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DNA Adducts as Biomarkers for Oxidative and Genotoxic Stress from Pesticides in Crop Plants

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Plant studies have been carried out to identify the nature and extent of the formation of adducts with DNA bases when treated with pesticide formulations. DNA extracted from crop plants after treatment with pesticide formulations has yielded evidence of adduct formation. The extent of DNA modification has been established by ^{32}P postlabeling studies. The radiochromatograms from ^{32}P postlabeling of isolated plant DNA from grapes, bush beans, soybeans, pumpkins, and cucumbers show elevated adduct levels in treated vegetable plants as compared with untreated controls. A number of different adduct spots appear, likely indicating adduct formation with pesticide molecules or their metabolites. The DNA adducts from hexenal and 4-hydroxy-2-nonenal were clearly observed, indicating oxidative stress and lipid peroxidation in the plant.

KEYWORDS: DNA adducts; pesticide; pesticide formulation; ^{32}P postlabeling; hexenal; 4-hydroxy-2-nonenal; oxidative stress; plant lipid peroxidation; 1,N^2-propanodeoxyguanosine adducts; genotoxicity; chlorothalonil; esfenvalerate; carbaryl; diazinon

INTRODUCTION

The formation of adducts between base components of DNA and various xenobiotic molecules has been well-established as a primary source of mutation in biological organisms (*1*−*4*). The generation of such adducts is a significant event in the progression of carcinogenesis and mutagenesis. Frequently, these xenobiotic compounds are electrophilic agents that can bind covalently at one or more of the various nucleophilic sites in the nucleic acid−bases. In addition, the presence of a specific adduct may not represent direct binding to the pesticide molecule or a metabolite. The role of microsomal cytochrome P450 is known to generate reactive electrophilic compounds capable of binding to nucleic acids (*5, 6*). Thus, these xenobiotic agents may induce the formation of other reactive species, which then lead to DNA modification. The natural outcome is that normal base pairing is prevented with concomitant misreading of the genetic code during transcription, leading to base transversions, lesions, DNA strand breaks, deletions, and frameshift mutation. This mechanism has been well-documented in animal systems and is believed to play a key role in human carcinogenesis (*2, 7*−*10*).

Because most chemical pesticides also consist of electrophilic moieties or can be metabolically bioactivated to electrophilic intermediates, it could be expected that interaction of pesticide molecules with DNA might be a likely outcome. In addition, data on genotoxicity and mutagenicity are routinely collected on pest control agents as part of the regulatory and approval process for the agricultural use of pesticide formulations. Such statistics are available in large databases (*11, 12*). Adducts of pesticide molecules with DNA bases have been detected and isolated, principally in mammalian tissues (*13*−*24*).

In contrast, relatively little is known about the risk to plant DNA posed by adduct formation by pesticide molecules or their metabolites (*20, 25, 26*). Even though some pesticide formulations may degrade so as not to present long-term hazard to humans or to ecosystems, contact of the plant with an active pesticide is probably of sufficient duration to allow attack on the genetic material of the plant by the pesticide molecule or its metabolites. Although adduct formation has been detected in plant systems, there are few studies in the literature, and many are only inferential. Adducts from heptachlor were detected by ^{32}P postlabeling studies of fields with declining hops (*25*). Brown et al. have reported adducts from alachlor metabolites (*22*). DNA damage has also been implicated in studies on strawberries where organic and nonorganic maintenance were compared (*26*). The exposure of crops to a variety of agrochemicals in Italy also shows evidence of harm to DNA (*15*). ^{32}P postlabeling has also been utilized to determine DNA adduct levels in the urine of open-field farmers and fruit growers exposed to triazoles, captan, and chlorothalonil (*16*) and organophosphates, including azinphos methyl and chlorpyrifos (*27*). Similarly, blood samples from farmers exposed to pesticide formulations were shown to contain genotoxic DNA adducts by postlabeling analysis (*24*). Yellowing and damaged conifer trees

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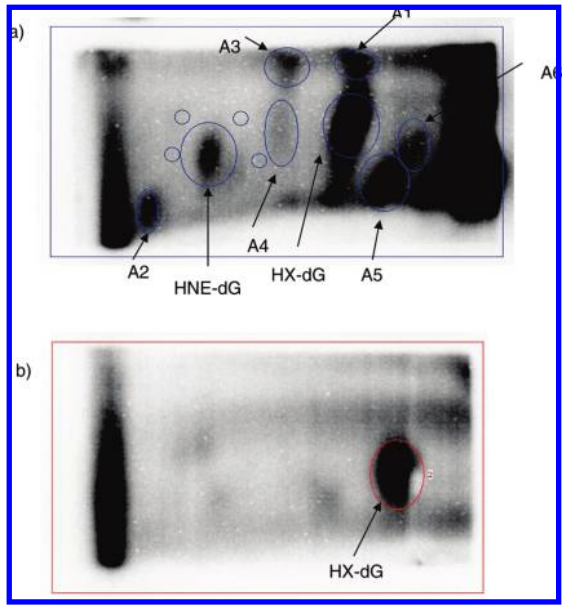


Figure 1. Radiochromatograms of ^{32}P-labeled mononucleotide adducts from bush beans (**a**) treated with chlorothalonil and (**b**) untreated plants (controls). Samples spiked with 50 fmol of HX-dG internal standard. The first dimension (D1) eluted with 1.7 M ammonium formate buffer at pH 3.5. The second dimension (D2) eluted with 2.7 M sodium phosphate. Small circles indicate background sampling.

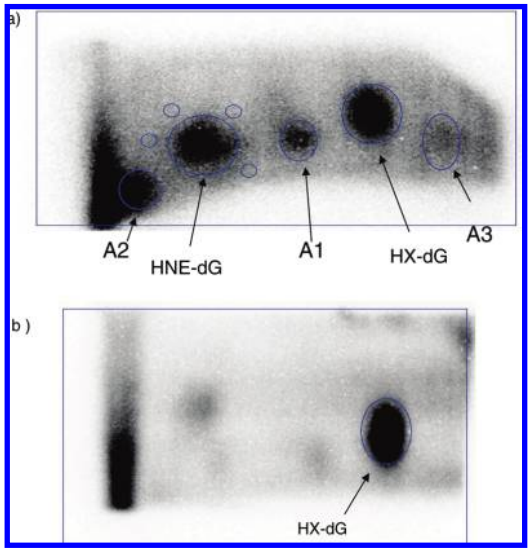


Figure 2. Radiochromatograms of ^{32}P-labeled mononucleotide adducts from cucumber (**a**) treated with chlorothalonil and (**b**) untreated plants (controls). Samples spiked with 50 fmol of HX-dG internal standard. The first dimension (D1) eluted with 1.7 M ammonium formate buffer at pH 3.5. The second dimension (D2) eluted with 2.7 M sodium phosphate. Small circles indicate background sampling.

