Bioconcentration and Metabolism of Thiabendazole [2-(4-Thiazolyl)-1*H*-benzimidazole] in Bluegill Sunfish, *Lepomis macrochirus*

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The steady-state bioconcentration of thiabendazole (TBZ) in the bluegill sunfish was determined in a dynamic (flow-through) bioconcentration test. Fish were exposed to 2.24 ppb of ^{14}C-labeled TBZ. The TBZ concentration was monitored in both the fish and water during a 28-day uptake period and the subsequent 14-day depuration period. Uptake rate constants (k_1), depuration rate constants (k_2), and steady-state bioconcentration factors (BCF, k_1/k_2) were calculated for whole fish, viscera, and edible tissues. The resulting BCF values were 96.5 (whole fish), 637.6 (viscera), and 23.0 (edible portion). Clearances of TBZ residue of >95% for the whole fish and >98% for the viscera occurred by the end of the depuration period. Homogenates of edible and visceral tissues were incubated with Glusulase; the ethyl acetate extracts of the incubates were purified and analyzed by HPLC for TBZ and 5-hydroxy-TBZ (5-HO-TBZ), the major mammalian metabolite of TBZ. Of the radioactivity isolated from the edible tissues, 59% was associated, on the basis of its HPLC behavior, with TBZ; smaller portions (32 and 7%, respectively) were associated with 5-HO-TBZ and an unidentified TBZ-related compound. With respect to the radioactivity isolated from the visceral tissues following sequential use of Glusulase and acid hydrolysis, approximately half was associated with 5-HO-TBZ and ~8% with TBZ. Low-level peaks of radioactivity were observed at five other retention times; these unidentified species accounted for about 28% of the radioactivity isolated from the visceral tissues, with none accounting for more than 10% of the isolated radioactivity.

Keywords: *Thiabendazole; bluegill sunfish; bioconcentration; metabolism; 5-hydroxythiabendazole*

INTRODUCTION

Thiabendazole [2-(4-thiazolyl)-1*H*-benzimidazole, TBZ], Figure 1, initially developed as an anthelmintic agent for use in food-producing animals (Brown et al., 1961), also possesses fungicidal activity (Robinson et al., 1969). It is thus used as the active ingredient in a variety of preparations (Spencer et al., 1981) currently employed for treatment of plant-derived food products to prevent their deterioration and, to a small extent, for applications to foliage (Edgington et al., 1971; Logan et al., 1975; Meredith, 1977). The possibility exists that agricultural application of a fungicide could result in introduction of the agent into nearby bodies of water, leading to its uptake by aquatic organisms. The 1-octanol/water partition coefficient (K_{ow}) of a compound is often the primary screening value for initial prediction of bioconcentration in aquatic organisms (Barron, 1990). It is assumed that hydrophobicity, as indicated by the K_{ow}, is the principal determinant for the potential of a chemical to bioconcentrate in fish (Barron, 1990). On the basis of laboratory experiments, mathematical relationships have been derived to estimate bioconcentration (e.g., Chiou, 1977; Neely et al., 1974; Veith et al., 1979; McCarty, 1986). Using a regression equation derived by Veith et al. (1979)

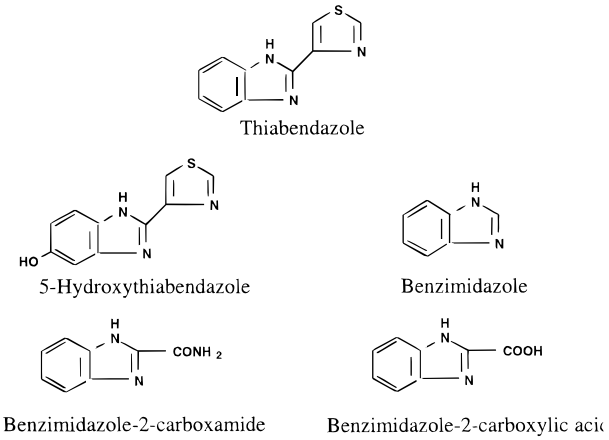

Figure 1. Structures of TBZ, 5-HO-TBZ, benzimidazole, benzimidazole-2-carboxamide, and benzimidazole-2-carboxylic acid.

$$\log \mathrm{BCF} = 0.76 \log K_{ow} - 0.23$$

we estimate the BCF of TBZ to be approximately 33. 1-Octanol/water partitioning is but a simple laboratory measurement, and it is generally recognized that the correlation of K_{ow} with bioconcentration is only an

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approximation (Opperhuizen et al., 1988; Swackhamer and Hites, 1988; Barron, 1990). Another physical property also looked upon as a predictor of bioconcentration is water solubility. TBZ possesses a log K_{ow} of 2.3 and a relatively low water solubility of only 28 ppm at neutral pH.

