Metabolic Fate of Foliarly Applied [^{14}C]Thiabendazole in Three Growing Plants: Wheat, Soybeans, and Sugar Beets

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Following foliar application of [^{14}C]thiabendazole to growing wheat, soybean, and sugar beet plants, samples were taken 2 h post-treatment, at immature stages, and at normal harvest. Significant levels of [^{14}C]thiabendazole residues were found in straw and immature leaves (~10−22 ppm), but residues were far lower in wheat grain, soybean seeds, and sugar beet roots (~0.12−0.88 ppm), demonstrating very limited translocation of thiabendazole-derived residues. Thiabendazole was the major component (≥91%) of the total residues in early foliage. Samples harvested at later growth stages contained increased levels of polar (aqueous-soluble) and nonextractable residues and diminished levels of organic-extractable residues. Most of the extractable radioactivity consisted of thiabendazole. The major transformation product of thiabendazole found was benzimidazole, present mainly in conjugated form. Fractionation of nonextractable residues in wheat straw demonstrated some incorporation into tissue components.

Keywords: *Thiabendazole; fungicide; benzimidazole; benzimidazole conjugates; residues; HPLC; GC/MS*

INTRODUCTION

Thiabendazole [2-(4′-thiazolyl)-1*H*-benzimidazole; TBZ] (Figure 1) was originally developed for use as an anthelminthic (Brown et al., 1961) but later was recognized to also possess significant fungicidal activity (Robinson et al., 1969). It is the active ingredient in fungicidal products used for treatment of plants and harvested plant products and shows activity against a number of fungal genera including *Aspergillus, Colletotrichum, Cytospora, Fusarium, Penicillium, Sclerotinia, Thielaviopsis*, and *Phoma* (Spencer, 1981). The use of TBZ as a fungicide (Edgington et al., 1971; Logan et al., 1975; Meredith, 1977) provides a possible route for entry of its residues into the human food chain, either through direct exposure or through exposure of food-producing animals (e.g., chickens, cattle) to feed from TBZ-treated plants. The objective of this study was to determine the concentrations of [^{14}C]TBZ and related residues present in immature and mature wheat, soybean, and sugar beet plants following their exposure to foliarly applied [^{14}C]TBZ, and to characterize and identify the residues present in the various plant samples.

MATERIALS AND METHODS

In-Life Portion. The biological portions of the studies were conducted in a 30 × 60 ft test plot shelter covered with a corrugated Filon fiberglass roof to allow for light transmission and yet prevent precipitation from reaching the plots. The test plots (sandy loam soil obtained from the Missouri River bottoms near McBaine, MO) were planted in May (soybeans and sugar beets) and June (wheat). Plots were irrigated manually at the soil surface to avoid the foliage being washed. The crops were maintained according to good agricultural practices throughout the studies.

Figure 1. Structures of thiabendazole and its major plant metabolite, benzimidazole.

Test Material. Reference standard TBZ (99.1% chemical purity) and [^{14}C]TBZ (uniformly labeled in the phenyl ring; >99% radiochemical purity) were supplied by Merck Research Laboratories, as were 5-hydroxythiabendazole (5-OH-TBZ) and benzimidazole-2-carboxamide (BNZ-$CONH_2$) (used as standards for identification of possible transformation products of [^{14}C]TBZ). Benzimidazole (BNZ), obtained from Sigma Chemical Co., was also used as a reference standard. After isotopic dilution with unlabeled TBZ, the specific activities of the radiolabeled TBZ were 21 325 dpm/μg (wheat) and 31 411 dpm/μg (soybeans and sugar beet). Pertinent data concerning the application of [^{14}C]TBZ and collection of samples from the treated plants are presented in Table 1.

Instrumentation. Combustion analyses were conducted with a Packard 306B Tricarb sample oxidizer or a Harvey OX-500 biological material oxidizer. Radioactivity was quantified by using either a Searle Model 300 liquid scintillation counting (LSC) system or a TM Analytic Delta 300 liquid scintillation counting system. Total [^{14}C]TBZ-equivalent residues in crop samples were determined by combusting samples in triplicate followed by LSC analysis of the trapped [^{14}C]CO_2. The scintillation fluids used for LSC were either Ready Gel or Hydrocount.

TLC plates were silica gel RP-18 F_{254} (E. Merck, glass-backed, 250 μm thick). Radioactive zones were observed by radioscanning using a Radiomatic Model RS radio thin-layer

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Table 1. Application to and Collection of Samples from Plants Treated with [^{14}C]TBZ

	wheat	soybeans	sugar beets
no. of applns	1	2	5
rate of appln, lb/acre/appln	0.71	0.30	0.36
times of appln, days after planting	48	59, 73	65, 79, 93, 107, 121
times (and types) of sample collections, days after initial treatment	2 h (foliage), 7 (foliage), 37 (haylage), 63 (straw and grain)	2 h (foliage), 27 (foliage),[a] 78 (straw and seeds)[b]	2 h (tops), 56(tops and roots),[c] 90 (tops and roots)[d]

[a] Thirteen days following final application. [b] Sixty-four days following final application. [c] Immediately following final application. [d] Thirty-four days following final application.

chromatography (RTLC) scanner. The solvent system used for development of the plates was $CH_3CN/H_2O/H_3PO_4$ (25/75/0.3% v/v/v). Authentic TBZ was applied to all plates to yield a reference R_f.

