JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Comparative Metabolism and Pharmacokinetics of Seven Neonicotinoid Insecticides in Spinach

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The metabolism of seven commercial neonicotinoid insecticides was compared in spinach seedlings (*Spinacia oleracea*) using HPLC-DAD and LC-MSD to analyze the large number and great variety of metabolites. The parent neonicotinoid levels in the foliage following hydroponic treatment varied from differences in uptake and persistence. The metabolic reactions included nitro reduction, cyano hydrolysis, demethylation, sulfoxidation, imidazolidine and thiazolidine hydroxylation and olefin formation, oxadiazine hydroxylation and ring opening, and chloropyridinyl dechlorination. The identified phase I plant metabolites were generally the same as those in mammals, but the phase II metabolites differed in the conjugating moieties. Novel plant metabolites were various neonicotinoid-derived *O*- and *N*-glucosides and -gentiobiosides and nine amino acid conjugates of chloropyridinylcarboxylic acid. Metabolites known to be active on nicotinic acetylcholine receptors included the desnitro- and descyanoguanidines and olefin derivatives. The findings highlight both metabolites common to several neonicotinoids and those that are compound specific.

KEYWORDS: Clothiandin; comparative metabolism; imidacloprid; LC-MSD; neonicotinoid insecticide; neonicotinoid pharmacokinetics; plant metabolism; thiamethoxam

INTRODUCTION

Seven neonicotinoids (**Figure 1**) account for about 17% of the world insecticide market value. They are used primarily as systemics undergoing absorption and translocation for protection of crops (*1*−*5*), including spinach (*6*−*10*). Plant metabolism is a major factor governing their uptake, persistence, and effectiveness. Identification of the metabolites is not only of academic interest but is also an important aspect of registration for use and assignment of tolerance values. The metabolism of the seven commercial neonicotinoids has been determined in mice (*11, 12*). Similar studies with spinach would allow a better understanding of structure−biodegradability relationships in plants versus mammals. This study compares the fate of the seven neonicotinoids in spinach using LC-MSD and authentic standards for structural assignment and HPLC-DAD for quantitation.

MATERIALS AND METHODS

Chemicals. Sources were described earlier for the chloropyridinyl (*11*) and chlorothiazolyl and tetrahydrofuranyl (*12*) derivatives.

Treatment of Plants. Spinach seedlings (Bloomsdale variety, from Berkeley Horticultural Nursery, Berkeley, CA) in 2 × 2 in. plastic boxes containing commercial soil mix of 2.5 in. depth were grown in a greenhouse under long-day conditions (16 h of light /8 h of darkness) at 20 °C. The seedlings were not treated with any pesticide except the experimental neonicotinoid. The six plants in each box were watered on a daily basis until the desired height (4 in.) was achieved, following which the soil was removed by gentle rinsing of the roots with distilled water. The test compounds were administered hydroponically with 50 mL of 100 ppm neonicotinoid made in quarter-strength modified Hoagland's solution for nutritional support [mmol/L: 0.4 $NH_4H_2PO_4$, 2.4 KNO_3, 1.6 $Ca(NO_3)_2$, 0.8 $MgSO_4$, 0.1 Fe as Fe-chelate, 0.023 B as $B(OH)_3$ [boric acid], 0.0045 Mn as $MnCl_2$, 0.0003 Cu as $CuCl_2$, 0.0015 Zn as $ZnCl_2$, 0.0001 Mo as MoO_3 or $(NH_4)_6Mo_7O_{24}$ and Cl as chlorides of Mn, Zn, and Cu]. Care was taken to minimize neonicotinoid contamination of the leaves. For compound loading experiments, 5 plants per treatment were grown for 13 days in 0 ppm (control) or 100 ppm of neonicotinoid, replenishing with the same solutions as required (up to 30 mL overall). For dissipation experiments, 5 plants were grown, as above, in neonicotinoid solution for 3 days (loading phase) and then transferred to the modified Hoagland's solution, containing no neonicotinoid, for an additional 10 days. In a supplemental experiment, 50 ppm of chloropyridinylcarboxylic acid (CPCA) was coadministered for 3 days with individual amino acids at 50 ppm to test for possible conjugate formation.

Extraction of Leaves. Whole leaves (1 g fresh weight) were either analyzed fresh or held for up to 3 days at −80 °C. The procedure for extraction of mouse tissue (*11, 12*) was adapted for spinach. Leaves were ground in a prechilled (−20 °C) mortar and pestle containing 5 mL of prechilled HPLC-grade acetonitrile, 1.5 g of white quartz sand (both from Sigma-Aldrich, St. Louis, MO), and the internal standard (IS). THI was used as the IS for the other six neonicotinoids, and DIN was used for THI-treated spinach. The leaves were ground for up to 3 min to ensure complete tissue disintegration. The resulting mixture was placed in a 50 mL plastic tube, vortexed (high setting) for 3 min, and

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10.1021/jf8020909 CCC: $40.75
Published on Web 10/16/2008

