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Temporal Changes in Purity and Specific Activity of Tritium-Labeled 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin: Radiopurity Model for Toxicology

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The specific activity (***S***) and radiopurity (***R***) of tritium labeled 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, [^{3}H]TCDD, were measured by gas chromatography/mass spectrometry (GC/MS) while attempting to accurately characterize TCDD doses received by invertebrates, fish, and fish embryos during several toxicology studies conducted over a 3 year period. The [^{3}H]TCDD sample was found to consist of six TCDD analogues involving hydrogen, deuterium, and tritium substitution at the 1,6-dibenzo-*p*-dioxin carbon positions and a complex mixture of impurities (with and without tritium labels). Planar aromatic impurities were identified as tolyl-TCDD adducts and appeared to result from the decay of ^{3}H radiolabels to produce TCDD carbocations which reacted with the solvent, toluene. Formation of TriCDD and tolyl-TriCDDs, from both TCDD and tolyl-TCDDs, probably resulted from radiolysis-induced loss of a chlorine to form TriCDD free radicals which reacted with toluene. The measurement of ***S*** for [^{3}H]TCDD by GC/MS was accurate and precise (±3%) because relative, rather than absolute, amounts of the analogues were determined. Changes in ***S*** over time were accurately modeled as a function of the conversion of each [^{3}H]TCDD analogue to a solvent-TCDD analogue at a rate determined by ^{3}H loss due to decay. Storage, purification, and use of tritiated chemicals for toxicology studies requires consideration of the ^{3}H decay-related phenomena. For example, hydroxylated TCDD is an expected decay reaction product of [^{3}H]TCDD in tissues and may be misidentified as a metabolite.

Introduction

Tritium (^{3}H) labeled compounds are often used in toxicology studies because of analytical advantages that include rapid sample throughput associated with liquid scintillation counting (LSC), the ability to analyze a large number of samples at low cost, and low limits of detection due to high specific activity. For example, investigations of the toxicity (*1, 2*), pharmacokinetics (*3*−*5*), and metabolism (*6*−*10*) of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), in addition to analytical methods, development for polychlorinated dibenzo-*p*-dioxins and dibenzofurans (*11*) have used ^{3}H-labeled preparations to measure concentrations of TCDD without the need for slower, more expensive gas chromatography/mass spectrometry (GC/MS) analysis. In aquatic toxicology, [^{3}H]TCDD has been especially useful for the determination of bioconcentration factors (*12, 13*) and delivered dose to small fish (*12, 14*) and/or early life stages of fish (*15*−*19*) that require analysis of tissue samples too small for use of radiocarbon (^{14}C) labeled TCDD with its lower specific activity. [^{3}H]TCDD typically is reported to be [1,6-$^3H_2$]TCDD, but actually is a complex mixture of unlabeled and single tritiated as well as double tritiated TCDD molecules (*20*). The radiopurity of [^{3}H]TCDD has generally been reported to be ≥97%; however, information concerning when or how radiopurity or specific activity were determined are frequently not provided by authors.

While the use of ^{3}H-labeled chemicals, including [^{3}H]-TCDD, offers practical advantages, characteristics of the labeling need to be accurately determined to ensure proper interpretation of qualitative and quantitative analytical results. Of particular concern is the determination of the labeling site(s), the radiochemical purity, and the amount of label (specific activity) associated with the chemical (*21*). Although these attributes should be quantified regardless of the radioisotope, ^{3}H-labeled chemicals require careful attention, especially during long-term experiments or storage of chemical stocks or samples. The 12.3 year half-life ($t_{1/2}$) of ^{3}H, associated with the natural radioactive decay of $^3_1\mathrm{H} \rightarrow {}^3_2\mathrm{He}^+ + \beta^-$ (*22*), has clear implications for the need to reconfirm specific activity and radiochemical purity on a regular basis.

Other unique aspects of ^{3}H labeling contribute to added concerns regarding radiochemical purity. For example, ^{3}H atoms can exchange with hydrogen (^{1}H) in biological studies at rates which are dependent on the site of the ^{3}H label and the solvent environment (*23, 24*). Fortunately, ^{3}H-labeled aromatic chemicals, such as TCDD, are less susceptible to ^{1}H exchange. In preparations with high specific activity and/or solutions where labeled molecules are very concentrated, ^{3}H can cause intramolecular or intermolecular decomposition through autoradiolysis and radiolysis, respectively. As a consequence, storage conditions and specific activities of different preparations may influence rates at which purity is degraded.

Mass spectrometry has been useful for quality control assessments of radiolabeled compounds (*25, 26*). From measurements of the amount of label on a chemical of interest and knowledge of the decay rate constant (λ, where $\lambda = \ln 2/t_{1/2}$) of the radionuclide(s) used, the specific activity (***S***) of the chemical can be calculated. ***S***, here in units of Curies per millimole (Ci/mmol), is the critical conversion factor for relating LSC counts to mass of the analyte present in the sample. A comparison of the calculated radioactivity associated with the chemical of interest in a unit of volume or mass of sample to the radioactivity measured by LSC determines the degree of radiopurity (***R***). The degree to which ***R*** in a sample is less than 100% determines the magnitude of the overestimation of the analyte mass through use of ***S***.