Models based on regression equations may not be predictive of compounds outside the chemical classes of the original data bases. Further, models are useful mainly for neutral organic compounds. TBZ is ionizable and likely would not be expected to have a predictable bioconcentration factor (BCF). The tendency for a chemical to accumulate in an aquatic species can be best estimated by actually determining its BCF. This is clearly a superior approach, as it generates data from an actual biological system. Determination of the BCF is a key element of an environmental risk assessment (Barron, 1990). To assess whether [^{14}C]TBZ will be bioconcentrated by a representative fresh-water fish, the bluegill sunfish (*Lepomis macrochirus*), and, if so, how rapidly it would be depurated, a 42-day dynamic study was undertaken.

TBZ is known to undergo metabolism to form the phenol 5-hydroxy-TBZ (5-HO-TBZ; Figure 1) in numerous species of farm and laboratory animals and humans, usually with subsequent conjugation to the *O*-sulfate and/or *O*-glucuronide (Tocco et al., 1964, 1965, 1966; Chukwudebe et al., 1994). The hydroxylation of aromatic rings of endogenous compounds and xenobiotics has also been observed to occur in fish, e.g., rainbow trout and cod (Kleinow et al., 1987; Stegeman, 1989; Goksøyr and Förlin, 1992), as has the formation of glucuronide and sulfate conjugates of phenols (goldfish, rainbow trout; Kleinow et al., 1987; Kleinow, 1995). Metabolic alteration of the parent compound by fish to more-polar species can have an impact upon bioconcentration (Barron, 1990). Because of the known metabolism of TBZ in mammals and avians, and the ability of aquatic species to form conjugated phenols, homogenates of the sunfish tissues were exposed to hydrolysis conditions prior to extraction. The resulting isolates were examined by HPLC with off-line radioactivity monitoring using TBZ, 5-HO-TBZ, and several other TBZ-related compounds (Figure 1) as reference standards to investigate the metabolism of this agent in the sunfish.

MATERIALS AND METHODS

The bluegill sunfish had initial weights of ~3−5 g and initial lengths of ~6−7 cm. Pumps were used for the intermittent introduction of the stock solution of [^{14}C]TBZ ([*U*-^{14}C]phenyl; 57.4 μCi/mg; 99.0% radiochemical purity) in dimethylformamide (DMF) and diluent (control) water, resulting in a mean test concentration of 2.24 ± 0.09 μg/L during the 28-day uptake phase. Only the aqueous DMF was introduced into the control aquarium during the uptake phase; diluent water only was introduced into the two aquaria during the depuration phase. The test and control aquaria (the volume of liquid in each was ~94 L; 22 ± 2°; pH 7.0−8.5; oxygen concentration 7.4 ± 0.5 mg/L) received flows (~266 mL/min) resulting in approximately four volume changes per day throughout the study. The uptake phase of the study was initiated by introducing 86 fish into the test aquarium and a like number into the control aquarium. Water and fish (five at each time point) were sampled from both the test and control aquaria on days 0, 3, 7, 14, 21, and 28 during the uptake phase; in addition, on both days 21 and 28 an additional 10 fish were collected from the test and control aquaria for characterization of radioactivity in the tissues. Addition of [^{14}C]TBZ to the test aquarium was terminated on day 28 of the uptake phase, and the water was replaced by control water. The fish in both aquaria were then exposed to flowing control water for the 14-day depuration phase, during which fish and water were sampled on days 1, 3, 7, 10, and 14. Of the fish that were collected at each of the sampling times, three of the five were dissected and separated into edible tissues (muscle, skin, and skeleton) and visceral tissues (internal organs only). The collected edible and visceral tissues were weighed, composited, and then homogenized using a small volume of water. A portion of each homogenate was measured out and combusted to produce CO_2 (Packard Model 306 sample oxidizer), which was trapped and quantified for carbon-14 content by liquid scintillation counting (Packard Tricarb Model 4530 liquid scintillation spectrometer). The remaining two fish from the five fish taken at each time point were weighed, combined, and homogenized. Weighed portions of each homogenate were analyzed for carbon-14 content as described above. The limits of detection (LOD), in terms of nanogram equivalents of TBZ per gram of tissue, were 2.3 for visceral and 1.9 for edible tissues, respectively. Water samples from both aquaria and for each time point were analyzed for carbon-14 content by liquid scintillation counting (Ready-to-Use II Cocktail made by Eastman Kodak Co.). The LOD for nanogram equivalents of TBZ per milliliter was 0.02.