HPLC was performed using a Shimadzu gradient HPLC system with a Gilson Model 202C fraction collector for collection of the column eluate. A Waters μBondapak C_{18}, 3.9 × 300 mm steel column was used for the HPLC analysis with a flow rate of 1.0 mL/min in conjunction with one of several gradient solvent systems. Methanol and aqueous buffer (0.1 M NaH_2PO_4 and 0.01 M $NaC1O_4$) were used as the gradient components. Detection of TBZ and related compounds (possible transformation products) by UV detection was achieved by monitoring at 279 nm, while radioactivity detection was accomplished using LSC of the collected 1-min fractions.

GC/MS analysis of standards and sample isolates was accomplished by use of a Kratos MS 25 mass spectrometer equipped with a Carlo-Erba 4160 gas chromatograph and a DS-55 data system. Separation of isolate components was achieved using a DB-5 (J&W Scientific) phenylmethyl silicone, bonded phase fused-silica capillary column (25 m × 0.25 mm), with a 0.25-μm film thickness. Carrier gas (helium) flow was approximately 2 mL/min. The GC column extended directly into the ion source. Instrument operating conditions included the following: splitless injection (1 μL) with column temperature at 150 °C for 3 min followed by temperature programming at 10 °C/min to 320 °C (held for 5 min); spectrometer scan rate 1 s/decade with ion energy of 70 eV. A solvent blank was run after each analysis of a reference standard and before examination of the plant isolates to determine whether any carryover of reference material occurred; none was observed.

Isolation and Characterization of ^{14}C Residues. Samples from the three varieties of plants, collected at various times (see Table 1), were analyzed for TBZ and related residues. As a general isolation scheme, solvent extraction and hydrolysis techniques were employed to release the radioactive residues from the plant samples. Two-hour and 7-day wheat samples and 2-h soybean and sugar beet samples were extracted using 6 N HCl/DMF (1/1 v/v). Methanolic KOH (3 N; room temperature or refluxing) was employed for extraction of later samples, as it was found that this system extracted TBZ-related residues from plant tissues as effectively as HCl/DMF and at the same time avoided the occasionally adverse effects which DMF had on HPLC analysis of the plant extracts. The released residues were then analyzed for total radioactivity by LSC, followed by TLC and/or HPLC. Glucosidase (Sigma) treatment was carried out overnight at 37 °C and pH 6 using a shaking water bath. Quantitation of individual released radioactive residues was performed using a combination of HPLC and LSC.

RESULTS AND DISCUSSION

Total [^{14}C]TBZ Residues in Wheat, Soybean, and Sugar Beet Plants. [^{14}C]TBZ-equivalent residues found in wheat, soybean, and sugar beet plants after various time intervals are presented graphically in Figures 2, 3, and 4, respectively. The average residue level found in wheat foliage immediately after treatment was 67.4 ppm. This level dropped to 41.2 ppm in forage wheat and to 21.9 ppm in haylage wheat (7 and 37 days post-treatment, respectively), most likely as a result of growth dilution and dissipation by natural mechanisms.

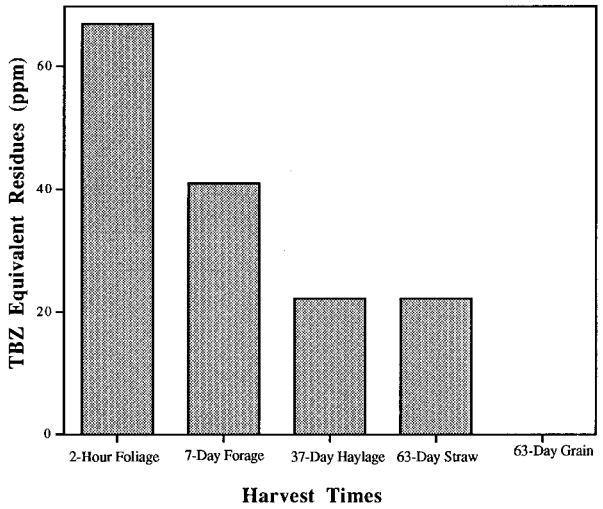


Figure 2. Concentrations of total [^{14}C]TBZ residues (ppm equivalents) in wheat at various harvest times after application (2-h foliage, 7-day foliage, 37-day haylage, 63-day straw and grain).

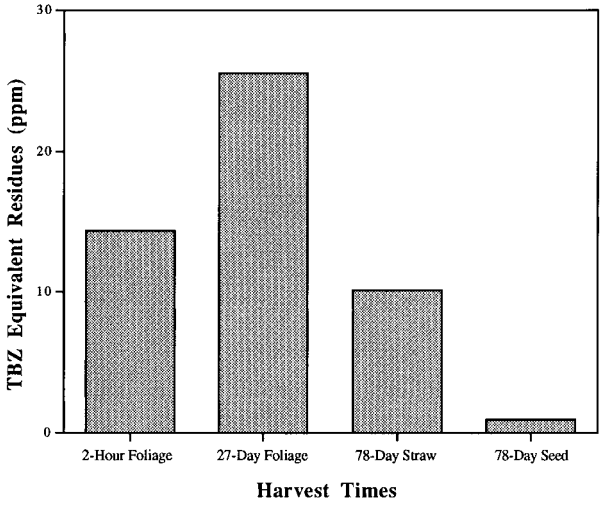


Figure 3. Concentrations of total [^{14}C]TBZ residues (ppm equivalents) in soybeans at various harvest times after final application (2-h foliage, 27-day foliage, 78-day straw and seeds).