The objective of this report is to describe a comprehensive analysis of ***R*** and ***S*** for [^{3}H]TCDD recently used in our laboratory to conduct several toxicity studies with aquatic organisms. These studies included a partial life cycle test (*27*−*29*) with brook trout (*Salvelinus fontinalis*); full life cycle tests (*30*) with an insect (*Chironomus tentans*) and a oligochaete worm (*Lumbriculus variegatus*); and a series of early life stage toxicity tests with seven fish species (*31*). Dietary exposures of [^{3}H]TCDD were used in the brook trout

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and invertebrate studies, while aqueous exposures were employed in the fish early life stage bioassays. In each study, dose−response relationships were based on whole-body or tissue-specific accumulation of [^{3}H]TCDD as determined by tissue combustion or digestion followed by LSC. The concentrations of TCDD measured in tissues were dependent on assumptions concerning the purity and specific activity of the [^{3}H]TCDD over the course of the experiments and the storage of samples prior to analysis. In support of these studies, GC/MS was used to (1) establish ***R*** and ***S*** of [^{3}H]-TCDD and (2) confirm the LSC-measured concentrations of TCDD in tissues from the aquatic organisms. In the course of the GC/MS analyses, previously unreported deuterium labels and reactions of TCDD associated with ^{3}H decay were discovered. Since these factors impact the measurement and interpretation of radiochemical purity and specific activity of [^{3}H]TCDD preparations, they were incorporated into a mass balance model which was used to account for changes in ^{3}H, TCDD, and impurities over time.

Experimental Section

Chemicals. [^{3}H]TCDD was acquired from Cambridge Isotope Laboratories, Andover, MA (lot no. AWN-729-87), as a toluene solution in several 5 mL ampules (hereafter referred to as solution A). The vendor reported that this lot contained [1,6-$^3H_2$]TCDD that was synthesized, purified, and analyzed in October 1987. The concentration of TCDD was reported to be 8 μg/mL toluene, with a ^{3}H activity of 1.0 mCi/mL, 97% radiochemical purity, and a specific activity of 40 Ci/mmol (1.48 TBq/mmol). In June 1993, prior to preparation of exposure solutions for aquatic toxicology studies, a check of solution A's purity by GC/MS indicated that other chemicals with both lesser and greater masses than TCDD comprised more than 50% of the chemical mass response measured for the sample. With the exception of approximately 4% trichlorodibenzo-*p*-dioxin (TriCDD) and <1% of tetra- and pentaCDD congeners, the contaminants were not readily identified. Because the mass spectra indicated only 60% of total ^{3}H activity measured by LSC was attributable to [^{3}H]TCDD and the amount of ^{3}H on the other PCDD congeners was small, the existence of additional ^{3}H activity associated with a large amount of unidentified contaminants was suspected.

A preparatory HPLC instrument was not immediately available in 1993 for purification of solution A with a procedure as described by Olson (1986). In an attempt to purify solution A, an activated-carbon chromatographic procedure, commonly used to isolate TCDD and other planar polychlorinated aromatic chemicals, was employed as originally described by Huckins et al. (*32*) with modifications of Marquis et al. (*33*). Because GC/MS analysis, following carbon chromatography, indicated a chemical purity <97%, the partially purified [^{3}H]TCDD in toluene was diluted 10-fold with high purity, unlabeled TCDD (lot no. R519 Cambridge Isotope Laboratories) to produce solution B which was used for exposure of brook trout (*27, 34*) and invertebrates (*30*). For fish early life stage studies (*31*), partially purified [^{3}H]-TCDD in toluene was transferred to acetone in 1994 (solution C) and used without dilution with unlabeled TCDD in order to achieve the LSC sensitivity required for detection of [^{3}H]-TCDD in small embryo samples. Because of the less than optimum radiochemical purity in solutions B and C, more intensive GC/MS analyses of the solutions and tissue samples from all three studies were required in order to determine the accuracy of LSC measured concentrations of TCDD in the organisms.

Liquid Scintillation Counting. [^{3}H]TCDD solutions (5 or 10 μL) were added to 20 mL glass scintillation vials containing 15 mL of either a pseudocumene cocktail (Monophase S, Packard Instruments, Meriden CT) or an alkylnaphthalene based, water miscible cocktail (Ultima Gold, Packard Instruments). Methods for preparation of tissue samples for LSC and counting solution and tissue samples were identical to those used for dose characterization in the toxicology studies (*27, 30, 31*).

Mass Spectrometry. Isolation of TCDD and Impurities from Tissue Samples. Tissues were prepared for analyses of TCDD concentrations with techniques similar to those described by Marquis et al. (*33*). Homogenized tissue samples (1−10 g) were mixed with sodium sulfate, spiked with $^{13}C_{12}$ 2,3,7,8-TCDD (Cambridge Isotope Laboratories), and Soxhlet extracted with hexane/dichloromethane overnight. Concentrated extracts of brook trout tissues were transferred to glass columns, containing sequential layers of sodium sulfate, sulfuric acid on Celite, potassium silicate, and silica gel and eluted with a mixture of hexane and dichloromethane. Concentrated eluants were transferred to columns of activated carbon on silica gel. After washing with dichloromethane and benzene, TCDD was eluted with toluene in the reverse flow direction. Since carbon column chromatography was found to only partially separate radioimpurities from [^{3}H] TCDD, HPLC was used to prepare tissue extracts for quantitative GC/MS analysis of [^{3}H]TCDD and radioimpurity concentrations in selected brook trout and fish embryo tissue samples. A Phenogel (Phenomenex, Torrance, CA) 100 Å gel permeation chromatography column was used with 100% dichloromethane mobile phase to remove lipids and other tissue matrix components from the extracts. Refined extracts were then fractionated with normal-phase HPLC on a Spherisorb (Phenomenex, Torrence, CA) 5 μm CN column using 100% hexane mobile phase.