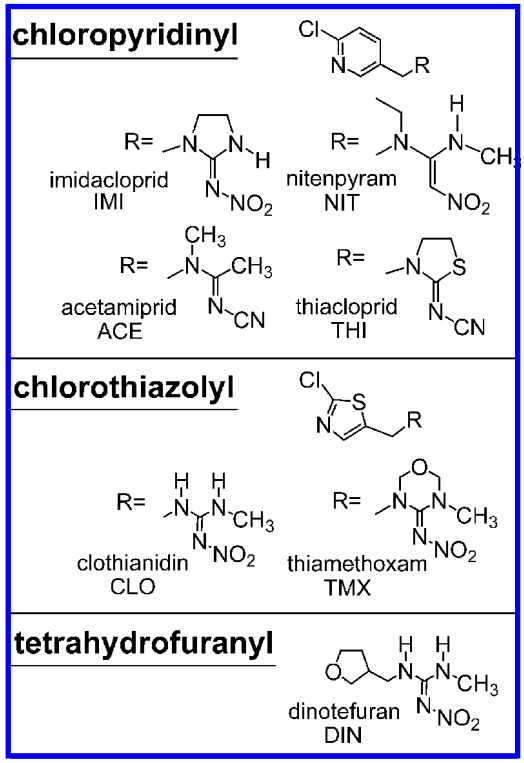

Figure 1. Neonicotinoid insecticides of three types showing names and abbreviations.

sonicated for 20 min at 20 °C. The tube was then centrifuged at 2000*g* for 15 min at 4 °C. The supernatant was placed in a glass tube on ice. The pellet was resuspended in 3 mL of acetonitrile, vortexed, sonicated, and centrifuged in the same way. The resulting supernatant was combined with the first supernatant, and the tube was placed in a Savant SVC 200 H Centrifugal Evaporator (Farmingdale, NY) for up to 3 h until dryness. The residue was dissolved in 200 μL of acetonitrile/water/trifluoroacetic acid (75:25:0.1) (all of HPLC grade) and filtered through a nylon membrane (0.45 μm) (Pall Life Sciences, East Hills, NY).

Analysis of Neonicotinoids and Metabolites. HPLC-DAD and LC-MSD conditions using trifluoroacetic acid and formic acid, respectively, were the same as in our previous studies (*11, 12*). All retention times (t_R) in this paper are for the LC-MSD conditions. Metabolites of the parent neonicotinoids were quantified as parts per million equivalents (ppm equiv) based on the absorbances at 254 nm and recovery values of the parent compounds (*11, 12*).

Table 1. New Neonicotinoid Plant Metabolites

precursors and metabolites[a]	MW	LC-MSD t_R (min)
IMI-5-OH and IMI-urea		
IMI-5-OH-gluc	433.8	14.8
IMI-urea-gluc	373.8	17.6
IMI-urea-gent	535.9	25.1
NIT		
NIT-dm-de	228.6	19.6
NIT-desCN[b]	211.7	10.8
ACE		
ACE-NH	197.7	2.6
ACE-$NCONH_2$	240.7	8.2
DIN		
THFOL[b]	278.3	15.3
CPCA[c]		
CPCA-Me[b]	171.6	10.7
6-HS-PCA[b]	155.2	3.9
pyridone[b]	153.1	10.3
amino acid conjugates of CPCA[d]		
CPCA-Ala	228.7	19.6
CPCA-Asn	271.7	21.5
CPCA-Asp	272.7	5.3
CPCA-Cys	260.8	14.8
CPCA-Leu	270.8	25.1
CPCA-Phe	304.8	21.1
CPCA-Pro	254.7	19.2
CPCA-Thr	258.7	26.4
CPCA-Val	256.8	22.7
6-HO-PCA-Asp	254.2	3.2

[a] For structures see the corresponding figures. [b] Identical to authentic standards from the indicated sources: THFOL, CPCA-Me, 6-HS-PCA, and pyridone from Sigma-Aldrich (St. Louis, MO); NIT-desCN from ref *11*. [c] CPCA-*N*-oxide (prepared by treating 10 mg of CPCA with 100 μL of 30% hydrogen peroxide in trifluoroacetic acid (750 μL) for 18 h at 20 °C (MW 173.6 and t_R 9.7 min) was not observed as a CPCA metabolite. [d] No amino acid conjugates were observed for CPCA with the following amino acids: Arg, Gln, Glu, Gly, His, Iso, Lys, Met, Ser, Trp, and Tyr.