Mature wheat straw had a residue level of 22.3 ppm, similar to that found for haylage wheat. Evidently, desiccation of the wheat plants by the time of final harvest had a significant effect on residue levels. Mature wheat grain was found to contain 0.121 ppm of [^{14}C]TBZ-equivalent residues, almost 200-fold less than that found in straw at the same time point. Thus, while high plant residues were found in the foliage samples (as expected, due to direct foliar application of the fungicide), relatively little was found to be translocated within the plants. The average residue level found in soybeans immediately after the first treatment was 14.3 ppm. This value increased to 25.4 ppm in 27-day

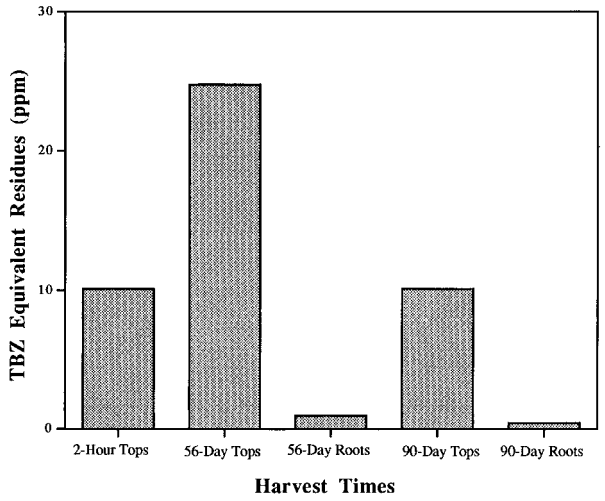


Figure 4. Concentrations of total [^{14}C]TBZ residues (ppm equivalents) in sugar beets at various harvest times after final application (2-h tops, 56-day tops and roots, 90-day tops and roots).

soybean forage and then decreased to 10.1 ppm in 78-day soybean straw. The increase between 2 h and 27 days resulted from the second application of TBZ to the plants, which occurred 13 days before the day-27 harvest. The subsequent decrease between 27 and 78 days was most likely due to growth dilution and dissipation by natural mechanisms. Soybean seeds were found to contain 0.877 ppm of TBZ-equivalent residues, more than 11-fold less than found in the straw. Thus, as with wheat, while some translocation within the plant was shown to occur, the extent of translocation was limited in magnitude. The average residue level found in sugar beet tops immediately after initial treatment was 10.1 ppm. This level rose to 24.6 ppm in immature sugar beet tops (56 days, immediately following the fifth and final application) and then dropped to 10.0 ppm in mature sugar beet tops (90 days). Immature sugar beet roots were found to contain only 0.855 ppm of TBZ-equivalent residues, almost 30 times less than found in the tops of the same harvest time. The sugar beet root residue level declined to 0.399 ppm at the time of mature harvest, approximately 25 times lower than the residue level found in mature sugar beet tops. High residues were found in plant tops (as expected, due to direct foliar application of the fungicide), but little was translocated within the plant to the roots.

Characterization of Residues in Immature Wheat, Soybean, and Sugar Beet Plants. *Wheat.* Information relating to the extractability and nature of these residues is presented in Table 2. TBZ was found to comprise the majority of the residue, and essentially all of the extractable residues, found in the 2-h and 7-day wheat samples. On the basis of TLC analysis, nearly all of the 2-h wheat residue (97.2%; 100% of the organic-extractable residue) was TBZ. With the 7-day residue, on the other hand, TBZ accounted for 79.3% (32.67 ppm) of the total radioactivity. The remainder of the 7-day ^{14}C residue was divided between the aqueous-soluble and nonextractable, tissue-associated fractions, which contained 6.8% (2.80 ppm) and 14.0% (5.77 ppm) of the total 7-day wheat residue, respectively. This indicated that degradation or metabolism had proceeded fairly quickly (within a week of application). As the aqueous-soluble and nonextractable residue fractions were minor, they were not examined by chromatography (rather, analogous fractions from later samples, in which these fractions constituted a larger proportion of the residue, were examined; see below).