Instrumental. GC/MS analyses were performed with a Finnigan-MAT Model 95S double focusing high-resolution GC/MS system. A 30-m DB5 capillary column (J&W Scientific, Folsum CA) was used in the Varian 3400 gas chromatograph. Using a Finnigan A200S autosampler, injections were made on-column via a Varian Septum-equipped Programmable Injector (SPI).

Preliminary detection of impurities in solution A was performed in the electron impact ionization mode (70 eV) at low mass-resolution (1000), while scanning between 20 and 900 amu. Concentration measurements for [^{3}H] TCDD and impurities were made in the selected ion monitoring (SIM) mode and were complicated by the presence of analogues of each chemical with two, one, or no ^{3}H labels. The analogues coelute from the gas chromatograph and, therefore, are analyzed simultaneously in the mass spectrometer. Each analogue produces a cluster of molecular ions which result from the presence of the naturally occurring isotopes of chlorine and carbon (Figure 1a). The analytical challenge arises from the similarity between the differences in mass units for ^{35}Cl and ^{37}Cl (1.9970 amu) and ^{1}H and ^{3}H (2.0082 amu). The major ions of these clusters occur at the even nominal masses in the range from m/z 320 to 330 (Figure 1b). To mass resolve these ions so that the ^{3}H contribution can be determined requires that the mass spectrometer be operated at a resolving power of 30 000 (M/ΔM, 10% valley definition). This level of resolution was at the upper limit of mass spectrometer capability when in the GC/MS configuration and reduced the instrument's sensitivity to a degree that did not meet the chemical mass detection requirements of the toxicological studies. Therefore, the mass spectrometer was operated in the low resolution SIM mode which merges all ion signals from each analyte to the nominal mass for the mixture of analogues.

TCDD concentrations were determined from the response of ions in the mass range 320 to 330 daltons. The concentration of unlabeled TCDD was measured at m/z 320 directly since this ion is exclusive to that analogue. In a process earlier utilized by Wendling et al. (*20*), the concentrations of the tritiated analogues were calculated from the difference

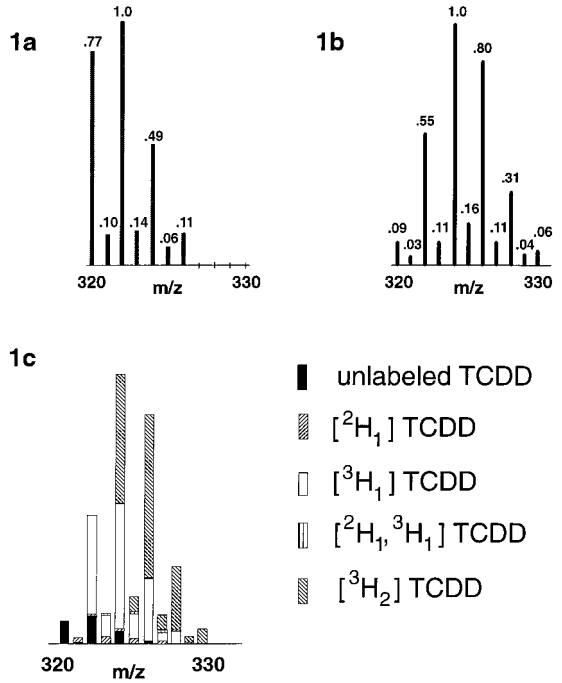

FIGURE 1. (a) Relative intensities of the ions of the molecular cluster of unlabeled TCDD with natural combinations of ^{35}Cl, ^{37}Cl, ^{12}C, and ^{13}C. (b) Relative combined intensities of the ions of the molecular clusters associated with combinations of ^{1}H, ^{2}H, and ^{3}H substitution at the 1,6 carbon positions of [^{3}H]TCDD in June, 1997. (c) Mass spectral basis for determination of the relative amount of [^{3}H]TCDD analogues associated with combinations of ^{1}H, ^{2}H, and ^{3}H substitution.

between the theoretical relative abundances and the measured responses at each of the isotopically determined m/z values (Figure 1c). That is, the amount of singly tritiated TCDD was calculated from the total response at m/z 322 minus the response due to the $(M + 2)^+$ ion ($C_{12}O_2{}^{35}Cl_3{}^{37}ClH_4$) of the native TCDD molecular ion cluster. Similarly, the amount of doubly tritiated TCDD is calculated from the total response at m/z 324 minus the response due to the $(M + 4)^+$ ion ($C_{12}O_2{}^{35}Cl_2{}^{37}Cl_2H_4$) of native TCDD plus the response due to the $(M + 2)^+$ ion ($C_{12}O_2{}^{35}Cl_3{}^{37}ClH_3$3H) of the singly tritiated species. To confirm the validity of this approach, high mass-resolution ($R = 30\,000$) SIM measurements were made of a concentrated aliquot of the radiolabeled TCDD solution in which exact masses for all native and labeled components contributing to the nominal masses of the molecular cluster were monitored.

Quantitative Analysis. Quantitative determination of TCDD concentrations in tissue samples was based on the relative response of the analyte (unlabeled + deuterated + tritiated TCDD) to that of the method internal standard ($^{13}C_{12}$ 2,3,7,8-TCDD). Quality assurance criteria were as described previously (*33*). Method efficiency was determined from the relative response of the method internal standard to that of the instrument internal standard ($^{13}C_{12}$ 1,2,3,4-TCDD). Relative response factors were developed from the analysis of a series of calibration solutions containing the internal standards and varied amounts of unlabeled 2,3,7,8-TCDD. Minimum limits of detection were calculated on the basis of a 3:1 signal-to-noise criterion for each analyte in each sample.