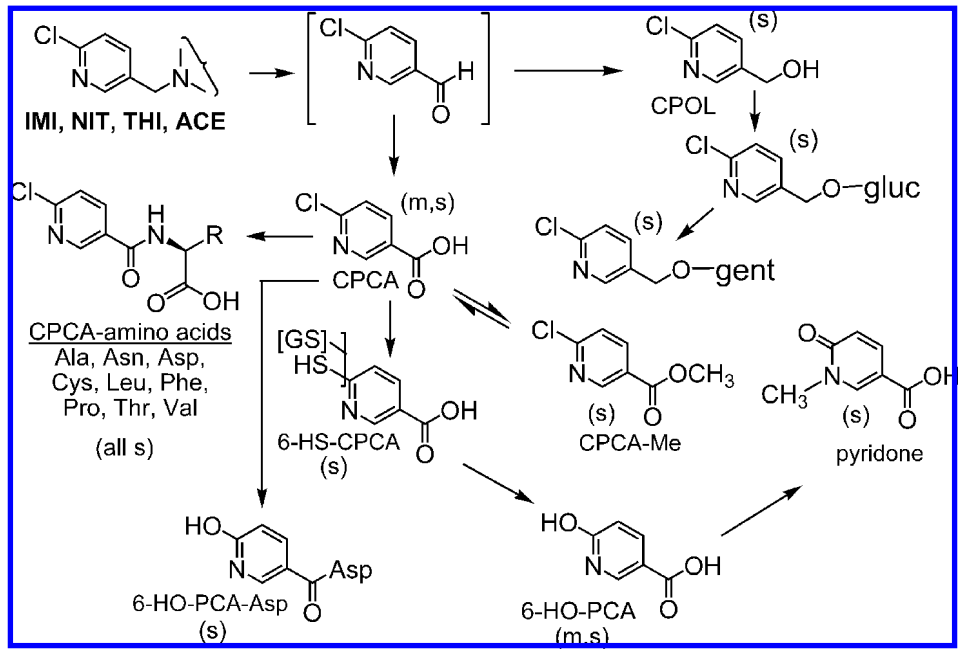

Figure 2. Partial metabolic pathways for CPCA in spinach (s) (this study) and metabolites in mice (m) (*11*). CPCA−amino acids refers to conjugates of CPCA with each of the nine indicated amino acids.

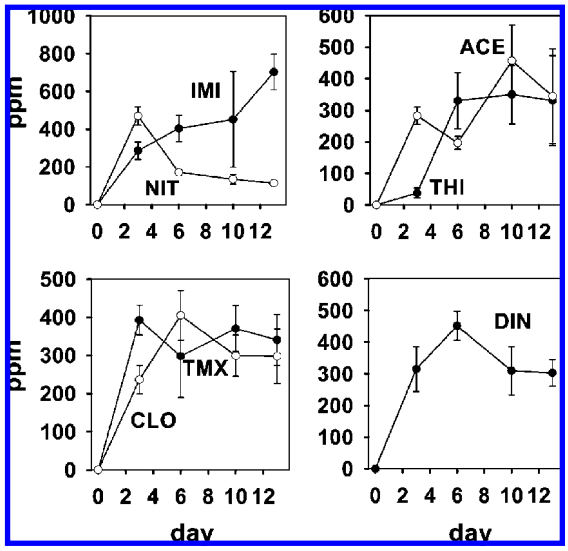

Figure 3. Uptake and persistence of seven neonicotinoids in spinach.

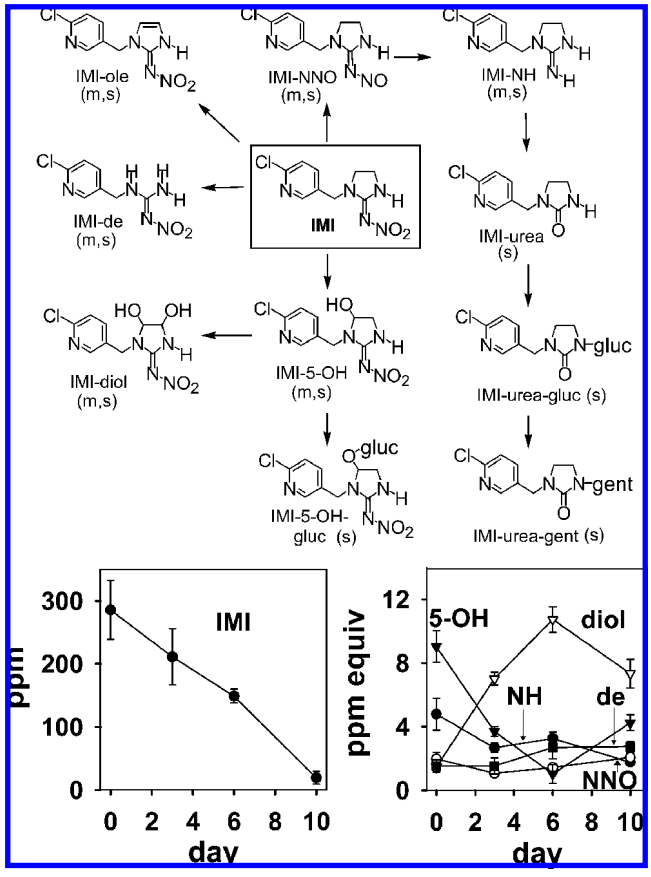

Figure 4. Partial metabolic pathways and pharmacokinetics for IMI in spinach (s) (this study) and metabolites in mice (m) (*11*).

RESULTS

Chromatography and Structural Assignments. The HPLC-DAD system for parent neonicotinoids and metabolites absorbing at 254 nm and the LC-MSD system for additional metabolites were the same as used before with reconfirmation here to give the same t_R values previously reported (*11, 12*). Metabolites were recognized by new peaks (t_R, [M^+] and $^{35}Cl/^{37}Cl$ ratio) in treated compared with control samples. Criteria for metabolite identification or tentative assignment of structure including comparison with authentic standards were as given earlier (*11, 12*) except for proposed new metabolites listed in