Table 2. Distribution/Characterization of [^{14}C]TBZ Residues in Plants (Percentages of Total Radioactive Residues)

	Wheat[a] time after initial treatment				
				63-day	
fraction	2-h foliage	7-day forage	37-day haylage	straw	grain
organic extractable	97.2	79.3	46.3	60.1	41.5
TBZ	97.2	79.3	36.8	33.1	23.2
polars	ND[b]	0	3.2	19.1	18.3
aqueous soluble	1.1	6.8	39.3	23.1	13.7
polars	0	0	19.9	14.4	0
BNZ-related	0	0	23.1	33.5	18.3
nonextractable, tissue-associated	1.8	14.0	11.7	16.8	17.5

	Soybeans[c] time after initial treatment			
			78-day	
fraction	2-h foliage	27-day forage	straw	seeds
organic extractable	93.3	60.8	47.3	63.2
TBZ	93.3	60.6	43.6	42.9
polars	0	0	0	0
aqueous soluble	1.4	35.5	41.4	33.3
polars	0	1.4	7.3	0
BNZ-related	0	1.4	7.3	0
nonextractable, tissue-associated	5.4	3.8	11.2	1.0

	Sugar Beets[d] time after initial treatment				
		56-day		90-day	
fraction	2-h tops	tops	roots	tops	roots
organic extractable	91.1	54.1	56.4	28.5	29.0
TBZ	91.0	52.2	55.6	27.1	25.8
polars	0	<1	0	0	0
aqueous soluble	2.1	39.2	43.3	60.4	65.0
polars	0	11.5	6.8	14.1	10.8
BNZ-related	0	11.5	6.8	14.1	10.8
nonextractable, tissue-associated	6.9	6.8	0.2	11.0	6.0

[a] Total residues in foliage, forage, haylage, straw, and grain averaged 67.4, 41.2, 21.9, 22.3, and 0.121 ppm, respectively. [b] Not determined [c] Total residues in foliage, forage, straw, and seeds averaged 14.3, 25.4, 10.1, and 0.877 ppm, respectively. [d] Total residues in 2-h tops, 56-day tops and roots, and 90-day tops and roots averaged 10.1, 24.6, 0.855, 10.0, and 0.399 ppm, respectively.

[^{14}C]TBZ-equivalent residues in wheat haylage harvested 37 days after treatment averaged 21.9 ppm. As with the earlier samples, [^{14}C]TBZ comprised the majority of the organic-extractable residue; however, the percentage of organic-extractable residue was lower than that found for the earlier samples. The organic-extractable fraction was found to contain 46.3% (10.2 ppm) of the total 37-day residue. The percentage of the residue found to be nonextractable and tissue-associated was 11.7% (representing 2.56 ppm), which is similar to the percentage for the same fraction at day 7. The aqueous-soluble fraction presented the primary difference in extraction profile between the 7- and 37-day wheat samples. This fraction was found to contain 39.3% (8.62 ppm) of the 37-day wheat residue (an additional 2.6% or 0.570 ppm was found in a second aqueous-soluble fraction). For comparison, the aqueous-soluble fraction contained <10% of the total 7-day residue. These data suggest that a primary metabolic pathway for TBZ in wheat involves the production of aqueous-soluble materials, possibly conjugates.

The 37-day organic-extractable fraction was analyzed by HPLC. [^{14}C]TBZ was found to be the major component, along with small amounts of material in the more polar region of the radiochromatogram, plus low-level radioactivity spread over various retention times both before and after the TBZ peak, most likely representing multiple metabolites or degradates. TBZ represented 36.8% (8.07 ppm) of the total wheat haylage residue. In general, throughout the course of this work, the presence of radioactivity over a wide range of retention times was found. It was concluded that this radioactivity most likely represented close association between low-level TBZ metabolites or degradates and soluble matrix components. The aqueous-soluble fraction was lyophilized and the residue reconstituted in methanol for HPLC analysis. Polar materials predominated in this fraction, although there was a small amount of radioactivity spread over a fairly wide range of retention times in the nonpolar region of the radiochromatogram. It was shown, using mature wheat straw, that these polar materials are likely sugar conjugates and that [^{14}C]BNZ represented a large part of the aglycons (vide infra). No further characterization analyses were conducted on 37-day plants or their extracts. Incomplete HPLC recovery was a common problem with extracts containing large amounts of matrix materials (as was typical of aqueous-soluble fractions). As discussed below, low-level radioactive residues are associated with many endogenous plant components. This can result in the elution of components containing nonquantifiable amounts of radioactivity.

Soybean. As with wheat, [^{14}C]TBZ was found to comprise the majority of the ^{14}C residue in the 2-h and 27-day soybean samples. On the basis of TLC analysis, virtually all of the 2-h soybean residue (93.3%; 100% of the organic-extractable residue) was [^{14}C]TBZ. With the 27-day samples, on the other hand, TBZ accounted for 60.6% (15.4 ppm) of the total radioactive residue. The remainder of the ^{14}C residue was divided between aqueous-soluble and nonextractable, tissue-associated fractions, which contained 35.5% (9.03 ppm) and 3.8% (0.967 ppm) of the total 27-day soybean residue, respectively. Thus, within a month of the first application of TBZ (within 2 weeks of the second application), degradation or metabolism had proceeded to a significant extent. Table 2 presents the extraction results for 2-h and 27-day soybeans.

TLC analysis of the 2-h soybean organic-extractable fraction showed [^{14}C]TBZ to be the only radioactive material present. Characterization of the aqueous-soluble and nonextractable residues was not pursued (rather, analogous fractions from later samples were examined; see below).