Concentrations of TCDD in solutions A, B, and C were determined by adding equal amounts of $^{13}C_{12}$ 1,2,3,4-TCDD to aliquots of the solutions and to standard solutions of native 2,3,7,8-TCDD obtained from the National Institute of Standards and Technology (NIST). $^{13}C_{12}$ 1,2,3,4-TCDD was used as the internal standard because the chromatographic resolution of the two isomers prevents the interference between $^{13}C_{12}$ 2,3,7,8-TCDD and [^{3}H]TCDD at mass 332 which was monitored in order to confirm that [$^{3}H_3$]TCDD was not present. A comparison of the relative responses of 2,3,7,8-TCDD to $^{13}C_{12}$ 1,2,3,4-TCDD in solutions A, B, and C with those in the standard solutions was used to estimate the total 2,3,7,8-TCDD concentrations.

HPLC fractions of solution A provided by Dr. James Olson allowed GC/MS analysis of pure samples of TCDD and a major impurity in order to compare instrumental response factors. Comparison of LSC counts to counts predicted from analogue molar concentrations determined by GC/MS for each chemical showed that the response factor of the contaminant was approximately the same as measured for TCDD. Thus, equivalent response factors for TCDD and each major impurity were assumed.

Specific Activity Determinations. The amount of radioactivity in disintegrations/minute (DPM) directly associated with [^{3}H]TCDD (or other ^{3}H-labeled chemicals) can be calculated from the GC/MS determined concentrations (C_i) of $n + 1$ different analogues of the chemical and the decay rate constant in minutes (λ) for ^{3}H. The ratio of radioactivity associated with the radiolabeled compound to mass of the compound (***S***) is determined as

$$S = \frac{\lambda N \sum_{i=0}^{n} (i)\left(\frac{C_i}{M + 2i}\right)}{k_1 k_2 \sum_{i=0}^{n}\left(\frac{C_i}{M + 2i}\right)} \qquad (1)$$

where i = number of ^{3}H labels on a specific analogue molecule, n = maximum number of ^{3}H labels possible on an analogue molecule, M = molecular weight of the unlabeled molecule, N = Avogadro's number, k_1 = constant adjusting DPM to activity; e.g. $k_1 = 2.22 \times 10^{12}$ DPM/Ci, and k_2 = constant adjusting mole units; e.g. $k_2 = 1000$ mmol/mol.

Results and Discussion

Hydrogen Isotope Ratios Associated with [^{3}H] TCDD. The three analogues of TCDD that result from ^{3}H labeling at the 1,6 dibenzo-*p*-dioxin positions, [$^{3}H_{0,1,2}$]TCDD, alter the typical molecular ion distribution produced by ^{35}Cl and ^{37}Cl in the mass spectrum as shown in Figure 1a,b. High mass resolution ($R = 30\,000$) SIM analysis of solution A resulted in good agreement with mole ratios for the three [^{3}H]TCDD analogues determined with the low resolution method, which was required for detection of trace concentrations in tissue samples. Values of ***S*** were 40.8 and 41.8 Ci/mmol for low and high mass resolution determinations, respectively.

Extraordinary amounts of deuterium (^{2}H) associated with the ^{3}H radiolabeled material were detected in the mass spectra of [^{3}H]TCDD in exposure solutions (Figure 1c). ^{2}H was probably present because separation of ^{3}H from hydrogen gas results in 2−3% $^{2}H_2$ impurity in $^{3}H_2$ used for molecular labeling reactions. The likelihood that ^{2}H was introduced during the preparation of [1,6-^{3}H]TCDD is indicated by the occurrence of ^{2}H only as a substitute for ^{3}H on [^{3}H]TCDD and its impurities. We are not aware of reports of similar deuterium complications associated with other samples of chemicals with ^{3}H labels. Two analogues, [^{1}H,^{2}H]TCDD and [^{2}H,^{3}H]TCDD, were present at concentrations which accounted for only 3% of the total amount of TCDD in solution A, however, neglecting the presence of these analogues resulted in a 14% underestimation of the total TCDD mass and caused an overestimation of ***S*** and an underestimation of ***R***. [$^{2}H_2$]TCDD was not mass resolved from [^{1}H,^{3}H]TCDD, however, the amount of this analogue should be negligible. Systematic errors in ***S*** and ***R*** were a consequence of the initial

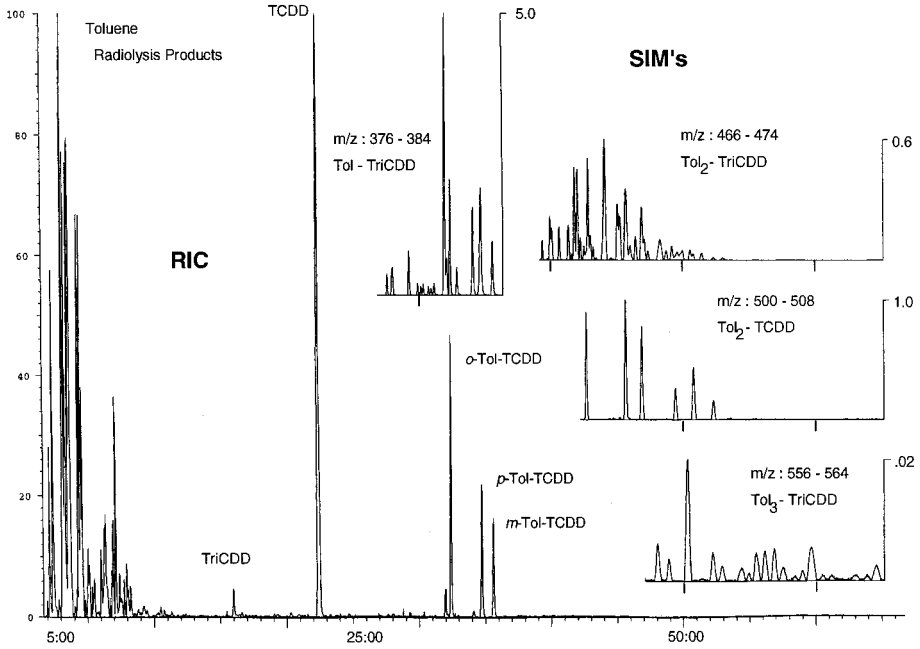

FIGURE 2. GC/MS analysis of an eight year old solution of [^{3}H]TCDD. TCDD and major contaminants are represented in the reconstituted ion chromatogram (RIC). Other decay and radiolysis produced impurities are presented in selected ion monitoring (SIM) mass chromatograms.