were accompanied by observation of adducts by ^{32}P postlabeling (*28*). The exposure of rye grass seedlings to atmospheric pollutants (benzene, toluene, xylene, etc.) also leads to DNA adducts, which have been detected by ^{32}P postlabeling (*29*). Furthermore, the incidence of stress to crop plants by pesticide formulations may also be related to DNA damage. A case in point is oxidative stress, which manifests itself by the formation of DNA adducts. Although pesticide stress may involve other biochemical systems, assaults on the genetic material may be among the several factors responsible for reported stress or damage to particular crops, for example, strawberries (*26*), peaches (*30*), cranberries (*31*−*37*),

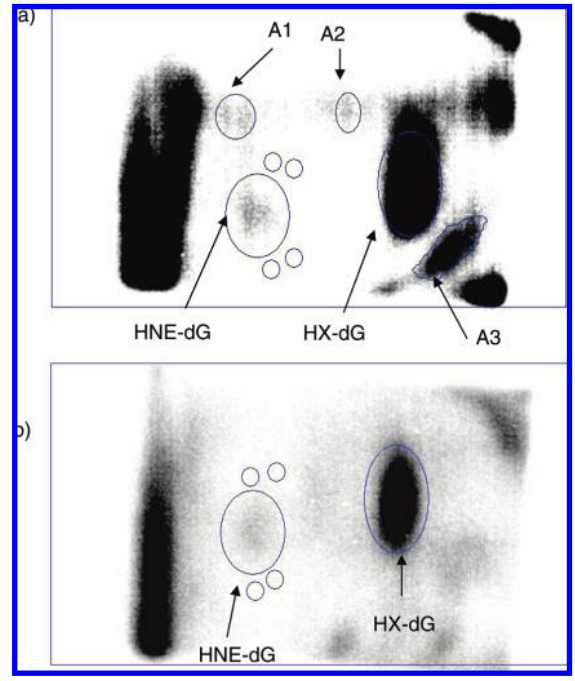


Figure 3. Radiochromatograms of ^{32}P-labeled mononucleotide adducts from grapes (**a**) treated with chlorothalonil and (**b**) untreated plants (controls). Samples spiked with 50 fmol of HX-dG internal standard. The first dimension (D1) eluted with 1.7 M ammonium formate buffer at pH 3.5. The second dimension (D2) eluted with 2.7 M sodium phosphate. Small circles indicate background sampling.

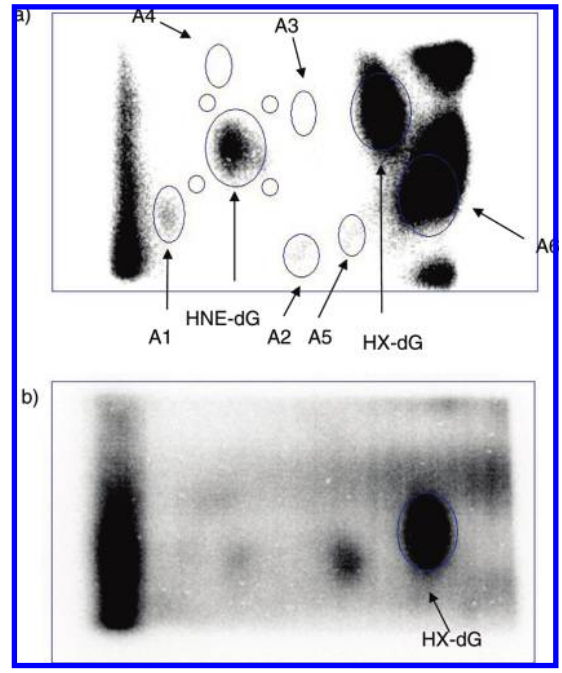


Figure 4. Radiochromatograms of ^{32}P-labeled mononucleotide adducts from pumpkin (**a**) treated with chlorothalonil and (**b**) untreated plants (controls). Samples spiked with 50 fmol of HX-dG internal standard. The first dimension (D1) eluted with 1.7 M ammonium formate buffer at pH 3.5. The second dimension (D2) eluted with 2.7 M sodium phosphate. Small circles indicate background sampling.

citrus (*38*), raspberries (*39*), tomatoes (*40*), sweet potato (*41*), corn (*42*), oats, and millet (*43*).

In this study, we have investigated the risk to DNA posed by treatment with several agrochemical pest control agents in a variety of crop plants. After treatment, DNA was isolated from the plants.

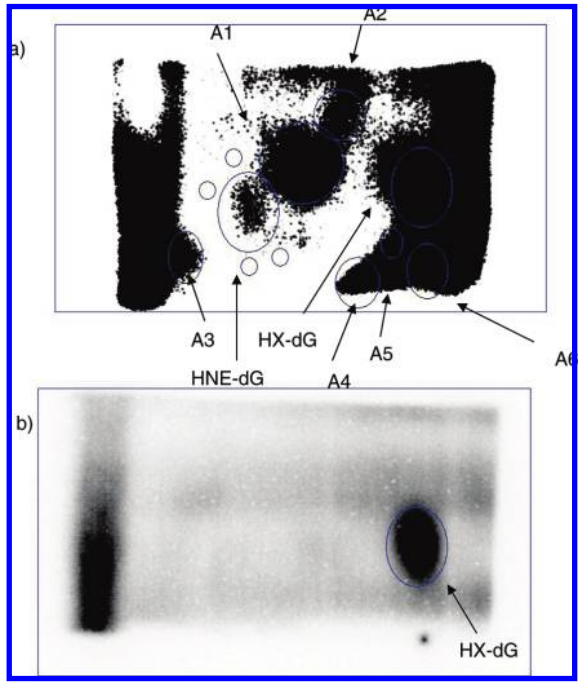


Figure 5. Radiochromatograms of ^{32}P-labeled mononucleotide adducts from soybeans (**a**) treated with chlorothalonil and (**b**) untreated plants (controls). Samples spiked with 50 fmol of HX-dG internal standard. The first dimension (D1) eluted with 1.7 M ammonium formate buffer at pH 3.5. The second dimension (D2) eluted with 2.7 M sodium phosphate. Small circles indicate background sampling.