The organic-extractable (3 N methanolic KOH) fraction of the 27-day soybean samples was found to contain 59.4% (15.1 ppm) of the total 27-day residue. HPLC analysis of the organic-extractable fraction showed [^{14}C]-TBZ to be the only radioactive component present. The aqueous-soluble fraction contained 27.2% (6.92 ppm) of the original 27-day soybean residue (an additional 8.3% or 2.11 ppm was found in the second aqueous-soluble fraction; see below). Lyophilization of the initial aqueous-soluble fraction, followed by reconstitution of the residue in methanol and HPLC analysis, revealed the presence of a radioactive component with a retention time shorter than that of TBZ, suggesting that it represented a polar metabolite, possibly a conjugate of BNZ (vide infra).

The percentage of the residue found to be nonextractable following the initial extraction was 13.4% (representing 3.41 ppm). This fraction was further extracted using refluxing 3 N methanolic KOH, releasing ~10% of the previously nonextractable residue (~1.3% of total residues) as organic-extractable material; TBZ was shown by HPLC to comprise the majority of the released residue. Thus, TBZ was shown to represent a total of about 60.6% (15.4 ppm) of the 27-day soybean residue. Refluxing methanolic KOH released the majority (61.9%) of the nonextractable fraction as aqueous-soluble materials, representing 8.3% or 2.11 ppm [^{14}C]TBZ equivalents relative to the initial 27-day residue. HPLC analysis (after lyophilization and reconstitution of the resulting material in methanol) again revealed the presence of the polar metabolite (later shown to be BNZ-related; see below). The remaining nonextractable, tissue-associated fraction contained only 3.8% (0.967 ppm) of the total original 27-day soybean residue and was not further investigated.

Sugar Beet. Nearly all of the total radioactive residue present in the sugar beet tops harvested 2 h following the initial application of [^{14}C]TBZ was organic extractable, and all of the extracted radioactivity exhibited the chromatographic behavior of TBZ (Table 2). However, 56 days after the initial treatment (i.e., 2 h following the fifth and final application of [^{14}C]TBZ), only 54% and 56% of the total radioactive residue in tops and roots, respectively, was organic extractable. This is not unexpected, as four of the five applications were made weeks prior to the extraction (Table 1) and thus most of the residue had been on the leaves and exposed to the environment for a long time. TBZ accounted for >95% of the extracted radioactivity with both 56-day tops and roots. With the 56-day samples about 40% of the total radioactive residue was characterized as aqueous soluble, far more than with the 2-h tops (2%) (Table 2). Nonextractable, tissue-associated radioactivity accounted for nearly 7% of the total residue with the 2-h and 56-day tops; very little radioactivity in the 56-day roots was associated with this fraction.

Characterization of Residues in Mature Wheat, Soybean, and Sugar Beet Plants. *Wheat.* This procedure began with a methanol extraction of wheat straw. On the basis of the HPLC radiochromatogram for the methanol extract, TBZ was the predominant radioactive component. In addition, the extract contained a significant amount (29.9%) of radioactivity more polar than TBZ (i.e., shorter retention time). The nonextractable, tissue-associated fraction was subjected to refluxing methanolic KOH. The HPLC radiochromatogram of the organic-extractable fraction obtained when the aqueous fraction was basic demonstrated the release of additional TBZ along with higher percentages of the polar materials previously noted. After acidification of each aqueous phase, another organic fraction was obtained. It contained polar radioactive material(s) of retention time 4−8 min, along with radioactivity spread over a wide range of retention times >16 min. It is likely that this latter, broad band of radioactivity represents TBZ-related material(s) closely associated with endogenous matrix components. Fractionation of the nonextractable, tissue-associated 63-day wheat straw residue confirmed the broad association with matrix materials (see below). Collection of the earlier-eluting polar fraction and reanalysis by HPLC indicated the presence of at least two compounds.

The aqueous-soluble fraction was lyophilized and reconstituted in methanol for HPLC analysis, which showed the presence of both polar metabolites (between 4- and 8-min retention times) and the typical low-level, less polar materials described above.

Table 2 presents the overall summary for the quantitative extraction of mature wheat straw. Organic-extractable material(s) accounted for 60.1% (13.4 ppm) of the mature wheat straw residue; 33.1% (7.39 ppm) of the total residue was shown to be [^{14}C]TBZ. Of the remaining organic-extractable radioactivity (27.0%), 19.1% was shown by HPLC to be polar material. The majority of the aqueous-soluble radioactivity was polar materials, later shown to be conjugates (see below).

The extraction procedure for mature grain began with a methanol extraction, which was found to release 23.2% (representing 0.028 ppm) of the total grain residue. HPLC analysis was hampered by the very low level of radioactivity present in wheat grain; TBZ was the only observed radioactive component in the methanol extract. This result is very similar to findings with the analogous straw extracts. As only a small amount of polar radioactivity was detected in the methanol extract of grain, no peak corresponding to this material was observed. The presence of such polar material(s) strengthens the noted similarity between mature grain and straw.