TABLE 1. Specific Activity and Radiopurity Analyses of Toxicity Test Solutions

solution − date (no. samples)	analyte (mol % of TCDD)	[^{3}H] analogue ratios (mol % of analyte ± SD)					*R* TCDD % of obsd LSC counts	S specific activity (Ci/mmol)
		[^{1}H$_2$]	[^{1}H,^{2}H]	[^{1}H,^{3}H]	[^{2}H,^{3}H]	[^{3}H$_2$]		
A − June 1993 (3)	TCDD	7.9 ± 0.2		37.6 ± 0.1		54.5 ± 0.2	62.6 ± 1.7	42.4 ± 0.1
A − June 1993 predicted	TCDD	8.3	0.8	38.5	2.0	50.3	79	41
A − Mar 1996 (4)	TCDD	10.9 ± 0.1	1.0 ± 0.02	43.3 ± 0.4	1.9 ± 0.02	42.9 ± 0.3	74.0 ± 3.7	37.9 ± 0.07
	Tol-TCDD (71)	27.0 ± 0.8	4.7 ± 0.3	65.0 ± 7.2	3.1 ± 0.5	0 ± 0	34 ± 4.0	20 ± 2
	Tri-CDD (2.5)	9.4 ± 0.7	1.7 ± 0.6	47.0 ± 2.0	1.7 ± 0.3	40.0 ± 2.0	1.8 ± 0.2	37.3 ± 0.4
A − Mar 1996 predicted	TCDD	10.3	0.9	40.8	2.1	45.9	71	39
A − Jan 1997 (5)	TCDD	11.0 ± 0.2	1.0 ± 0.05	41.5 ± 0.4	2.1 ± 0.1	44.3 ± 0.6	68.3 ± 1.0	38.3 ± 0.3
	Tol-TCDD (68)	27.0 ± 0.8	4.6 ± 0.4	64.5 ± 0.01	2.8 ± 0.6	1 ± 1	21.0 ± 0.7	20.1 ± 0.4
	Tri-CDD (4)	12.6 ± 0.6	1.0 ± 0.3	45 ± 2	2.5 ± 0.5	39.0 ± 1.9	1.7 ± 0.05	36.2 ± 0.6
	Tol-Tri-CDD (18)	28 ± 1	6 ± 1	56 ± 5	3 ± 1	7 ± 6	8.8 ± 0.6	22 ± 2
	Tol$_2$-TCDD (8)	83.0 ± 1.4	13.2 ± 0.4	1 ± 1	0.5 ± 0.4	2 ± 2	0.03 ± 0.001	2 ± 1
	Tol$_2$-TriCDD (6)	45 ± 9	6 ± 4	40 ± 8	5 ± 3	3 ± 4	2.4 ± 0.2	15 ± 4
	Tol$_3$-TriCDD (0.1)	71 ± 5	17 ± 5	0.1 ± 0.4	0.2 ± 0.4	11 ± 8	0.002 ± 0.001	6 ± 5
B − June 1993 (2)	TCDD	92.2 ± 0.004		3.3 ± 0.02		4.5 ± 0.02	75 ± 2	3.5 ± 0.01
B− June 1993 predicted	TCDD	91.5	0.1	3.4	0.2	4.7	45	3.8
B − Jan 1997 (5)	TCDD	94.6 ± 0.6	0 ± 0	4.2 ± 0.5	0 ± 0	1 ± 1.0	41 ± 10	1.9 ± 0.5
	Tol-TCDD (4.3)	28.0 ± 0.4	5.1 ± 0.4	60.9 ± 0.7	1 ± 1.0	4 ± 1.5	16 ± 1.4	20.7 ± 0.5
	Tri-CDD (0.6)	70 ± 6	0 ± 0	13 ± 8	2.5 ± 1.7	14 ± 13	1.2 ± 0.5	12 ± 5
C − June 1994 (3)	TCDD	10.3 ± 0.3		42.2 ± 0.7		47.5 ± 0.9	72 ± 1.7	39.7 ± 0.4
C − June 1994 predicted	TCDD	11.4	0.8	41.3	1.7	44.6	100	38.4
C− Jan 1997 (5)	TCDD	14.0 ± 0.4	1.0 ± 0.04	43.2 ± 0.4	1.8 ± 0.2	40.0 ± 0.5	91 ± 1.8	36.2 ± 0.2
	Tol-TCDD (16)	32 ± 5	4.9 ± 0.5	59 ± 10	1.1 ± 0.4	3 ± 5.2	6.4 ± 0.5	17 ± 3
	Tri-CDD (2.3)	15.0 ± 0.4	0.9 ± 0.5	48.1 ± 0.5	4 ± 2	32 ± 1	1.6 ± 0.1	33.6 ± 0.3

[^{3}H]TCDD signal subtraction method, which ignored odd masses. The presence of ^{2}H required modification of the method to account for the two additional TCDD analogues measured at m/z 321−329.