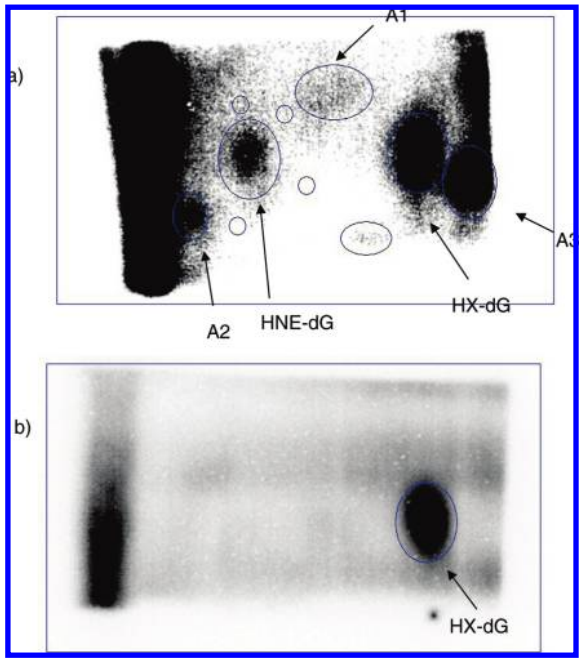


Figure 6. Radiochromatograms of ^{32}P-labeled mononucleotide adducts from soybeans (**a**) treated with esfenvalerate (with no added internal standard) and (**b**) untreated plants (controls) (with 50 fmol of HX-dG internal standard). The first dimension (D1) eluted with 1.7 M ammonium formate buffer at pH 3.5. The second dimension (D2) eluted with 2.7 M sodium phosphate. Small circles indicate background sampling.

The extent of DNA modification was evaluated by ^{32}P postlabeling studies. Plant DNA was hydrolyzed and subjected to γ-^{32}P phosphorylation by the standard procedure of Randerath et al. (*44*). In comparison with untreated controls, radiochromatograms from

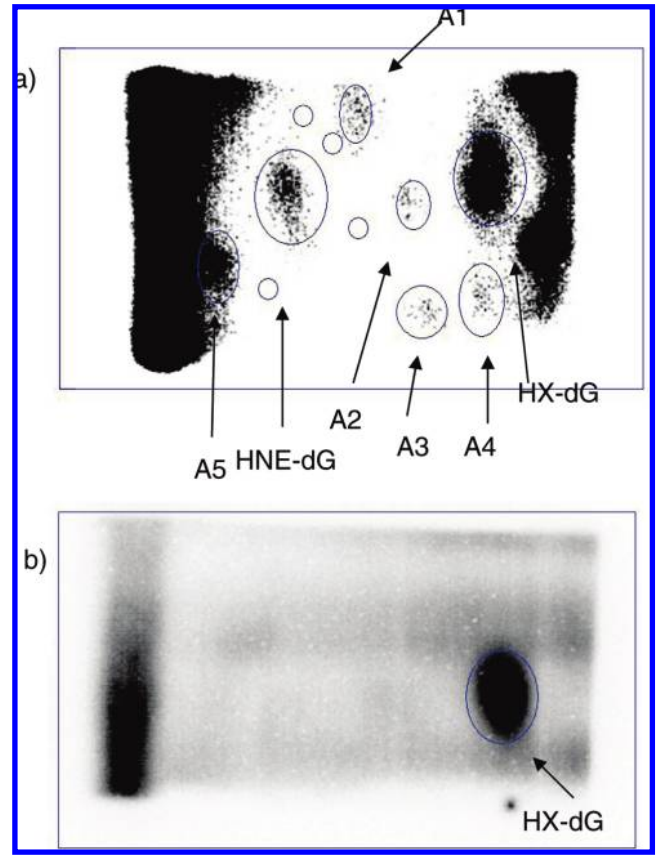


Figure 7. Radiochromatograms of ^{32}P-labeled mononucleotide adducts from soybeans (**a**) treated with carbaryl (with no added internal standard) and (**b**) untreated plants (controls) (with 50 fmol of HX-dG internal standard). The first dimension (D1) eluted with 1.7 M ammonium formate buffer at pH 3.5. The second dimension (D2) eluted with 2.7 M sodium phosphate. Small circles indicate background sampling.

^{32}P postlabeling plant DNA from treated vegetable plants were analyzed for evidence of modified mononucleotides.

MATERIALS AND METHODS

General. Qiagen DNeasy Plant Maxi Kits (Qiagen, Inc., Valencia, CA) were utilized to extract DNA from treated plants. Centrifugation was carried out on a centrifuge equipped with a swing-out rotor at 3000−5000*g*. UV spectrophotometric measurements were made on a Shimadzu UV−visible spectrophotometer. Polyethyleneimine (PEI) cellulose thin-layer autoradiochromatograms were read with a Packard Canberra InstantImager and interpreted with Imager software.

The Packard InstantImager is a microchannel array detector consisting of an array of 200000 microchannels of 400 μm diameter. Incoming electrons from each radioactive event are multiplied in avalanches from the microchannel holes and are counted. The instrument design with high-speed signal processing and pulse height analysis accomplishes highly accurate imaging and quantization in real time.

Treatment with Pesticide Formulations. The pesticide formulations used in this study were obtained from commercially available sources: chlorothalonil from Ortho Daconil (29.6% chlorothalonil), carbaryl from Garden Tech Sevin (22.5% carbaryl), diazinon from Ortho Diazinon Ultra (22.4% diazinon), and esfenvalerate from Ortho Bug B Gon (esfenvalerate 0.425%). All commercial pesticide formulations were analyzed and compared favorably to analytical samples of the active pesticide. Concentrations of the formulations were found to be within ±2% of the manufacturers' stated values with the exception of one formulation, which varied by 4% from the stated concentration. Concentrate pesticide formulations were diluted to manufacturers' specifications as follows: chlorothalonil, Ortho Daconil concentrate diluted 1:256 with water; carbaryl, Garden Tech Sevin concentrate diluted 1:64 with