Edible tissues from the additional fish collected on day 28 and visceral tissues from the fish collected on days 21 and 28 of the uptake phase were processed for HPLC-based characterization of the radioactive compounds present in them. Portions of the homogenates were weighed into centrifuge tubes, 0.1 M sodium acetate solution was added, and the pH was adjusted to 5.0. Glusulase enzyme preparation, glucuronidase to sulfatase activity ratio 13.5 to 1 (NEN Products, Du Pont), was added to each sample, and the mixture was shaken. A drop of toluene was then added to each tube and the sample incubated overnight in a water bath at 37 °C. The sample was then heated at 60 °C for 5 min, the pH was adjusted to 6.3, and the sample was extracted with ethyl acetate. Following centrifugation, the ethyl acetate layer was removed and the extraction repeated two more times (pH readjusted to 6.3, if required). The three ethyl acetate extracts were combined, and the residual tissue was saved for counting and posssible later treatment. The ethyl acetate solution was extracted with 0.1 M HCl. Following centrifugation, the aqueous layer was transferred to a centrifuge tube; the extraction was repeated, and the two aqueous layers were combined. This acidic solution was then washed, successively, with ethyl acetate, dichloromethane/hexane, and hexane. These wash solutions were retained for counting. The pH of the HCl phase was adjusted to ~7.2 (Na_2HPO_4) and the solution extracted with ethyl acetate. The resulting organic phase was taken to dryness (N_2) and the residue reconstituted in 0.5 mL of dimethylformamide (DMF) containing unlabeled TBZ and other reference compounds (benzimidazole, benzimidazole-2-carboxylic acid, benzimidazole-2-carboxamide, and 5-HO-TBZ). These compounds were added to the reconstituted sample to provide UV-detectable retention time markers for the recognition of [^{14}C]TBZ-related compounds during the HPLC analysis of the radioactive isolates. One-half milliliter of water was added to the DMF-solubilized samples, which were then analyzed by HPLC as described below.

The visceral tissue samples retained after their ethyl acetate extraction were subjected to refluxing dilute HCl (pH 1.0−1.5) for 1 h; the pH of the solution was readjusted (if necessary) to 1.0−1.5 and the hydrolysis procedure repeated. The sample was cooled to room temperature, its pH was adjusted to 6.3 (NaOH), and then the sample was extracted with ethyl acetate. This organic extract was subjected to the same procedure as the ethyl acetate extracts from the Glusulase-treated tissue samples, ultimately yielding analogous isolates for HPLC analysis.

Samples of liquid extracts, wash solutions (excluding the HCl solutions), and HPLC fractions were added to Ready-to-Use II scintillation cocktail made by Kodak. Samples of the HCl solutions were added to Trucount scintillation cocktail. Measurement of radioactivity was achieved using a Packard Model Tricarb-460 CD. Samples of residual fish tissue homo-

Table 1. Summary of [^{14}C]Thiabendazole-Related Concentrations (ng equiv/g of Tissue) of Radioactivity in Sampled Fish Tissues[a] and Water[b]

day	whole fish	edible portion	viscera	water
		Uptake Phase		
0	7.7 ± 0.3	4.5 ± 1.6	36.8 ± 0.9	2.20 ± 0.05
3	192.6 ± 1.6	38.1 ± 0.9	1428.9 ± 13.3	2.24 ± 0.01
7	183.6 ± 2.7	46.0 ± 0.9	842.0 ± 279.4	2.28 ± 0.07
14	159.1 ± 34.4	41.0 ± 1.2	867.8 ± 41.3	2.27 ± 0.02
21	187.1 ± 2.1	40.2 ± 3.1	1483.6 ± 1.9	2.09 ± 0.02
28	198.8 ± 1.5	71.1 ± 0.8	1095.8 ± 3.0	2.35 ± 0.08
		Depuration Phase		
1	250.5 ± 2.7	28.9 ± 0.6	2028.8 ± 25.7	ND[c]
3	80.6 ± 0.4	19.6 ± 0.5	715.6 ± 4.3	0.05 ± 0.04
7	16.2 + 0.8	12.2 ± 1.5	65.4 ± 2.0	ND[c]
10	10.7 ± 6.2	13.2 ± 0.3	31.5 ± 5.8	0.04 ± 0.04
14	10.6 ± 0.4	6.1 ± 1.8	21.6 ± 0.5	0.06 ± 0.02

[a] Each value represents a mean of three replicates of a single homogenate, limits of detection of 1.9 and 2.3 ppb for edible and visceral tissues. [b] Each value represents a mean of four replicates, limit of detection of 0.02 ppb. [c] Not detected (less than the detection limit).