The nonextractable fraction, which contained 76.8% (0.093 ppm) of the grain residue, was subjected to refluxing methanolic KOH. Extraction of the aqueous fraction while basic yielded no radioactivity; only extraction under acidic conditions resulted in isolation of radioactivity. This latter fraction contained 18.3% of the original grain residue, representing 0.022 ppm of [^{14}C]TBZ equivalents. HPLC analysis of this fraction showed only a polar radioactive component with a retention time similar to that of the polar species found in wheat straw, which suggested that the grain also contained a BNZ conjugate. The aqueous-soluble fraction from the extraction under acidic conditions was found to contain only 13.7% (0.016 ppm) of the total radioactive residue in grain. Together these two fractions accounted for 32.0% of total radioactive residue in grain. The final nonextractable, tissue-associated fraction (after subjection to refluxing methanolic KOH) was found to contain only 17.5% (0.021 ppm). In addition, 27.3%, but representing only 0.033 ppm of [^{14}C]TBZ equivalents, was not accounted for after this treatment. Further investigation of mature wheat grain residues was not undertaken because of the very low residue levels remaining.

Soybeans. Mature soybean straw was extracted using methanolic KOH. The organic-extractable fraction was found to contain 44.6% (4.53 ppm) of the initial 78-day soybean straw residue, while the aqueous-soluble and nonextractable, tissue-associated fractions contained 34.3% (3.48 ppm) and 21.1% (2.14 ppm), respectively. HPLC analysis of the initial organic-extractable fraction showed TBZ as the major radioactive component. The minor radioactive components of the organic-extractable fraction represented a total of ~3% of the mature straw residue. The initial aqueous-soluble fraction was also analyzed by HPLC (after lyophilization and reconstitution of the residual material in methanol), which again revealed the presence of a polar metabolite. In addition, as was noted for all of the aqueous-soluble fractions analyzed by HPLC, a significant amount of radioactivity was spread over a wide range of retention times. This radioactivity is probably associated with many different substances (aqueous-soluble matrix components).

The nonextractable, tissue-associated fraction was further characterized following treatment with refluxing methanolic KOH. The resulting organic-extractable and aqueous-soluble fractions were found to contain 12.8% and 33.7% of the nonextractable fraction, respectively, representing 2.7% or 0.274 ppm and 7.1% or 0.720 ppm of TBZ equivalents relative to the original straw residue. Most of the organic-extractable fraction (~74%, representing 0.203 ppm) was shown to be TBZ by HPLC analysis. The aqueous-soluble fraction was shown to contain predominantly the same polar metabolite found in the previous aqueous-soluble fractions. The final nonextractable fraction contained 11.2% (1.14 ppm) of the mature soybean straw residue.

Mature soybean seeds were extracted using methanolic KOH. The organic-extractable residue was found to comprise 53.7% (0.471 ppm) of the initial 78-day soybean seed residue, while the aqueous-soluble and nonextractable tissue-associated fractions contained 21.9% (0.192 ppm) and 21.8% (0.191 ppm), respectively. HPLC analysis of the organic-extractable fraction indicated that TBZ was the only detectable radioactive component. TLC analysis of the organic-extractable fraction provided results that paralleled those from HPLC. On the basis of the HPLC data, TBZ comprised 42.9% (0.376 ppm) of the total mature soybean seed residue. The aqueous-soluble fraction was lyophilized and the residual materials were reconstituted in methanol prior to analysis by HPLC. Only the polar peak associated with BNZ conjugates was observed.

The tissue-associated fraction was further investigated by treatment with refluxing KOH in methanol. Most of the radioactivity associated with this fraction was released; the residue partitioned approximately equally between the organic-extractable (43.6% of the tissue-unextractable fraction, representing 9.5% or 0.083 ppm of TBZ equivalents in the original seed) and aqueous-soluble (52.3% of the tissue-unextractable fraction, representing 11.4% or 0.100 ppm of TBZ equivalents in the original seed) fractions. Only 1% of the original seed residue (representing 0.009 ppm) remained associated with the tissue after exhaustive extraction.

Thus, TBZ represented very similar percentages of the total radioactive residue in mature straw (43.6%, representing a total of 4.42 ppm) and mature seeds (42.9%, representing 0.376 ppm). However, straw was found to contain significantly more tissue-unextractable residue (11.2%) than seeds (1.0%). Both straw and seeds were found to contain aqueous-soluble polar metabolite(s), which were shown to be mainly BNZ-related (see below).

Sugar Beets. In a qualitative sense, the results found for 90-day sugar beets parallel the results from the 56-day samples closely (Table 2). TBZ was found to be the only significant component of the organic-extractable residue from leaves or roots. The aqueous-soluble fractions contained primarily a very polar component, similar in retention behavior to the component identified in the wheat and soybean studies as conjugate(s) of BNZ. In a quantitative sense, the organic-extractable fractions from the 90-day plants represented smaller percentages of the total radioactive residues in tops and roots compared to 56-day plants, while the aqueous-soluble and nonextractable, tissue-associated fractions contained increased percentages.

Isolation and Identification of Residues. Larger scale extractions of wheat and soybean straw permitted isolation of sufficiently large quantities of TBZ-related compounds for further investigation and identification.