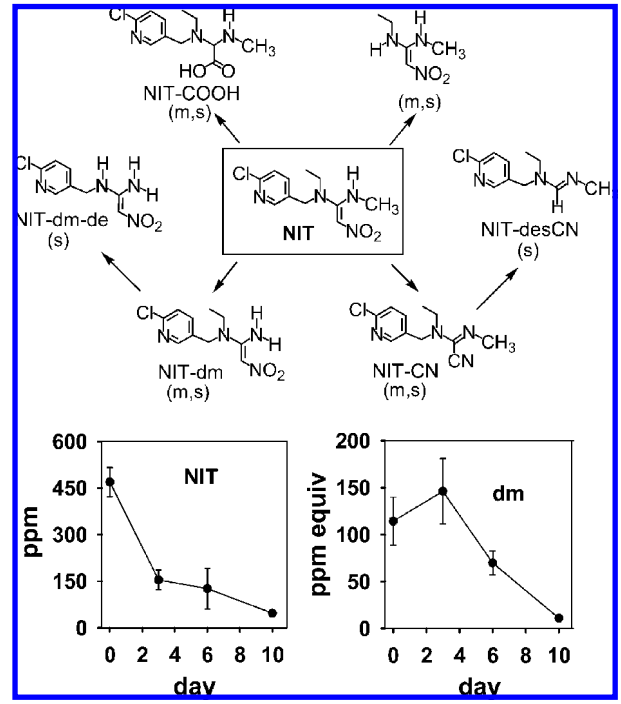

Figure 5. Partial metabolic pathways and pharmacokinetics for NIT in spinach (s) (this study) and metabolites in mice (m) (*11*).

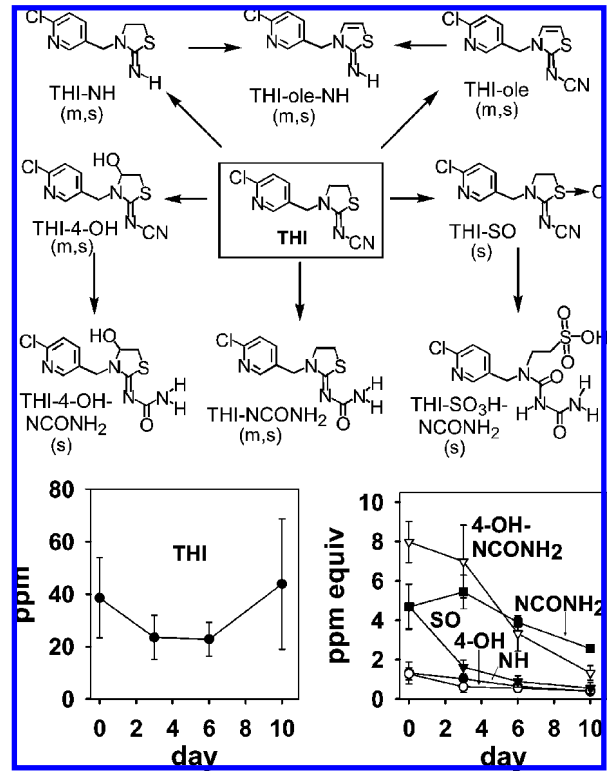

Figure 6. Partial metabolic pathways and pharmacokinetics for THI in spinach (s) (this study) and metabolites in mice (m) (*11*).

Table 1 along with their MW, t_R, precursor, and basis for structural assignment. Unidentified metabolites are noted in Table 1 of the Supporting Information.

Metabolism of CPCA (Figure 2). IMI, NIT, THI, and ACE on hydroxylation at the *N*-methylene substituent were cleaved to the carboxaldehyde (not observed), which was reduced to chloropyridinylmethanol (CPOL) or oxidized to CPCA. CPOL in turn was conjugated with glucose or gentiobiose [6-*O*-*β*-D-