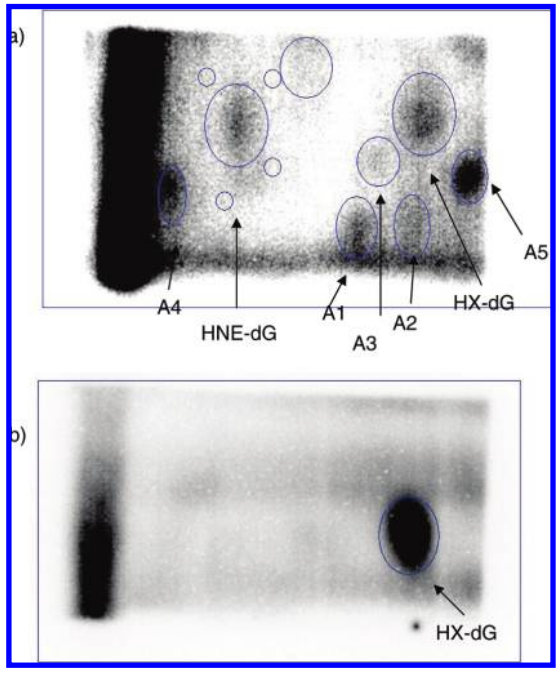
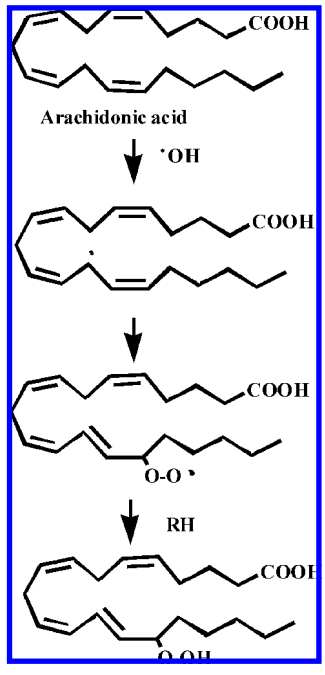


Figure 8. Radiochromatograms of ^{32}P-labeled mononucleotide adducts from soybeans (**a**) treated with diazinon (with no added internal standard) and (**b**) untreated plants (controls) (with 50 fmol of HX-dG internal standard). The first dimension (D1) eluted with 1.7 M ammonium formate buffer at pH 3.5. The second dimension (D2) eluted with 2.7 M sodium phosphate. Small circles indicate background sampling.

Scheme 1. COX and LOX Peroxidation



water; diazinon, Ortho Diazinon Ultra concentrate diluted 1:384 with water; and esfenvalerate, Ortho Bug B Gon concentrate diluted 1:128 with water.

Plants, including corn, soybean, pumpkin, cucumber, and grapes, were grown under greenhouse conditions from seed. After growth for 2−6 weeks, groups consisting of 2−4 plants were isolated and sprayed on four occasions over a period of 2 weeks with pesticide formulations described above. Use of these formulations was approved for application with these plants. Controls were prepared by spraying untreated plants with water.

DNA Isolation. Solutions and columns were used from DNeasy Plant Maxi Kits (Qiagen). These solutions included buffer AP1 [1−2.5% edetic acid and 1−2.5% sodium dodecyl sulfate (SDS)], buffer AP2 (10−20% acetic acid), RNase A (solution 100 mg/mL), buffer AP3/E (50% guanidine hydrochloride), buffer AW (ethanol solution), and buffer AE (10 mM Tris and 0.5 mM ETA, pH 9.0). QIA Shredder Maxi Spin Columns and DNeasy Spin Columns were obtained from Qiagen as components of the DNeasy Plant Maxi Kits.

The following general protocol was followed for all samples: 1 g of plant material (in most cases from leaves) was ground to powder with the assistance of liquid nitrogen. After evaporation of the liquid nitrogen, 5.0 mL of AP1 buffer (preheated to 65 °C) and 10 μL of RNase A stock solution were added to the ground plant material. The mixture was vortexed and heated for 10 min at 65 °C to lyse the cells. Removal of non-DNA plant materials (proteins, polysaccharides, pigments, RNA, etc.) by precipitation was accomplished by incubation with 1.8 mL of AP2 buffer for another 10 min at 0 °C, followed by centrifugation for 5 min at room temperature. After removal of the resulting pellet, the supernatant was transferred onto a shredder maxi column and again centrifuged for 5 min. The collected liquid was transferred to another tube, and an equal volume plus 50% of AP3/E buffer was added and mixed by vortexing. The liquid sample and any precipitate were placed on a spin column and were centrifuged for 5 min. After the eluant liquid was discarded, 12 mL of AW buffer was added to the spin column and the procedure was repeated. The resulting spin column was placed in a new 50 mL tube, and 1 mL of AE buffer was then introduced onto the column. After this rested for 5 min at room temperature, the mixture was again centrifuged with collection of the eluant. This last step was repeated with another 1 mL of AE buffer, and the eluants were combined.

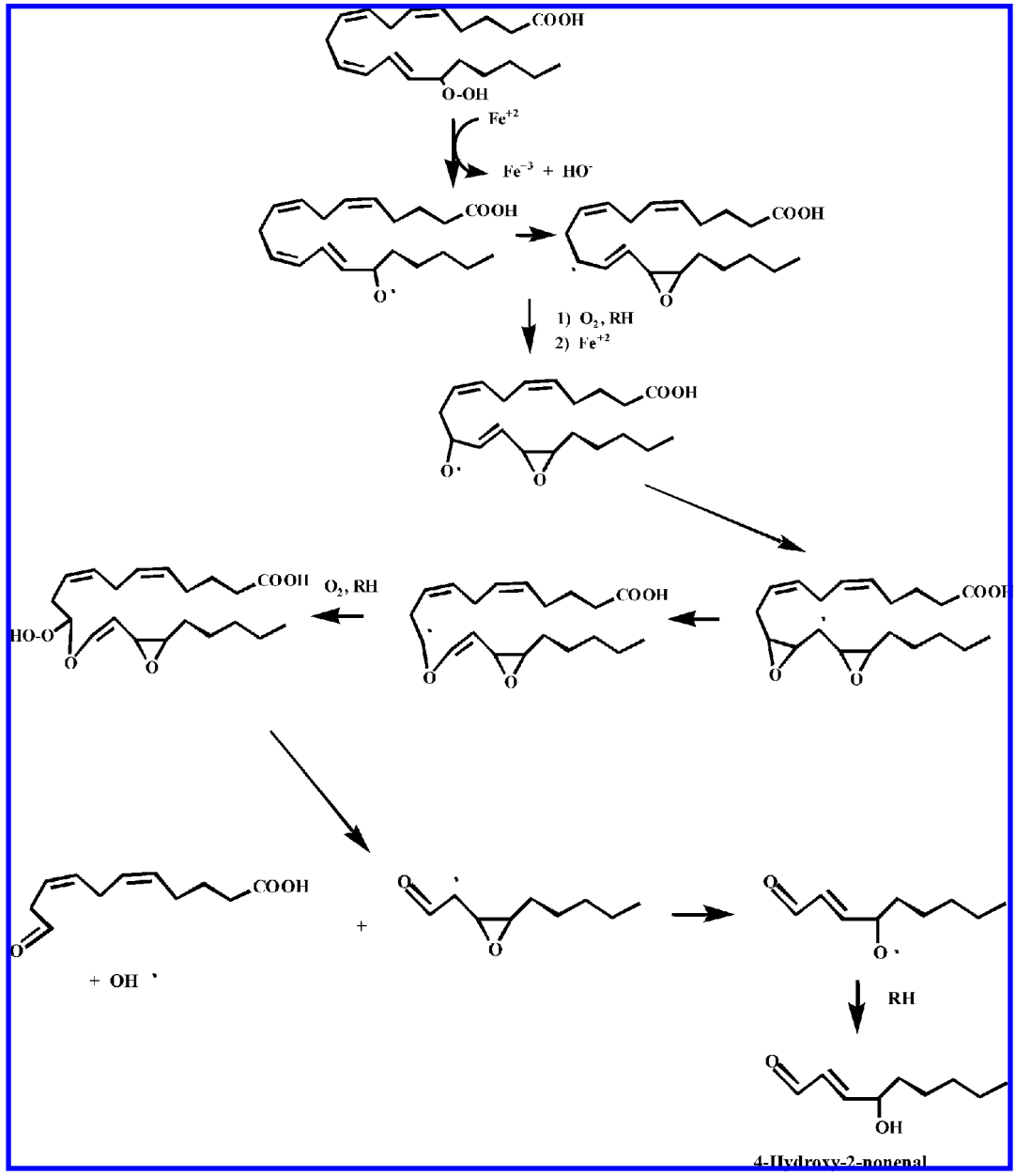
The isolated DNA in AE buffer solution was analyzed for purity and yield by UV spectrophotometry. DNA quantity was evaluated at λ = 260 nm where an absorbance of 1.00 was equivalent to 50 mg/mL of DNA. Absorbance ratios A_{260}/A_{280} and A_{230}/A_{260} were determined to check for complete removal of RNA, plant proteins, and other plant materials. Ratios of A_{260}/A_{280} in the range of 1.7−2.0 and A_{230}/A_{260} in the range of 0.36−0.44 were considered acceptable.