Table 2. Uptake (28 Days; k_1) and Depuration (14 Days; k_2) Rate Constants and Calculated (k_1/k_2) BCF Values[a]

tissue	k_1 (day^{-1})	k_2 (day^{-1})	BCF
whole fish	29.90	0.31	96.5
edible portion	7.59	0.33	23.0
viscera	153.03	0.24	637.6

[a] Based on concentrations of TBZ-related radioactivity.

genates were solubilized using Unisol tissue solubilizer (Isolab, Inc.) for 24 h or until the sample was visually homogeneous; methanol and Unisol Complement scintillator solution were added, and the sample was counted.

The radioactive isolates were examined using a reverse phase column (Supelco 25-cm LC18-DB) with a Varian 5060 liquid chromatograph, Perkin-Elmer ISS-100 auto sampler, and a Kratos 757 UV detector (254 nm). A gradient solvent system (0.01 M potassium phosphate, pH 7.0; methanol) was employed at a flow rate of 1 mL/min. The column eluate was collected in 1-mL fractions using a Pharmacia Frac-100 fraction collector, and their radioactivity content was measured as described above. A single radioactive zone at a given retention time does not, of course, guarantee that the radioactivity is associated with a single labeled compound. For the purposes of this work, however, a radioactive peak is assumed to represent one compound.

RESULTS AND DISCUSSION

The mean water concentration of [^{14}C]TBZ, as determined by radiochemical analysis, of the exposure solution (uptake phase) was 2.24 ± 0.09 ppb, with no value deviating more than 6.7% from the mean. Summaries of the total concentrations (means and standard deviations) of [^{14}C]TBZ-related radioactivity in fish tissues at each sampling time are presented in Table 1. The uptake (k_1) and depuration (k_2) rate constants, and the corresponding BCF values (calculated from k_1/k_2), were calculated from the water and tissue concentrations of TBZ-related radioactivity for each time point using the "ProcNLIN" program for the SAS Statistical Software System (SAS, 1985). The rate constants calculated using the program are presented in Table 2.

The kinetic model used to determine k_1 and k_2 from the raw data predicts that by approximately 10 days into the depuration period the concentration of TBZ-related compounds in the whole fish should be reduced by 95%. Greater than 95% clearance of the radioactivity from the whole fish was observed by 14 days into the depuration phase. Among the three tissue types, the viscera, with a calculated BCF of ~637 (measured value at day 28, 466.3), accumulated TBZ-related compounds to the greatest extent. Although the bioaccumulation of these materials was much greater than that in either the whole fish or the edible tissues, well over 50% of the total radioactive residue was cleared from the viscera by only 3 days into the depuration period, and there was greater than 98% clearance by 14 days. The bioconcentration of radioactivity in viscera likely results from the presence in internal organs of residues which undergo rapid depletion when exposure to TBZ ceases. The very low BCF (~23) in edible tissues indicates that TBZ does not accumulate in tissue that would be consumed by humans. Further, the low BCF in whole fish (~96) demonstrates that TBZ is unlikely to accumulate in fish consumed by fish predators. The corresponding measured day 28 values are 30.3 and 84.6, respectively. Under laboratory conditions it is apparent that TBZ residues do not persist to a significant extent in bluegill sunfish and would not be expected to biomagnify in the food chain.

Seventy-one percent of the [^{14}C]activity in the day 28 edible tissue homogenate was extracted by ethyl acetate following treatment with Glusulase. The reconstituted sample for HPLC analysis, arising after a series of cleanup steps from the initial extract, contained 57% of the total radioactive tissue residue. In all, 93% of the radioactivity present in the original sample was accounted for in the tissue homogenate (after extraction), side-stream extracts, washes, and reconstituted samples. HPLC analysis of the isolated [^{14}C]activity from the day 28 edible tissues and counting of the collected HPLC fractions demonstrated that the radioactivity was distributed among three components. On the basis of comparisons with retention times of the cochromatographed reference standards, the radioactivity in the reconstituted samples consisted of TBZ (59%) and 5-HO-TBZ (32%) and an unidentified compound, X (6%).