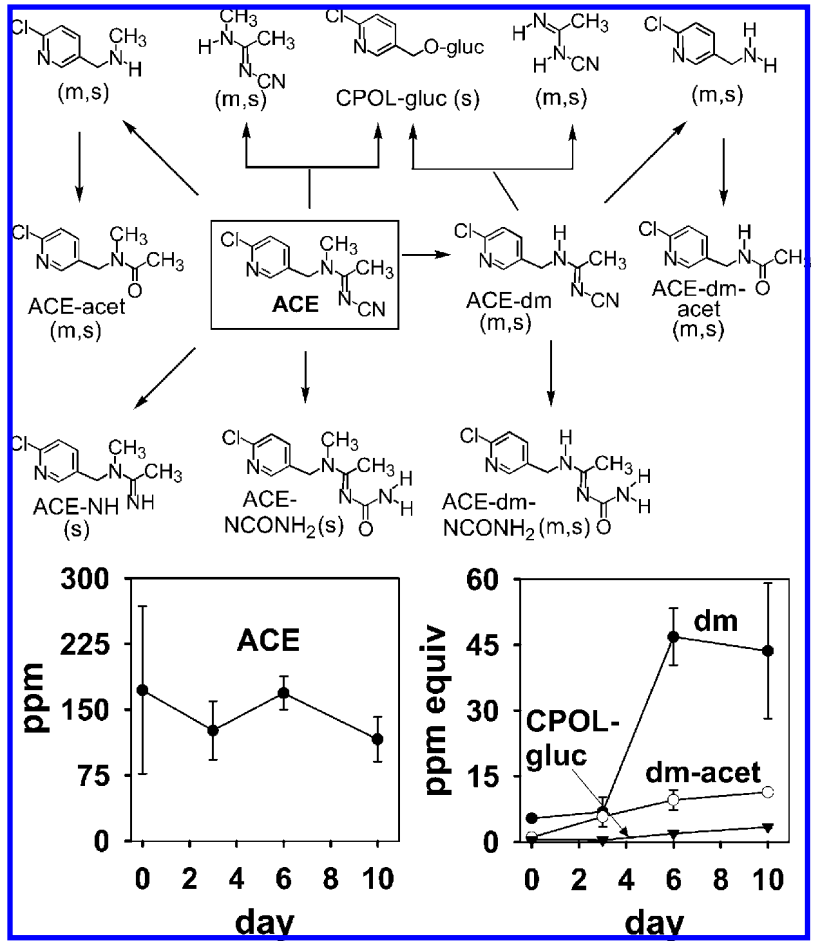

Figure 7. Partial metabolic pathways and pharmacokinetics for ACE in spinach (s) (this study) and metabolites in mice (m) (*11*).

glucopyranosyl-D-glucose]. Each of the chloropyridinyl neonicotinoids gave CPCA and CPOL-gluc with higher levels from ACE than the other compounds. CPCA underwent three types of reactions: conversion to the glutathione (GSH) conjugate (not observed) with cleavage to 6-mercaptopyridinylcarboxylic acid (6-HS-PCA); conjugation with nine different amino acids but not as CPCA-gluc; or methylation to the methyl ester. 6-HS-PCA was oxidized to 6-hydroxypyridinylcarboxylic acid (6-HO-PCA), which was converted by N-methylation and oxidation to the pyridone, 1,6-dihydro-1-methyl-6-oxo-3-pyridinylcarboxylic acid. Each amino acid conjugate of CPCA was validated by enhanced formation on coadministering the specific amino acid. 6-HO-PCA-Asp was observed from treatment with both CPCA and aspartate.

Uptake and Persistence (Figure 3). Neonicotinoid uptake from 100 ppm solutions was studied over a period of 13 days. IMI levels steadily increased to day 10, whereas NIT levels declined after 3 days. Although ACE levels initially increased more quickly than THI levels, they were essentially the same at 6, 10, and 13 days. The chlorothiazolyl compounds (TMX and CLO) gave essentially the same uptake curves, which were similar to that of the tetrahydrofuranyl compound, DIN. In a parallel experiment to the uptake study, the persistence of individual neonicotinoids was followed after loading for 3 days by removal of the source for residue dissipation and analyses at 0, 3, 6, and 10 days thereafter, with the results given below.

Metabolism and Pharmacokinetics of IMI (Figure 4). The residue dissipation curve showed a steady loss with a half-life of about 6 days. Five sites of metabolic attack were observed. Initial nitro reduction yielded sequentially the nitrosoguanidine (IMI-NNO), guanidine (IMI-NH), urea, urea-gluc, and urea-gent derivatives as minor metabolites. IMI-5-OH was present early at high amounts with some IMI-5-OH-gluc but declined as IMI-diol increased, implying the sequence IMI → IMI-5-OH → IMI-diol as an important pathway. Minor products arose from desaturation (IMI-ole) and opening or dethylenation of the imidazolidine ring (IMI-de).

Metabolism and Pharmacokinetics of NIT (Figure 5). NIT was the least persistent neonicotinoid examined, possibly related, as for its photoinstability (*13*), to its nitromethylene (=$CHNO_2$) structure compared to the nitroimine (=NNO_2) or cyanoamidine (=NCN) substituent of the other compounds. The principal pathway was demethylation to NIT-dm, which then was deethylated in small part to NIT-dm-de; no initial deethylation was apparent. The nitromethylene substituent was converted to the cyano (NIT-CN) and ultimately the descyano derivative (NIT-desCN) or oxidized to the carboxylic acid (NIT-COOH). The nitromethylene-containing fragment liberated on chloropyridinylmethyl cleavage was also observed.

Metabolism and Pharmacokinetics of THI (Figure 6). THI was present at relatively low levels for the 10 day period. The conversions of THI involved five different initial sites: cyano hydrolysis to THI-$NCONH_2$; sulfoxidation to THI-SO, which underwent ring opening with further S-oxidation and cyano hydrolysis to THI-SO_3H-$NCONH_2$; hydroxylation at the 4-position to THI-4-OH, which then gave the urea THI-4-OH-$NCONH_2$; conversion to the olefin THI-ole; loss of the cyano