The AE buffer solutions of plant DNA were frozen and stored until further workup. Alternatively, an ethanol/NaCl solution was used to pelletize the DNA. The DNA pellets were washed, dried, and stored at 0 °C until processed with the postlabeling protocol.

Reagents for Postlabeling Analysis of DNA Adducts. Micrococcal nuclease (168 mill units/μg) from *Staphylococcus aureus* was provided by Sigma (Deisenhofen, Germany). Phosphodiesterase from calf spleen (spleen phosphodiesterase; 4 units/mL) was purchased from Boehringer Mannheim (Mannheim, Germany). NP1 (7 units/μL) from *Penicillium citrinum* was obtained from Fluka (Deisenhofen, Germany). Cloned T4 polynucleotide kinase (30 units/μL) was obtained from USB Amersham (Braunschweig, Germany), and [γ-^{32}P]ATP (>7.000 Ci/mmol, 167 μCi/μL) was obtained from ICN (Eschwege, Germany).

DNA Hydrolysis and NP1 Treatment. The DNA extraction solution or resuspended pellet was subjected to the DNA hydrolysis and postlabeling protocol of Wacker et al. (*45*), a modified procedure of Randerath, Reddy, and Gupta (*46*). Polypropylene reaction tubes of 1.5 mL (Sarstedt, Nümbrecht, Germany) were used for the DNA hydrolysis and all subsequent steps. Samples of 10 μg of DNA were incubated for 4 h at 37 °C with 2.50 μL of micrococcal nuclease solution (0.2 unit/μL; 1 μg/μL), 2.50 μL of spleen phosphodiesterase solution (0.002 unit/μL; 1 μg/μL), and 2 μL of DNA digestion buffer [25 mM $CaCl_2$ and 50 mM sodium succinate (pH 6.0)] in a total volume of 20 μL (0.5 μg/μL DNA hydrolysate). This treatment released the 3′-monophosphates of the adducted and the 3′-monophosphates of the unmodified nucleotides from the DNA. To decrease the ratio of unmodified to modified nucleotides, a NP1 treatment was carried out. A volume of 6 μL of NP1 mixture was added to the solution, consisting of 1.2 μL (8.4 units) of NP1 solution, 1.8 μL of 0.3 mM $ZnCl_2$, and 3 μL of 250 mM sodium acetate (pH 5.0). The NP1 treatment removed the 3′-phosphate from mostly all unmodified nucleotides. Therefore, polynucleotide kinase was not able to add a ^{32}P-phosphate group from the ATP. The mixture was incubated for 45 min at 37 °C, and the reaction was stopped by adding 2.4 μL of 0.5 M Tris base. The solution was desiccated to dryness and redissolved in 10 μL of water before the postlabeling reaction.

Scheme 2. Fe^{+2} (or Cu^{+})-Mediated Decomposition



^{32}P Postlabeling of the Adducts. A volume of 2.0 μL of labeling mixture was added to the sample solution containing 10 μg of NP1-enriched DNA. The labeling mix was made of 1.5 μL of kinase buffer [100 mM DTT, 100 mM $MgCl_2$, 10 mM spermidine, and 400 mM bicine/NaOH (pH 9.5)], 0.3 μL of 23 μM [γ-^{32}P]ATP (>7000 Ci/mmol; 1.9 MBq, 50 μCi, 6.9 pmol), and 0.2 μL (6 units) of T4 polynucleotide kinase. The sample was incubated for 45 min at 37 °C, and the reaction was stopped by application of the entire sample solution to a prewashed PEI-cellulose sheet (Macherey & Nagel, Düren, Germany). This sheet was developed in two directions for the determination of the adducts as given under thin-layer chromatography (TLC) conditions. From this chromatogram, the amount of 4-hydroxy-2-nonenal (HNE)-dGp adducts and 2-hexenal (HX)-dGp adducts/sample was determined by means of a calibration curve with the synthesized adduct standards.

TLC Conditions. Ammonium formate buffer (1.7 M; pH 3.5) was used for the development from bottom to top after attaching 4 cm wicks (Whatman #1) to 16 cm × 20 cm (height × width) to prewashed PEI-cellulose sheets. The first 7 cm from the bottom of the plate and the wick at the top of the plate were excised after the first development and discarded. The plate was soaked in running water for 4 min, air-dried, and rotated 90° for chromatography in the next direction. The development from left to right was carried out in 2.7 M sodium phosphate buffer (pH 3.8) into a 6 cm wick (Whatman #1), which was excised and discarded after the development. The resulting plate was air-dried.