Identification of Impurities Associated with Intramolecular and Intermolecular Reactions Caused by Radioactive Decay in [^{3}H]TCDD Solutions. Three major impurities in solution A had GC retention times greater than [^{3}H]TCDD (Figure 2) with molecular ions at 410 daltons. None of the impurities contained two ^{3}H (Table 1) and each mass spectra showed losses corresponding to chlorine and C_7H_7. The two impurities with more similar mass spectra had longer retention times than the third, presumably due to their greater planarity. These observations were consistent with formation of tolyl-[^{3}H]TCDD adducts. Such reactions probably occur as the result of a rate determining step involving the radioactive decay of ^{3}H on [^{3}H]TCDD analogues followed by release of helium (^{3}He) to form an electron deficient carbocation that rapidly reacts with the solvent (Figure 3). Spontaneous nuclear decay of ^{3}H on organic molecules has similarly been observed to form unstable carbocations that reacted to form adducts with benzene and other solvents (*35*). Of the several S_N1 reaction products possible between the TCDD carbocation and toluene, the most likely products involve formation of a biphenyl linkage between the carbon at position 1 of the TCDD and any of the five unsubstituted positions of the aromatic ring of toluene (Figure 3). Although the methyl group can act as a weak ortho and para substitution directing group on an aromatic ring, the strength of the carbocation-toluene ring interaction appears to allow adducts to form at all hydrogen substituted positions on the aromatic ring of toluene. The three peaks in the gas chromatogram associated with a m/z of 410 were assigned in the order of retention times as *o*-, *p*-, and *m*-tolyl-TCDD

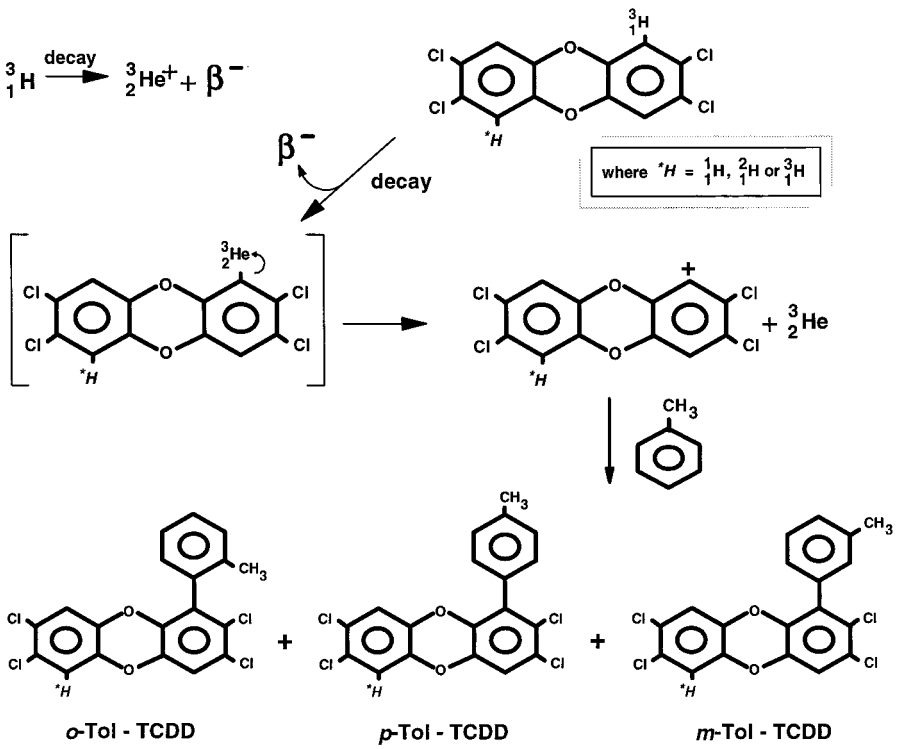

FIGURE 3. Mechanism for reactions initiated by the decay of ^3H on [^3H]TCDD and resulting products in a toluene solution.

on the basis of increasing planarity, decreasing polarity, and the expected greater peak area and effectiveness of separation of the less planar *o*-tolyl-TCDD from TCDD by carbon chromatography.

Large amounts of other impurities without tritium labels were observed by GC/MS at *m*/*z* 182, 198, and 212 (Figure 2) and thought to be dimethylbiphenyls, tolyl benzyl ethers, and methylphenylesters of benzoic acid, respectively. Radiolysis in the toluene solution probably created tolyl and benzyl free radicals that reacted with toluene or oxygen and toluene to form the impurities. Analysis of pure toluene showed only trace amounts of dimethylbiphenyls. Similar reactions of tolyl or benzyl free radicals with [^3H]TCDD, although possible, are limited by the relatively small number of [^3H]TCDD molecules in comparison to toluene (approximately 1:30 000). The absence of detectable Tol-TCDD with two ^3H demonstrated that reaction of [^3H]TCDD with tolyl free radicals was not a significant route for Tol-TCDD formation.