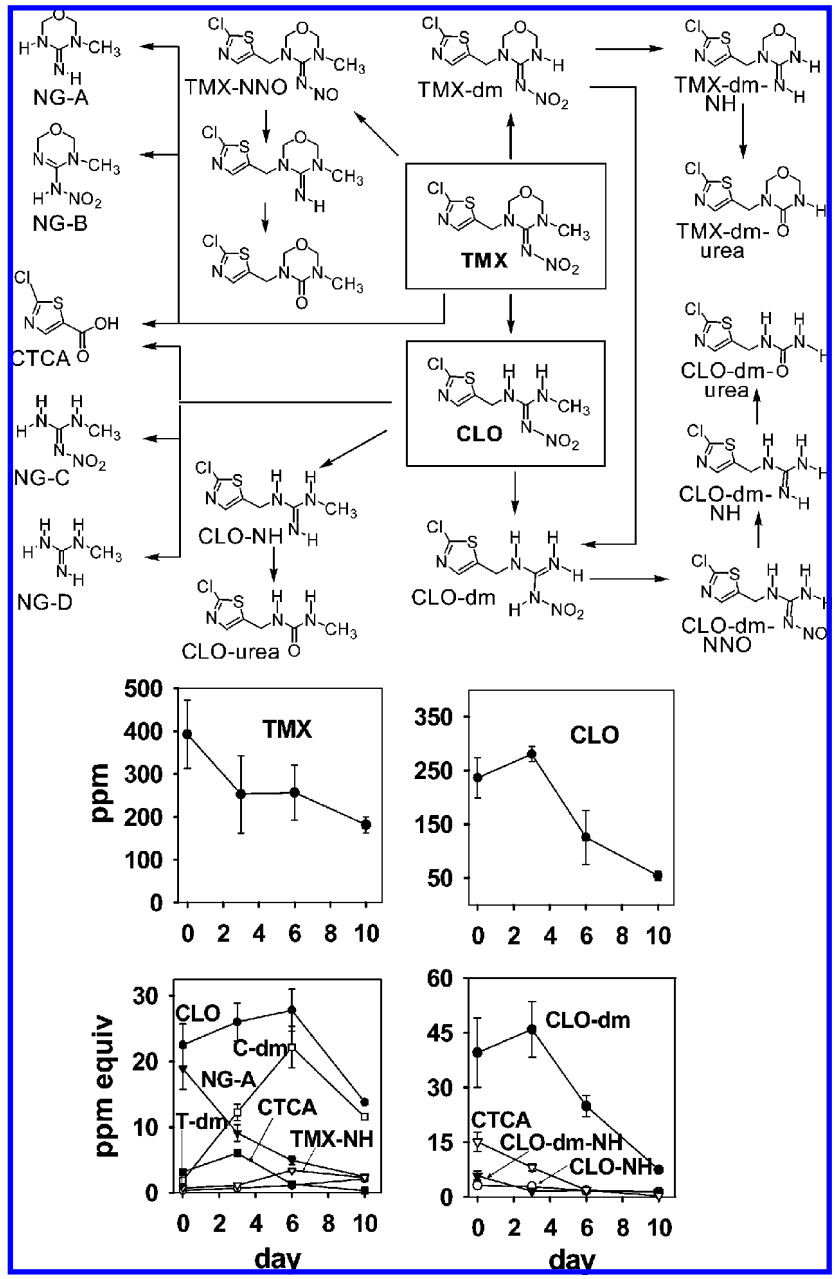

Figure 8. Partial metabolic pathways and pharmacokinetics for TMX and CLO in spinach. Each of the individual compounds was also observed as a mouse metabolite (*12*).

group for THI giving THI-NH and for THI-ole, ultimately yielding THI-ole-NH. The ureas and THI-SO were present in higher amounts than other metabolites quantitated. THI-SO was not detected in the hydroponic THI solution exposed to the same light conditions with no plant present and was therefore probably a plant metabolite rather than a photoproduct.

Metabolism and Pharmacokinetics of ACE (Figure 7). ACE was fairly persistent with half of the day 0 level present at day 10. Metabolism involved several initial sites of attack: N-demethylation to ACE-dm, the most prominent metabolite of those analyzed; cyano hydrolysis to ACE-$NCONH_2$; cleavage of the N−CN linkage to ACE-NH; hydroxylation at the *N*-methylene substituent to yield the cyanoamidine-containing fragment and ultimately CPOL-gluc; cleavage at the N(CH_3)−C(CH_3)═N linkage to *N*-methylchloropyridinylmethylamine, which was acetylated to ACE-acet. ACE-dm underwent similar pathways to ACE to yield ACE-dm-$NCONH_2$, chloropyridinylmethylamine and its acetyl derivative ACE-dm-acet, and CPOL-gluc, plus the corresponding cyanoamidine-containing fragment.

Metabolism and Pharmacokinetics of TMX and CLO (Figure 8). The metabolic pathways of TMX and CLO are shown together because under the test conditions TMX was the more persistent compound and was slowly converted to CLO. Thus, TMX yielded two sets of metabolites, one with the oxadiazine ring intact and the other the ring-opened CLO derivatives. In the first set, TMX was converted to the nitroso compound (TMX-NNO), then the guanidine and urea. Alternatively, TMX underwent N-demethylation to TMX-dm, proceeding to the desmethyl-NH and urea derivatives. On quantitation, TMX gave CLO in larger amounts than TMX-dm or TMX-NH. CLO formed from TMX or administered directly