Quantitation of DNA Adducts. Each chromatogram was visualized and counted by a Packard InstantImager with an exposure time of 1−2 h. The relative counting error of a spot was <3% at the end of the counting period. The unspecific radioactive background was subtracted by sampling background from spots that were placed adjacent to the adduct spot. A template was saved for all further determinations. The net cpm value of the spot was given by the Imager software. Calibration of the cpm values with known molar quantities of HNE-dGp and the HX-dGp was accomplished by spiking varying amounts in the range of 1−10 fmol of HNE-dGp adduct standard and 50 fmol of HX-dGp adduct standard to samples of calf thymus DNA. After hydrolysis, postlabeling with ^{32}P phosphate, TLC, and autoradiography of samples, a calibration plot (cpm vs fmol of nucleotide) yielded standard cpm/fmol values for HNE-dGp and the HX-dGp. The analytical variation of the method was <5% under these conditions.

Calculation of Relative Adduct Levels (RAL). Adduct levels in plant samples were measured with internal or external standards of HNE-dGp and/or HX-dGp. With an internal standard of HX-dGp, cpm

Scheme 3

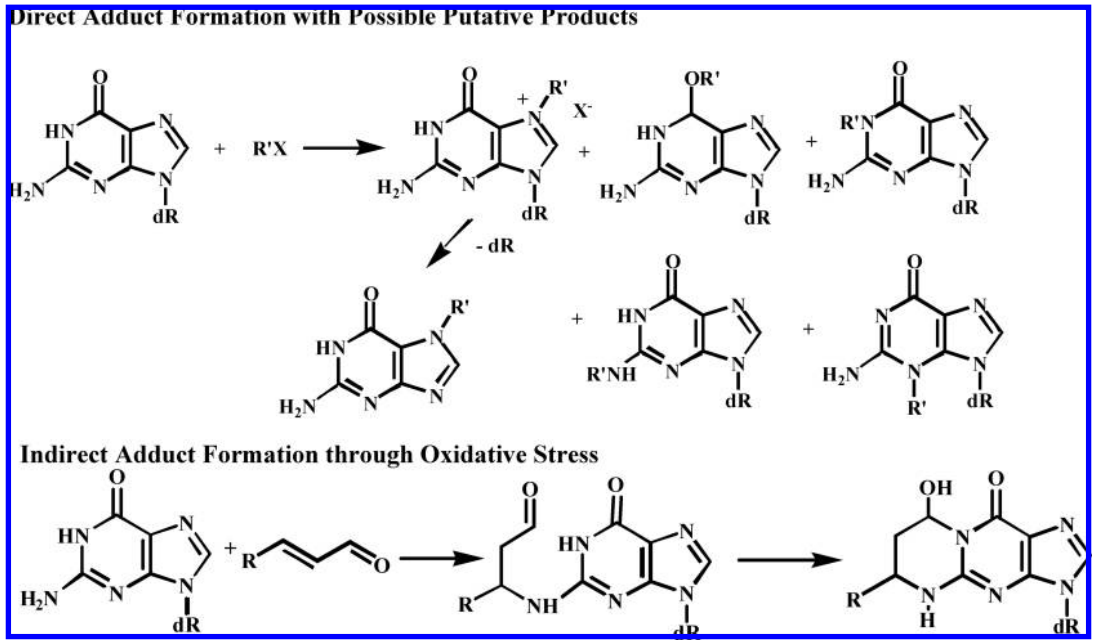


Table 1. Adduct Levels in Plants Treated with Chlorothalonil[a]

plant species	HNE-dG adducts in untreated plants[b] ^{32}P levels (cpm)[c]	HNE-dG adducts in treated plants ^{32}P levels (cpm)[c]	HNE-dG adducts in Untreated Plants[b,d] (per 10^9 nucleotides)	HNE-dG adducts in treated plants[e] (per 10^9 nucleotides)
bush beans	1029	8178 ± 1928	54	235 ± 55
cucumber	423	9982 ± 2928	22	214 ± 63
grape	4644	4826 ± 1454	76	79 ± 24
pumpkin	1014	6899 ± 5295	54	109 ± 31
soybeans	34	8163	2	160

[a] Reported as mean ± standard deviation for values from multiple samples. [b] Controls. [c] Normalized counts/min (cpm) using HX-dG internal standard. [d] Calibration standard, 493 cpm/fmol of HNE-dG. [e] Calibration standards: bush beans, 907 cpm/fmol of HNE-dG; cucumber, 1215 cpm/fmol of HNE-dG; grape, 1589 cpm/fmol of HNE-dG; pumpkin, 1325 cpm/fmol of HNE-dG; and soybeans, 1325 cpm/fmol of HNE-dG.

values for all spots were first normalized based to a cpm count of 50000 cpm/fmol HX-dGp corresponding to 50 fmol of HX-dGp. The normalized cpm values (for samples with internal standards) or raw cpm values for samples with an external standard were then multiplied by the calibration standard value for HNE-dGp obtained as described in the previous paragraph. Because 12 μg samples of DNA were used for the postlabeling analysis, this corresponds to 3.84×10^{-8} mol of nucleotides (from a standard value of 3.2 nmol of nucleotides equivalent to 1 μg of DNA). Consistent with the usual notation, the number of adducts was expressed as adducts per 10^9 nucleotides.

The following equation expresses the complete conversion:

$$\text{adducts per } 10^9 \text{ nucleotides} = \frac{\text{cpm}_{\text{dNp}}}{\text{cpm/fmol}_{\text{dNpStandard}}} \times \frac{10^{-15} \text{ mol/fmol}}{3.84 \times 10^{-8} \text{ mol}} \times 10^9 \text{ nucleotides}$$

This directly gives the number of the HNE-dG adducts per 10^9 nucleotides. In cases with no HX-dG internal standard, numbers of HX-dG adducts can be calculated directly using the standardized HX-dG calibration value. DNA levels for other adducts (A1−A6) in each sample were quantified by the same equation and were reported as RAL in a manner similar to that used recently for DNA adducts found in the urine of farm workers (*16*).

RESULTS AND DISCUSSION

Figures 1–8 display the resulting autoradiograms from ^{32}P postlabeling of modified DNA isolated from several crop plants after treatment with pesticide formulations containing chlorothalonil, carbaryl, diazinon, and esfenvalerate. In all cases of the pesticide formulations considered in this study, radiochromatograms of ^{32}P-labeled DNA mononucleotides from extracts of DNA from treated plants displayed one or more adduct spots. Nonadducted nucleotides, along with unspent ATP, phosphate, and other compounds, do not appear since they either flow into attached paper wicks or remain at the origin and are removed before the autoradiographic assay. The number of spots and their radiointensities on the PEI-cellulose plates varied depending upon the pesticide formulation used and the type of plant subjected to the particular pesticide formulation. All autoradiograms of mononucleotides from treated plants were compared with those from plants that did not receive treatment with pesticide formulation (controls sprayed with distilled water only). The control autoradiograms displayed either no detectable adduct spots or only faint spots from adducts formed from endogenous compounds. It is important to note that only DNA adducts appear in the thin-layer field. All other non-DNA plant material (proteins, polysaccharides, pigments, RNA, etc.) is removed in the DNA extraction process. Quality control of the DNA is achieved by UV spectroscopic analysis as described earlier. Unmodified nucleotides are chromatographed into the wicks along the edges in the two-dimensional thin-layer process and are removed.