HPLC analysis of the initial organic-extractable fractions indicated that TBZ was the major radioactive component. Preparative HPLC allowed for the isolation of quantities of TBZ from both wheat straw and soybean straw, and its identity was confirmed by GC/MS. The major fragmentation pathway for TBZ involves elimination of HCN from the thiazole ring to give an ion of *m/z* 174 (Ellsworth et al., 1976; VandenHeuvel et al., 1977). Loss of 27 mass units is also observed in the mass spectrum of BNZ, but in this case the HCN is likely expelled from the imidazole ring. The presence of M − 27 ions facilitates the recognition of TBZ-related compounds by GC/MS.

The aqueous-soluble TBZ-related residues isolated via the larger scale extractions were also investigated. With respect to the samples from wheat, the fraction was acidified and further extracted with ethyl acetate. HPLC analysis gave results very similar to those found with the previously discussed fractions. Lyophilization and HPLC analysis gave a similar result. Both of these extracts were purified by HPLC, with collection of the radioactivity eluting between 2 and 8 min. The collected fractions were combined, concentrated, and again purified by HPLC. In this case two zones were collected, the region from 3.6 to 5 min, "polars A", and the region from 5 to 11 min, "polars B". The majority of the radioactivity was associated with polars A. The major peak found in polars B had a retention time similar to that of BNZ under the HPLC conditions used. Each of these fractions was further purified using TLC.

Polars A, the bulk of the polar radioactivity, was divided into two portions for further investigation. A glucosidase enzyme hydrolysis of one aliquot of polars A was conducted in an attempt to effect further characterization. HPLC analysis of the resulting hydrolysate showed a radioactive component with a retention time similar to that of BNZ. This suggested that polars A consisted of one or more sugar conjugates of BNZ.

A second aliquot was derivatized with acetic anhydride in pyridine. HPLC analysis of the reaction product indicated the presence of a single radioactive component of longer retention time (hence lower polarity) than BNZ—presumably an acetyl derivative. The minor polar fraction, polars B, was also subjected to acetic anhydride in pyridine. Subsequent HPLC analysis indicated the presence of a radioactive component with the same retention time as the derivative for polars A, as well as several minor radioactive components. One of the latter coincided in retention time with BNZ under the gradient conditions used to analyze the derivatives. Treatment of BNZ under the same derivatization conditions gave a major component that possessed the same retention time as the major acetylation product of polars A and B. Thus, acetylation of both the polar fraction (polars A) and the polar metabolite suspected to be BNZ (polars B) gave the same major acetyl derivative, suggesting either that two different acetyl derivatives had the same retention time or that acetic anhydride in pyridine effected hydrolysis of BNZ conjugate(s) prior to formation of the acetyl derivative.

Each acetyl derivative was treated with glucosidase. On the basis of HPLC, BNZ was the product of both reactions. Thus, glucosidase hydrolysis conditions effected the cleavage of BNZ acetate (from polars B) as well as the polars A acetate (a derivatized sugar conjugate or BNZ acetate). The identity of the BNZ liberated from polars A was confirmed using GC/MS. The isolated metabolite possessed the same retention time and mass spectrum (M, *m/z* 118; M − 27, *m/z* 91) as authentic BNZ. BNZ was also released from the polars A acetate by treatment with potassium carbonate solution.

The aqueous-soluble TBZ-related residues isolated from soybean straw were thought to contain BNZ−sugar conjugates, on the basis of their HPLC and hydrolytic behavior and comparison to the results from wheat straw. The aqueous-soluble radioactive fractions were lyophilized and reconstituted in methanol and then further purified using C_{18} solid phase extraction (SPE) columns. The initial eluate (unretained material) was collected; the radioactive material remaining on the column was eluted with methanol. Both of the fractions collected from the SPE columns were examined by HPLC with radioactivity detection. The unretained eluate contained the polar metabolites, which were combined and subjected to acetylation conditions (acetic anhydride/pyridine, room temperature, 20 h) in an attempt to convert the polar compound(s) to less polar species. Following the acetylation step, two less polar components represented the majority of the radioactivity, while a third component was found at a retention time that coincided with BNZ under the HPLC conditions used. The collected radioactive fractions were extracted using ethyl acetate and repurified using preparative HPLC. The purified acetyl derivatives were then treated with glucosidase to effect the removal of any conjugated sugars. HPLC analysis of the resulting mixture showed a radioactive component with a retention time similar to that of authentic BNZ. GC/MS analysis of the same fraction confirmed the presence of a compound that possessed the retention time behavior and mass spectrum of BNZ. The metabolic fate of TBZ in soybeans thus parallels that found in wheat.

Jacob et al. (1977) reported that only unchanged TBZ was recovered from sugar beet plants treated with TBZ and grown under artificial light. However, when grown in sunlight, BNZ and benzimidazole-2-carboxamide (BNZ-$CONH_2$), as well as TBZ, were identified as residue components. Likewise, photolysis experiments using thin films of TBZ on glass plates confirmed the photolytic decomposition of TBZ to both BNZ and BNZ-$CONH_2$. The results of the present study have shown the presence of BNZ and BNZ conjugates in wheat and soybean plants treated foliarly with TBZ. On the basis of the literature precedent, it is likely that the BNZ found in wheat and soybeans was formed photolytically from TBZ, with the conjugation of BNZ occurring enzymatically within the plant. BNZ has also been shown to be a minor metabolite of TBZ in animals (Chukwudebe et al., 1994). The major metabolic pathway for TBZ in animals is hydroxylation at the 5-position followed by conjugation through the hydroxyl group (Tocco et al., 1964, 1965, 1966; Chukwudebe et al., 1994); neither this compound nor BNZ-$CONH_2$ was found in the current study.