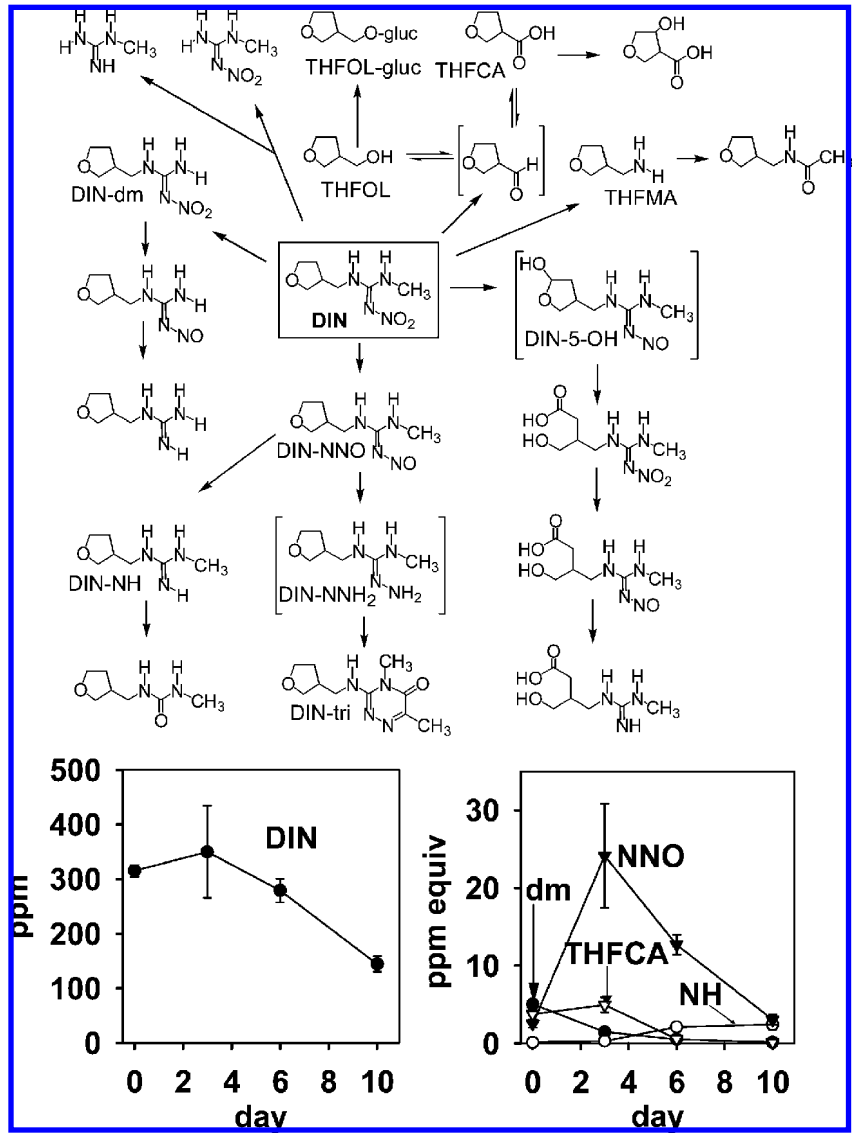

Figure 9. Partial metabolic pathways and pharmacokinetics for DIN in spinach. Each of the individual compounds, except for THFOL and THFOL-gluc, was also observed as a mouse metabolite (*12*).

gave CLO-dm and its NNO, NH, and urea derivatives. CLO metabolism was dominated by CLO-dm formation. In another sequence CLO underwent *N*-nitro cleavage to CLO-NH and CLO-urea. Four cleaved guanidines (NG-A, NG-B, NG-C, and NG-D) and CTCA were observed from TMX or CLO. The overall pathway in spinach was also observed in mice (*12*).

Metabolism and Pharmacokinetics of DIN (Figure 9). The DIN levels dropped to half in about 10 days. The metabolism was characterized by multiple pathways. Nitro reduction yielded the nitroso compound (DIN-NNO), which was further reduced via the NNH_2 derivative (not observed) to yield the methyltriazinone (DIN-tri) on coupling with pyruvate (*14*). DIN-NNO was also hydrolyzed to DIN-NH and the urea. Initial N-demethylation to DIN-dm led to the corresponding nitrosoguanidine and guanidine. Hydroxylation of the tetrahydrofuranyl moiety to DIN-5-OH was followed by ring opening, nitro reduction to the nitroso compound, and cleavage to the NH derivative; the DIN-2-OH series of metabolites (*12*) was not observed. Cleavage of the overall DIN molecule yielded either tetrahydrofurfurylmethylamine (THFMA), which was acetylated, or the aldehyde (not observed), which underwent either reduction to THFOL or oxidation to THFCA leading to glucoside formation. Both methylnitroguanidine and methylguanidine were observed as metabolites. Four of the DIN metabolites were quantitated, with DIN-NNO in largest amount and smaller levels of DIN-dm, DIN-NH, and THFCA. The DIN pathway observed here in spinach was also applicable in mice except as specifically noted.

DISCUSSION

Relationship to Earlier Studies on Plant Metabolism. The findings of this investigation with spinach involve hydroponically adminstered neonicotinoids at 100 ppm. The high levels used here to facilitate analysis might yield a different balance of metabolites than more normal insecticidal levels, but the products overall are consistent with earlier reports on the metabolism or residues in plants under practical conditions for IMI (*15*), THI (*16, 17*), ACE (*18*), TMX (*19, 20*), CLO (*21*−*23*), and DIN (*24*). Some of the apparent metabolites may in fact be photoproducts (e.g., NIT-CN) (*25*), but in any case, photodecomposition would be minimal for compounds in the plants following root uptake compared to foliar spray applications. The results on neonicotinoid uptake and persistance in spinach are not necessarily predictive of other crops, for example, rice, where they provide excellent residual activity (*1*).