Impurities detected at *m*/*z* 376 correspond to tolyltrichlorodibenzo-*p*-dioxin (Tol-TriCDD) which could be a reaction product associated with decay of ^3H on the [^3H]-TriCDD, a contaminant present in [^3H]TCDD preparations. Twelve Tol-TriCDD products from the decay reaction of [^3H]-TriCDD were expected due to the asymmetry of the TriCDD structure. With the mass spectrometer operating in a multiple ion selection mode, 13 Tol-TriCDDs were identified, but the three most abundant of these also had the same amount of $^3H_2$ as [^3H]TCDD. This can be explained by a radiolysis reaction in which [$^3H_2$]TCDD was dechlorinated and a tolyl group added at a 2,3,7, or 8 dibenzo-*p*-dioxin position (Tol′) through a free radical reaction. A similar reaction (dechlorination and solvent adduct formation) has been reported for the photolysis of octachlorodibenzo-*p*-dioxin in benzene (*36*). Irradiation of TCDD in hexane with γ radiation also resulted in dechlorination but with addition of a ^1H through reaction of the [^3H]TriCDD free radical with the alkane solvent (*37*). Increasing concentrations of [^3H]TriCDD after purification of solutions indicated that the [^3H]TriCDD free radicals caused by radiolysis could also react with toluene by abstracting a hydrogen from the methyl group. Thus, radiolysis of [^3H]TCDD to produce [^3H]TriCDD, when followed by decay of ^3H, is responsible for the presence of an additional 12 Tol-TriCDDs, as shown in Figure 4 which diagrams all of the reaction pathways and products expected to occur as a result of ^3H decay and radiolysis of [^3H]TCDD.

In addition to TriCDD, the three Tol-TCDDs, the three Tol′-TriCDDs, and the 12 Tol-TriCDDs predicted as ^3H decay and radiolysis reaction products, six 1,6-ditolyl-TCDDs (Tol_2-TCDDs) with no ^3H labels, 36 ditolyl-TriCDDs (Tol′,Tol-TriCDDs and Tol_2-TriCDDs) with either one or no ^3H, and 36 tritolyl-TriCDDs (Tol′,Tol_2-TriCDDs) with no ^3H, were predicted as shown in Figure 4. All six peaks in the mass spectra at *m*/*z* 500 were identified as Tol_2-TCDDs with probable *o*-, *m*-, *p*-tolyl combinations at the 1,6-dioxin positions. Almost all of the other predicted reaction products were subsequently detected by GC/MS, as shown in Figure 2. The data unequivocally indicate the absence of any intermolecular or intramolecular ^3H exchange. Theoretically, radiolysis of the trichloro reaction products should result in minor amounts of dichlorodibenzo-*p*-dioxins with tolyl substituents. The presence of Tol-TCDDs and Tol_2-TCDDs indicates that the rate of radiolysis (R_r) was slower than the rate of ^3H decay (R_d) which is the rate at which tolyl adducts are formed without loss of chlorine. Autoradiolysis may have contributed to some of the impurities such as Tol-TriCDD and Tol′,Tol-TriCDD in solution A (Figure 4), but the rate of autoradiolysis (R_a) must have been slow in comparison to R_d and R_r because the amount of Tol-TCDD, TriCDD, and Tol′-TriCDD in the solution is greater than the amount of Tol-TriCDD and Tol′,Tol-TriCDD.

GC/MS analyses of pure [^3H]TCDD and Tol-[^3H]TCDD obtained by HPLC indicated that concentrations of contaminants could be quantified in solutions and tissues using the assumption that GC/MS response factors for the contaminants are similar to that of TCDD. Approximately 30% of the radioimpurity in solution A was estimated, on the basis of the pattern of ^3H substitution in reaction products and their relative amounts (Table 1), to result from radiolysis reactions. Prediction of amounts of reaction products in other

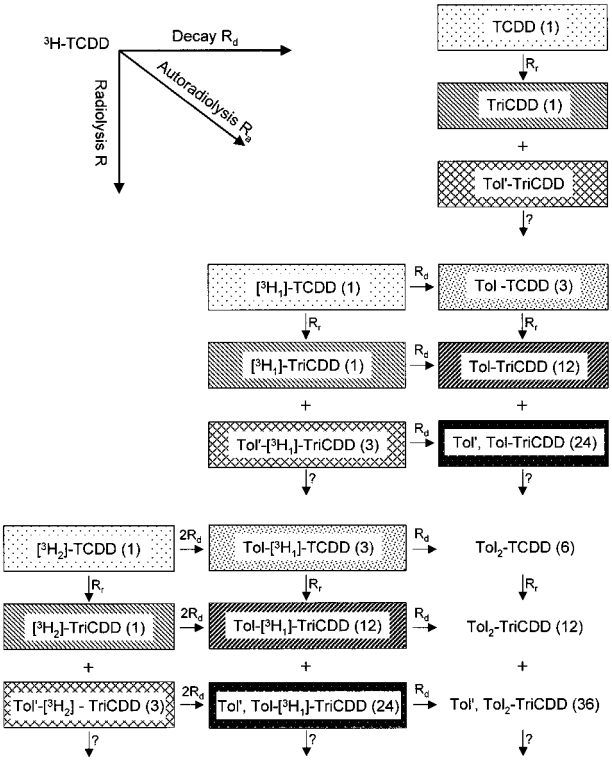

FIGURE 4. ^{3}H decay and radiolysis reactions of [^{3}H]TCDD in toluene solutions. Decay of ^{3}H on the dibenzo-*p*-dioxin structure results in tolyl adducts at the 1,6-dibenzo-*p*-dioxin positions (Tol), whereas each radiolysis reaction results in loss of a chlorine with and without substitution of a tolyl group (Tol′) at one of the 2,3,7,8-dibenzo-*p*-dioxin positions. The rate of radiolysis (R_r) in solution A was estimated to be ~30% of the rate of ^{3}H decay (R_d). Autoradiolysis was not detectable. These reactions were predicted to result in a complex mixture chemical structures observable by GC/MS. The shading indicates common structures with two, one, or no ^{3}H label. Almost all reaction products were subsequently detected by GC/MS.