Forty-two percent of the [^{14}C]residues in the day 28 visceral tissue homogenates were extracted following exposure to Glusulase, and 28% were isolated in the reconstituted samples (compared to 71% and 57% for the corresponding day 28 edible tissues, respectively) (see Table 3). The enzyme-hydrolyzed/extracted visceral tissue homogenate was exposed to hydrolysis conditions a second time (refluxing 0.1 M HCl) and then extracted. This yielded an additional 16% of the initial tissue radioactivity. Using the combination hydrolysis/extraction procedure, 58% of the [^{14}C]activity present in the original homogenate was extracted and 36% isolated in the reconstituted samples used for HPLC. Even though 42% of the radioactivity in the visceral tissues was not

Table 3. Radioactivity Balances for Homogenized Fish Tissues

		viscera[b]					
		21-day			28-day		
	28-day edible[a]	Glusulase	HCl	total	Glusulase	HCl	total
% extracted (ethyl acetate)[c]	71	41	13	54	42	16	58
% reconstituted sample[d]	57	22	9	31	28	8	36
% overall recovery[e]	93			95			95

[a] Glusulase only. [b] Glusulase and HCl. [c] The percentage of the total radioactivity in the tissue sample homogenate extracted into ethyl acetate. [d] The percentage of the total radioactivity in the tissue sample homogenate present in the final isolate used for HPLC. [e] Total accountability of all radioactivity initially present in the tissue sample homogenate.

Table 4. Radiochemical HPLC Analysis of Radioactivity Isolated from Homogenized Sunfish Tissues[a]

	28-day edible			21-day and 28-day viscera				
					HPLC fractions containing radioactivity		% of the total radioactivity recovered in all 42 fractions	
compound	t_R, min (UV)	HPLC fractions containing radioactivity	% of the total radioactivity recovered in all 42 fractions	t_R, min (UV)	Glusulase	HCl	Glusulase	HCl
benzimidazole-2-carboxylic acid[b]	~8			~8		8−9		6−8
unknown W				~11	9−15		10−13	
unknown Y				~19	18−20		4−5	
benzimidazole[b]	~21			~21				
unknown X	~23	23−24	6	~23	22−24	21−23	7−8	15−18
benzimidazole-2-carboxamide[b]	~25			~25				
5-hydroxythiabendazole[b]	~28	27−29	32	~28	27−32	28−29	57−64	13−14
unknown Z				~32		31−34		17−19
thiabendazole	~36	36−37	59	~36	36−37	36−37	4−6	14−17

[a] Only peaks resulting from fractions containing >50 dpm are listed. Percent HPLC recoveries of radioactivity >95. [b] Unlabeled reference standard.

extracted, clearance was rapid and elimination nearly complete at the end of the 14-day depuration period. In all, 95% of the radioactivity present in the original homogenate was accounted for in the residual extracted homogenate, side-stream extracts, washes, and reconstituted samples.

HPLC analysis of the [^{14}C]activity isolated from the day 28 visceral tissues and counting of the collected HPLC fractions showed few discernible peaks corresponding to components with fractions containing significant amounts (>50 dpm) of radioactivity. The reconstituted Glusulase/extraction samples contained a total of five. On the basis of retention time comparisons with cochromatographed reference compounds and the radioactivity recovered in all HPLC fractions, the radioactivity was associated with TBZ (4%), 5-HO-TBZ (64%), and unknown compounds W (12%), Y (4%), and X (8%).

The reconstituted samples resulting from extraction and cleanup following acid hydrolysis of the previously treated (Glusulase/extraction) tissue homogenates also contained five radioactive HPLC components. On the basis of retention time comparisons with the cochromatographed reference standards, these TBZ-related compounds (and percent of total radioactivity recovered from the column for each component) were TBZ (15%), 5-HO-TBZ (13%), unknown X (16%), unknown Z (19%), and a compound with the retention time of benzimidazole-2-carboxylic acid (8%). The latter is unstable, known to undergo facile degradation to benzimidazole in solution (Sanson and VandenHeuvel, unpublished observation). A closely related 5-substituted benzimidazole-2-carboxylic acid also undergoes decarboxylation in solution (VandenHeuvel et al., 1978). It is thus unlikely that benzimidazole-2-carboxylic acid would survive the isolation procedure intact. Further, no radioactivity with the retention behavior of benzimidazole was detected in the isolates by HPLC. For these reasons it can be reasonably concluded that benzimidazole-2-carboxylic acid should not be present in the reconstituted sample prepared for HPLC.