The DNA isolated from all plants in this study (bush beans, cucumber, pumpkins, grapes, and soybeans) treated with the fungicide chlorothalonil produced multiple adduct spots. At least six adduct spots are visible in the radiochromatograms of bush

Table 2. RAL for Other Adduct Spots in Plants Treated with Chlorothalonil[a] (Adduct Spots Labeled as in **Figures 1−5**)

plant species	RAL (A1) (adducts per 10^9 nucleotides)	RAL (A2) (adducts per 10^9 nucleotides)	RAL (A3) (adducts per 10^9 nucleotides)	RAL (A4) (adducts per 10^9 nucleotides)	RAL (A5) (adducts per 10^9 nucleotides)	RAL (A6) (adducts per 10^9 nucleotides)
bush beans	169 ± 60^c (5901 ± 2102)[b]	164 ± 20^c (5697 ± 694)[b]	145 ± 95^c (5038 ± 3292)[b]	81 ± 21^c (2814 ± 716)[b]	127 ± 61^c (4410 ± 2133)[b]	174 ± 79^c (6051 ± 2768)[b]
cucumber	116 ± 24^c (5420 ± 1106)[b]	268 ± 46^c (12506 ± 2154)[b]	229 ± 31^c (10667 ± 1449)[b]			
grape	36 ± 14^c (2201 ± 859)[b]	66 ± 6^c (4015 ± 395)[b]	155 ± 15^c (9447 ± 897)[b]			
pumpkin	73 ± 20^c (3731 ± 1016)[b]	26 ± 9^c (1323 ± 483)[b]	60 ± 15^c (3033 ± 741)[b]	13 ± 12^c (679 ± 621)[b]	63 ± 7^c (3195 ± 355)[b]	1409 ± 355^c (71701 ± 18053)[b]
soybeans	797^c (40547)[b]	172^c (8753)[b]	136^c (6932)[b]	134^c (6932)[b]	91^c (6823)[b]	324^c (16472)[b]

[a] Reported as mean ± standard deviation for RAL and cpm values from multiple samples (corrected cpm values in parentheses are based on HX-dG internal standard). [b] Normalized counts/min (cpm) using HX-dG internal standard. [c] Calibration standards: bush beans, 907 cpm/fmol of adducted nucleotide; cucumber, 1215 cpm/fmol of adducted nucleotide; grape, 1589 cpm/fmol of adducted nucleotide; pumpkin, 1325 cpm/fmol of adducted nucleotide; and soybeans, 1325 cpm/fmol of adducted nucleotide.

Table 3. Adduct Levels in Soybeans Treated with Pesticide Formulations[a]

pesticide formulation	HNE-dG adducts in untreated plants[b] ^{32}P levels (cpm)	HNE-dG adducts in treated plants ^{32}P levels (cpm)	HX-dG adducts in treated plants ^{32}P levels (cpm)	HNE-dG adducts in untreated plants[b] (per 10^9 nucleotides)	HNE-dG adducts in treated plants[e] (per 10^9 nucleotides)	HX-dG adducts in treated plants[f] (per 10^9 nucleotides)
chlorothalonil	34^c	8163^c	internal standard	2	160	internal standard
esfenvalerate[d]	34^c	3062 ± 1958	8264 ± 3509	2	60 ± 38	215 ± 91
carbaryl[d]	34^c	2956 ± 152	6310 ± 668	2	58 ± 3	164 ± 17
diazinon[d]	34^c	1679 ± 264	1823 ± 13	2	33 ± 5	47 ± 0.3

[a] Reported as mean ± standard deviation for values from multiple samples. [b] Controls. [c] Normalized counts/min (cpm) using HX-dG internal standard. [d] External HX-dG standard. [e] Calibration standard, 1325 cpm/fmol of HNE-dG. [f] Calibration standard, 1000 cpm/fmol of HX-dG.

Table 4. RAL for Other Adduct Spots in Soybeans Treated with Pesticide Formulations[a] (Adduct Spots Labeled as in **Figures 5−8**)

pesticide formulation	RAL (A1) (adducts per 10^9 nucleotides)	RAL (A2) (adducts per 10^9 nucleotides)	RAL (A3) (adducts per 10^9 nucleotides)	RAL (A4) (adducts per 10^9 nucleotides)	RAL (A5) (adducts per 10^9 nucleotides)	RAL (A6) (adducts per 10^9 nucleotides)
chlorothalonil[b]	797^c (40547)[b]	172^c (8753)[b]	136^c (6932)[b]	134^c (6823)[b]	91^c (4613)[b]	324^c (16472)[b]
esfenvalerate[d]	26 ± 8^c (1344 ± 413)	49 ± 4^c (2477 ± 197)	154 ± 56^c (7857 ± 2846)			
carbaryl[d]	11 ± 9^c (569 ± 446)	11 ± 1^c (571 ± 50)	16 ± 4^c (809 ± 192)	15 ± 7 (773 ± 359)	54 ± 1 (2767 ± 27)	
diazinon[d]	1 ± 0.04^c (46 ± 2)	49 ± 0.2^c (2483 ± 8)	27 ± 5^c (1377 ± 240)	18 ± 2^c (927 ± 125)	7 ± 3^c (341 ± 147)	27 ± 3^c (1396 ± 152)

[a] Reported as mean ± standard deviation for RAL and cpm values in parentheses from multiple samples. [b] Normalized counts/min (cpm) using HX-dG internal standard. [c] Calibration standard, 1325 cpm/fmol of adducted nucleotide. [d] External HX-dG standard.

beans, soybeans, and pumpkins, whereas only four are evident in the cases of cucumber and grapes. A possible seventh spot is visible in the chromatograms from chlorothalonil-treated pumpkin.

Soybeans were used to test the insecticides esfenvalerate, carbaryl, and diazinon, as well as the fungicide chlorothalonil. Multiple well-defined adduct spots appear in all cases. The least activity appears in the case of esfenvalerate, where two dark spots and two lighter spots appear. Chlorothalonil and carbaryl-treated soybeans reveal at least six distinct spots with a possible seventh weaker spot in each case. Diazinon shows seven spots although two are somewhat fainter. Although further study is warranted, evidence for adduct formation appears to be a common, general phenomenon upon treatment of plants with pesticide formulations. Transport of pest control agents into the plant organelles and nucleosomes does not appear to be an impediment to adduct formation.

