was detected in the ether extract along with 3-hydroxy-5-phenylisoxazole and an unidentified compound (presumably 3-hydroxy-5-(p-hydroxyphenyl) isoxazole). This suggests the presence of the same metabolic pathway of 3-hydroxy-5-phenylisoxazole in higher plants as in rats and soil microbes. However, the expected conjugates of benzoic acid such as

FIG. 7. Proposed Metabolic Pathways of Ioxathion in Plants.
1-O-benzoyl-β-D-glucopyranose\textsuperscript{14} and benzoyl-aspartic acid\textsuperscript{15} could not be identified owing to the low level of radioactivity.

The metabolism of isoxathion was characterized by rapid cleavage of the phosphoric acid linkage to afford 3-hydroxy-5-phenylisoxazole as an initial product. The isoxazole then underwent the rapid glucosidation to form 3-(β-D-glucopyranosyloxy)-5-phenylisoxazole and 2-(β-D-glucopyranosyl)-5-phenyl-4-isoxazolin-3-one. Apparently, the latter is readily oxidized to the principal metabolite, 2-(β-D-glucopyranosyl)-5-p-hydroxyphenyl-4-isoxazolin-3-one. The hydroxylation of the former glucoside may also occur among unidentified minor products. Another metabolic pathway of 3-hydroxy-5-phenylisoxazole via the formation of benzoic acid to its conjugates was negligible, and the isoxazole skeleton was quite stable in these plants.

Summarizing the above results, the metabolic pathways of isoxathion are proposed in Fig. 7.

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