Comparison of Spinach and Mice. This is the first study to directly compare the persistence and fate of the commercial neonicotinoids in plants. The same HPLC-DAD and LC-MSD methods were used for all of the compounds. Our earlier studies with mice (*11, 12*) enabled a spinach−mouse comparison as background for broader plant−mammal considerations. The phase I metabolites for all of the compounds in spinach were remarkably similar to those in mice except as noted in **Table 1**. The phase II metabolites were different, as expected. CPOL was conjugated as the glucoside and gentibioside and CPCA as several amino acid derivatives in spinach but only the glycine conjugate in mice. THI-SO on ring opening was S-methylated in mice, whereas in spinach only sulfonic acid formation was determined. In addition, 6-HS-PCA was observed as a significant metabolite of CPCA in spinach, whereas 6-methylthiopyridinylcarboxylic acid and the corresponding mercapturic acid were found in mice (*11*), implying a greater S-methylation preference in the mammalian system.

Neonicotinoids as Multifunctional Substrates. In plants, as in mammals, the neonicotinoids are converted to a large number and great variety of metabolites. The plant enzymes responsible for these conversions have not been specifically examined. In spinach, the $=NNO_2$ group was reduced to =NNO (DIN) and $=NNH_2$ (observed as the methyltriazinone). The =N−CN moiety was converted to the guanidine =NH or urea $-NH-C(O)NH_2$ (THI). N-Demethylation was observed with each substrate (CLO, DIN, and NIT). N-Dealkylation also removed an ethyl (NIT) or ethano (IMI) moiety. The ethano substituent of THI was converted to the 4-OH and olefin and that of IMI to the 5-OH and diol with a small amount of olefin. The heterocyclylmethyl moiety was cleaved in all cases, probably by *N*-methylene hydroxylation. The chloropyridinyl moiety of CPCA was converted to a mercaptopyridinyl group, presumably via GSH conjugation, and to 6-OH-PCA, which was methylated and oxidized to form the pyridone. The multifunctional nature of the neonicotinoids ensures their biodegradability in plants as well as mammals.

Concluding Remarks. The fate of a pesticide is dependent on its chemical and physical properties and the environment to which it is exposed. The toxicology of a pesticide in animal systems (e.g., mammals or insects) is a summation of that for the pesticide and its metabolites. A few of the neonicotinoid metabolites formed in spinach (e.g., desnitro- and descyanoguanidines and olefin derivatives) are potent agonists of nicotinic acetylcholine receptors from mammals (*3, 4, 26*) and both pest (*3, 4, 27*) and beneficial insect species (*28, 29*). When crop residues are considered, the safety evaluations include not only the mammalian but also the plant metabolites. The neonicotinoids examined here yield largely the same metabolites in mammals (mice) and plants (spinach), except for those formed by phase II reactions. There are some plant metabolites early in the degradation schemes unique to each neonicotinoid, and there are also some late in the pathways characteristic of the chloropyridinyl, chlorothiazolyl, and tetrahydrofuranyl types. Thus, there are metabolite markers for general or specific neonicotinoid exposure. The facile metabolism of the neonicotinoids ensures against environmental accumulation but also requires care in selecting and using compounds for maximum effectiveness.

ABBREVIATIONS USED

ACE, acetamiprid; acet, acetamide derivative; CLO, clothianidin; CLO-dm, desmethyl-clothianidin; CPCA, chloropyridinylcarboxylic acid; CTCA, chlorothiazolylcarboxylic acid; DAD, diode array detector; DIN, dinotefuran; DIN-dm, desmethyldinotefuran; dm, desmethyl; DIN-tri, methyltriazinone derivative of DIN-NNH_2; ESI, electrospray ionization; gent, gentiobioside; gluc, glucoside; GSH, glutathione; IMI, imidacloprid; IS, internal standard; MSD, mass selective detector; NG, nitroguanidine; NH, guanidine or imine derivative; NNH_2, aminoguanidine derivative; NIT, nitenpyram; NNO, nitrosoguanidine derivative; ole, olefin derivative; ppm equiv, parts per million equivalents based on the absorbance at 254 nm and recovery values of the parent compounds; SIM, selected ion monitoring; THFCA, tetrahydrofuranylcarboxylic acid; THFOH, tetrahydrofuranmethanol, THI, thiacloprid; TMX, thiamethoxam; TMX-dm, desmethyl-thiamethoxam; tri, methyltriazinone derivative.

ACKNOWLEDGMENT

We thank our Berkeley laboratory colleagues Motohiro Tomizawa, Shannon Liang, Rosa Hsieh, Tami Clark, and Alex Gulevich for advice and assistance. We acknowledge Barbara Rotz of the greenhouse facility and Rita Nichiporuk of the Mass Spectrometry Facility, Department of Chemistry at Berkeley, for valuable advice.

Supporting Information Available: Supplemental Table 1 listing unidentified neonicotinoid-derived metabolites in spinach. This material is available free of charge via the Internet at http://pubs.acs.org.

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Received for review July 9, 2008. Revised manuscript received September 3, 2008. Accepted September 4, 2008. This study was supported by Grant R01 ES08424 from the National Institute of Environmental Health Sciences, National Institutes of Health, a University of California Toxic Substances Research and Teaching Fellowship, and the William Muriece Hoskins Chair in Chemical and Molecular Entomology.

JF8020909