Although the structures of adducts in most of the spots are unknown at this time, two of the adducts visible on the autoradiograms could be identified due to previous experience with these particular adducts. These two spots correspond to deoxyguanosine adducts formed with HX and 4-hydroxynonenal (HNE) previously identified in ^{32}P postlabeling studies (*7, 47*). Both HX, HNE, and related compounds are products of lipid peroxidation via cyclooxygenases (COXs) or lipoxygenases (LOXs) (*48−57*). These compounds arise endogenously but may be induced by external agents such as xenobiotic materials, which produce reactive oxygen species as a result of actions of cytochrome P450, and similar proteins, which attempt to detoxify these xenobiotic compounds. It has been suggested that ozone might also mediate DNA modification. Ozone is known to pass through the stomata, enter intercellular spaces, and decompose to active oxygen species or other free radicals (*58*). These reactive species are documented to have genotoxic effects in animals and humans, as well as in plants (*59−61*). Besides interacting directly with DNA, these reactive species may activate other natural compounds found in cellular material. Ultimately, this leads to a cascade of DNA adduction, lesion, mispairing, and mutation (*62, 63*).

Schemes 1 and **2** display the sequence reactions between the lipid, arachidonic acid, and these oxygen radical species to HNE (*52*). Hexenal is formed by a similar sequence of reactions (*64, 65*). Reaction with deoxyguanosine (**Scheme 3**) provides an indirect route to adduct formation (*63, 66−69*). In all of the radiochromatograms, the dG-HX and dG-HNE derivatives are visible, indicating that the pesticide induced oxidative stress in the plant. Not only are these adducts genotoxic by themselves with possible deleterious mutations, but they are also signals that the plant has been affected in other ways, manifested by yellowing of leaves, retarded development of roots, stems, and foliage, stunted growth, delay in fruit set, etc. As such, the appearance of these adducts is evidence that stress has occurred, thus serving as a biomarker for oxidative stress. The numbers of modified nucleotides observed (**Tables 1** and **3**) are quan-

tification of the degree of stress to which the plant has been subjected (*70*−*74*). It is clear that some plant species are more susceptible to stress (e.g., cucumber). Grapes have significantly less HNE adducts, for instance. Such plants contain various biochemical systems to offset the effects of the pesticides, including glutathione S-transferases, cytochrome P450, and antioxidant molecules. Variability in the level of adduct formation is seen also from one pesticide formulation to another. From the pesticide formulations tested, diazinon produces the least HNE-dG and HX-dG, while esfenvalerate produces about three times the number of these modified nucleotides. In plants and animals, detoxifying defense systems such as cytochrome P450s, GSTs or other enzymes, antioxidants, or mixed cooperative systems are frequently in place to scavenge and detoxify reactive agents. However, once these systems are overwhelmed, further damage to the plant may occur, including the formation of DNA adducts, mutation, and assault on other biochemical systems in the plant (e.g., respiration, photosynthesis, growth and maturation, fruit set, etc.) Such oxidative damage to plant mitochondria has been reported from paraquat treatment of pea seedlings, which induces inhibition of glycine decarboxylase, impacting photorespiration (*75*). Further evidence of the cytotoxic effects of oxidative damage in plants has recently been found from HNE modification of several proteins in mitochondria, including damage to dehydrogenases, ion channel proteins, and respiratory chain proteins (*76*).

In every radiochromatogram studied, several additional spots were observed. In some cases, these are relatively small amounts, which manifest themselves as weaker spots or faint shadows. These most likely arise from direct adduct formation with the pesticide molecule or with one of its metabolites (**Tables 2** and **4**). Chlorothalonil in soybeans produces five times the number of other modified nucleotides as compared with the HNE adducts. Considerable modified nucleotides appear with chlorothalonil treatments of bush beans and cucumber. Because there are several reactive sites on each of the nucleic acid−bases and one or more reactive sites on pesticide molecules or their metabolites, the number of possible adduct structures is large (**Scheme 3**).

Although some nucleotide modifications from either direct or indirect adduct formation may be benign and of no consequence, alterations or DNA lesions of this type are usually considered to be mutagenic or cytotoxic if left unrepaired (*77*−*79*). For cells to preserve genome integrity, cells in both plants and animals utilize various monitoring and signaling mechanisms to control chromosome metabolism and repair damaged genes (*78, 80*−*82*). Transcription-coupled repair eliminates certain lesions as the template strand is transcribed. However, small changes to the DNA bases by adduct formation, deamination, oxidation, etc. alter their base-pairing characteristics but do not block RNA polymerase. Thus, incorrect nucleotides become incorporated into the RNA, leading to transcriptional mutagenesis (*83*). Failure of these control processes leads to significant genome instability, impairs normal cellular reproduction and processes, and even leads to cell death (*78, 79, 82*). This type of injury to DNA in mammalian cells can lead to the onset of cancer (*84*−*87*). Thus, extensive exogenous DNA damage can disrupt cellular processes and even be lethal to cells, posing a distinct threat to crops (*88*). In agriculture, such damage could be potentially detrimental to the quality of crop plants, impacting root, stem, leaf, and fruit development. In fact, accounts of stress to crops accompanying pesticide use (*4, 30*−*43*) may be related to DNA damage (at least in part), although other biological macromolecules and systems may be involved. Hence, genome injury poses a potential and distinct threat to crop integrity.

CONCLUSIONS

Numerous pesticide molecules or their metabolites are typically reactive electrophilic compounds, which like other xenobiotic agents are capable of binding covalently with nucleic acid−bases. Evidence has been presented for the formation of modified nucleotides resulting from the application of pesticides or pesticide formulations to crop plants. Products of reaction with DNA bases may take the form of either direct binding with the pesticide molecule (or an intermediate metabolite) or indirect binding with a product of lipid peroxidation or other stress to the plant. Direct adducts have been detected in the case of the following pesticide formulations: chlorothalonil, diazinon, esfenvalerate, and carbaryl. In addition, the appearance of known adduct products from interactions with HX and HNE indicates that pesticide formulations induce oxidative stress via lipid peroxidation. The detection of adducts from these unsaturated aldehyde derivatives serves as a useful biomarker for stress to the plants. Finally, adduct formation with pesticides appears to be widespread and pervasive in view of the incidence of adducts with all pesticide formulations tested. Furthermore, damage to the plant genome is an additional factor in pesticide stress to crop plants and, as such, presents a risk that must be taken into account when considered in any risk−benefit analysis in a program of integrated pest management.

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