Fractionation of the Tissue-Unextractable 63-Day Wheat Straw Residue. Even after extractions using methanolic KOH, the tissue-associated fraction of 63-day wheat straw contained an average residue level of 3.76 ppm ([^{14}C]TBZ equivalents). This residue (16.8% of the initial wheat straw residue) was fractionated using sequential hydrolytic techniques in an effort

Table 3. Distribution of [^{14}C]TBZ Equivalents in the Nonextractable, Tissue-Associated Fraction of 63-Day Mature Wheat Straw after Fractionation

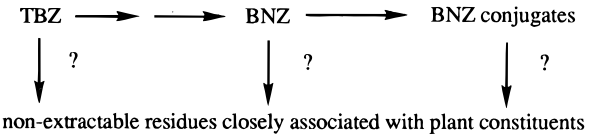
	aqueous-soluble	
description	ppm	%
tissue-unextractable residue	3.76	16.8
potassium phosphate buffer	1.97	8.8
α-amylase	0.134	0.6
Pronase E	0.179	0.8
EGTA	0.045	0.2
dioxane/2 N HCl	0.112	0.5
24% aqueous KOH	0.134	0.6
72% aqueous H_2SO_4	0.201	0.9
nonextractable, tissue-associated	0.022	0.1
unaccounted for	0.894	4.0

to further characterize the nature of the unextractable residue. The fractionation procedure involved solubilization and enzymatic and chemical hydrolysis. The initial procedures were quite mild, but the subsequent ones were very vigorous. The methods were employed in the following sequence: solubilization with potassium phosphate buffer (PPB), for release of soluble polysaccharides and proteins; α-amylase enzyme hydrolysis, for release of starch; Pronase E (a general protease) enzyme hydrolysis, for release of protein-associated residues; ethylene glycol bis(2-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) in sodium acetate buffer, for release of soluble pectins; 2 N HCl in dioxane, for release of lignins; 24% aqueous KOH, for release of noncellulosic polysaccharides; and 72% aqueous H_2SO_4, for release of cellulose. Table 3 presents the distribution of radioactive residues into the soluble fractions after each of these steps. By far, the largest amount of the unextractable residue was released in the initial solubilization step. The aqueous-soluble fraction from PPB solubilization was concentrated and analyzed by HPLC. This fraction apparently contained the same rapidly eluted polar conjugate found in the previous aqueous-soluble fractions (see above).

Each additional step in the fractionation procedure released a small amount of the unextractable residue, although no single fraction accounted for more than 6% of the tissue-associated residue (no more than 1% of the original wheat straw residue). The nonextractable, tissue-associated residue found after exhaustive fractionation was only 0.02 ppm, and no further characterization was undertaken.

Conclusions. Following foliar application of [^{14}C]-TBZ to wheat, soybean, and sugar beet plants, total radioactive residues diminish with time after final application and plant maturation. Residue levels in mature plants are much lower for grain, seeds, and roots than those found in the corresponding straw and leaves, the asymmetric distribution demonstrating little translocation of TBZ-related residues into storage tissues. Using organic extraction techniques, residues in plant tissues from applied [^{14}C]TBZ were broadly fractionated into three phases: organic extractable, aqueous soluble, and nonextractable, tissue associated. With each of the three species, the organic-extractable fractions, consisting predominantly of TBZ, diminished with maturation of the plants, whereas the levels of aqueous-soluble (and nonextractable, tissue-associated) fractions increased with plant maturation. These changes in concentration strongly suggest the conversion of TBZ into polar metabolites/degradates with increasing exposure time to environmental and metabolic factors. TBZ is known to undergo photodegradation to BNZ (Jacob et al., 1975). Metabolic transformations in animals include hydroxylation at the 5-position followed by conjugation through the 5-hydroxyl group (Tocco et al., 1964, 1965, 1966; Chukwudebe et al., 1994) and elimination of the thiazolyl group to produce BNZ (Chukwudebe et al., 1994). On the basis of chromatographic methods, the aqueous-soluble radioactive components from these three plant species are similar. Derivative formation and use of enzymatic hydrolysis suggest that the polar species are conjugates of BNZ, with the presence of the latter confirmed by GC/MS. Fractionation of the nonextractable residue in wheat straw showed that the radioactivity was associated with endogenous tissue components (e.g., protein, starch, pectin, and cellulose), no single fraction accounting for more than 1% of the original wheat straw residue.

Therefore, the fate of foliarly applied TBZ in wheat, soybeans, and sugar beets involves transformation of this fungicide to aqueous-soluble species, primarily conjugates of BNZ, and a tight association of residues with various endogenous components of plants. With these three plant species, TBZ comprises nearly all of the unconjugated plant residues and is the only significant residue component in the organic extractable fractions. There is little translocation of TBZ or BNZ conjugates into grain, seeds, or roots. A proposed pathway for the metabolism/degradation of TBZ in three representative plants is presented below.



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