The radioactivity balance for the day 21 fish visceral homogenate derived samples was very similar to that observed for the day 28 visceral tissues (see Table 3). The reconstituted samples arising from Glusulase and HCl hydrolysis of these day 21 tissues gave HPLC results, both qualitatively and quantitatively, also essentially the same as those for the day 28 samples.

A greater proportion of the [^{14}C]activity was extracted by ethyl acetate from the enzyme-hydrolyzed day 28 edible tissues (71%) than from the similarly treated day 21 and day 28 visceral tissues (41−42%) (see Table 3), suggesting for the latter increased polarity of metabolites and/or greater interaction of the TBZ-related species with the tissue matrix. As shown in Table 4, nearly three-fifths of the [^{14}C]activity extracted from the edible tissues was TBZ, with lesser amounts of 5-HO-TBZ (about one-third) and an unknown metabolite, X. As the unknown compound possessed a shorter retention time than 5-HO-TBZ using reversed phase HPLC, it is presumably a more-polar metabolite of TBZ than is 5-HO-TBZ. Of the [^{14}C]activity extracted from the enzyme-treated visceral tissue homogenates, 5-HO-TBZ was the principal component (~60%) in both the day 21 and day 28 reconstituted samples. Thiabendazole was also present (~5%) in these extracts arising from visceral tissues, as were several more-polar (based on HPLC behavior) unknown compounds: W, Y, and X (~12, ~5, and ~7%, respectively). With respect to the radioactivity (an additional ~15%) extracted by ethyl acetate from the HCl-treated residual visceral tissue homogenate (exposed previously to Glusulase), no predominant radioactive components were found by HPLC with radioactivity monitoring of the effluent. Rather, four compounds were found in approximately equal amounts (13−19% of the total radioactivity recovered in all HPLC fractions)—unknowns X and Z, TBZ and 5-HO-TBZ—plus one unknown (~7%) possessing the

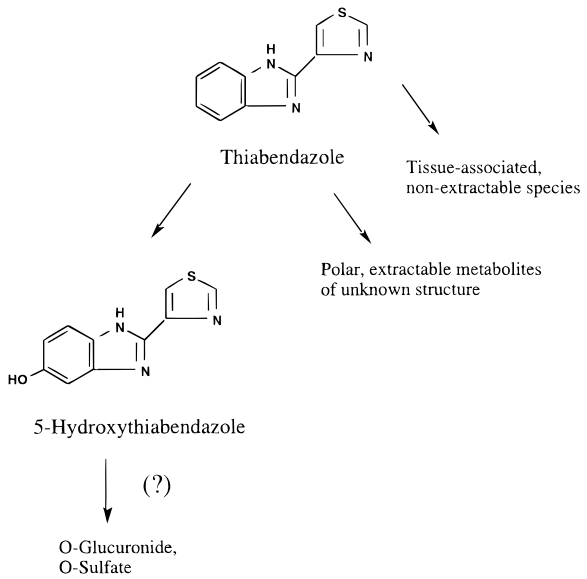

Figure 2. Proposed metabolic pathway of TBZ in the bluegill sunfish.

retention time of benzimidazole-2-carboxylic acid. Unknown Z was noted only following acid hydrolysis. The unknowns from both hydrolysis procedures account for a total of 28% of the [^{14}C]activity isolated from the visceral tissues, with none accounting for 10% or more.

On the basis of the number and relative amounts of isolated radioactive species, the proposed major metabolic pathway for TBZ in the sunfish is hydroxylation to form 5-HO-TBZ. This parallels the transformation found for mammals (Tocco et al., 1964, 1965, 1966; Chukwudebe et al., 1994) and the hen (Chukwudebe et al., 1994). Minor pathways leading to other metabolites more polar than TBZ are clearly operative in the fish, resulting in the production of the low levels of unknowns noted in this current work. Benzimidazole, recently shown to be a metabolite of TBZ in goat and hen tissues (Chukwudebe et al., 1994), was not found as a metabolic product of TBZ in the sunfish. The proposed metabolic pathway of TBZ in the bluegill sunfish is presented in Figure 2. Even though the polar TBZ-related species are more difficultly extracted from fish visceral tissues, the radioactivity associated with them (as well as that associated with TBZ, 5-HO-TBZ, and tissue-associated species) was cleared rapidly during the depuration phase of this study. This rapid clearance, plus the small BCF values for edible tissues and whole fish, indicate that TBZ and its biotransformation products will not accumulate and persist, nor biomagnify, in the food chain.

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