[^{3}H]TCDD solutions could be complicated by different rates of radiolysis associated with storage of [^{3}H]TCDD at different concentrations and temperatures.

Model Development. Because of the complex changes in the composition of [^{3}H]TCDD in solutions and tissues over time, regardless of original purity, a kinetic model was required to account for the ^{3}H decay-related reactions. Based on changes in [^{3}H]TCDD composition caused by decay of ^{3}H over time, eq 1 was adapted to calculate changes in (1) ***S***, the specific activity, (2) the concentration of TCDD, and (3) the concentration of radioimpurities resulting from ^{3}H decay. The intramolecular reactions caused by ^{3}H decay were relatively easy to model because ^{3}H labels remained at the 1,6-dibenzo-*p*-dioxin positions, and each decay event resulted in loss of the parent molecule. The rate at which a labeled molecule reacts thus depended on the decay rate of the radiolabel and the number of radiolabels on the molecule. The presence of molecules with different numbers of labels effects the rate of change in ***S*** and ***R*** for the solution. The decay model did not include increases in the concentrations of trichloro impurities associated with dechlorination reactions caused by radiolysis of TCDD or Tol-TCDD impurities. The rate of radiolysis-associated reactions depends on the concentration of both ^{3}H and TCDD or Tol-TCDD in the solution. The specific activity at any time (S_t) after purification of the solution or measurement of the analogue ratios was calculated using a reformulation of the equations described by Kogan et al. (*38*) and including the six analogues created by the combinations of ^{1}H, ^{2}H, and ^{3}H substitution at the 1,6-dibenzo-*p*-dioxin positions

$$S_t = \frac{\lambda N \sum_{i=0}^{2}\sum_{j=0}^{2-i}(i)\left(\frac{C_{ij}}{M+2i+j}\right)e^{-i\lambda t}}{k_1 k_2 \sum_{i=0}^{2}\sum_{j=0}^{2-i}\left(\frac{C_{ij}}{M+2i+j}\right)e^{-i\lambda t}} \quad (2)$$

where t = elapsed time from the initial analogue condition, i = number of ^{3}H labels on the analogue molecule, j = number of ^{2}H labels on the analogue molecule ($i + j \leq 2$), C_{ij} = concentration of analogue ij at $t = 0$, and λ = decay rate constant of the ^{3}H for the same unit of time as "t".

The concentration of TCDD over time was calculated from the analogue concentrations:

$$[\text{TCDD}]_t = \sum_{i=0}^{2}\sum_{j=0}^{2-i} C_{ij}\, e^{-i\lambda t} \quad (3)$$

Concentrations of the impurities produced from decay of ^{3}H on TCDD in the solution over time were calculated with Bateman equations (*39*). For example

$$[^3\text{H}_1\text{-Tol-TCDD}]_t = \frac{C_{2,0}}{M+4} 2\lambda \left(\frac{e^{-2\lambda t}}{(\lambda - 2\lambda)} + \frac{e^{-\lambda t}}{(2\lambda - \lambda)}\right) \quad (4)$$

Characterization of *S*, *R*, and Chemical Purity. [^{3}H]TCDD Solutions Used in Toxicity Tests. Based on the reported total ^{3}H radioactivity of 1 mCi/mL for stock solution A in October 1987, an activity of 0.77 mCi/mL was expected at initiation of toxicity tests in June 1993. A ^{3}H radioactivity of 1.00 ± 0.16 mCi/mL (n = two sets of five samples) was determined by LSC. A TCDD concentration of 4.6 ± 0.1 ng/μL (n = 3) was measured by GC/MS, which also indicated that the composition of the mixture of [^{3}H]TCDD analogues was 7.9 ± 0.2% unlabeled ([^{1}H$_2$]TCDD), 37.6 ± 0.1% single tritiated ([^{1}H,^{3}H]TCDD), and 54.5 ± 0.2% double tritiated ([^{3}H$_2$]TCDD) as presented in Table 1. This analogue composition gives a calculated ***S*** of 42.4 ± 0.1 Ci/mmol. The TCDD concentration and ***S*** indicated that only 62.6 ± 1.7% of the LSC measured radioactivity was attributable to [^{3}H]TCDD. If the GC/MS measurement of TCDD concentration was sufficiently accurate, the value for radiopurity would be an underestimate since deuterium analogues of TCDD were not measured in the 1993 analysis of solution A and the additional radioimpurities caused by radiolysis of TCDD were not considered. Even after carbon column clean up, only 80% of the total ^{3}H activity could be attributed to TCDD.

Changes in [^{3}H]TCDD analogue ratios, ***S***, ***R***, and chemical purity in solution A over the period of 1987−1997 were predicted with the ^{3}H decay model (eqs 2−4) under an assumption that both ***R*** and chemical purity were ≥97% in 1987 when solution A was prepared and purified. On the basis of the GC/MS analysis of solution A in January 1997 (Table 1), the model predicted original [^{3}H]TCDD analogue percentages in 1987 to be 5.2, 0.47, 33.1, 1.7, and 59.5 for [^{1}H$_2$], [^{1}H,^{2}H], [^{1}H,^{3}H$_1$], [^{2}H,^{3}H], and [^{3}H$_2$], respectively. This analogue composition indicated that solution A had ***S*** = 44.6 Ci/mmol in 1987. Figure 5 compares the model's predictions of analogue ratios, ***R,*** and ***S*** based on the 1987 analysis to GC/MS measurements of solution A in 1993 and 1996 (Table 1). The mole percent of each analogue with respect to total TCDD varied over time (Figure 5a) in accordance with the effect ^{3}H decay had on the number of analogue molecules present with respect to total TCDD. For example, the relative amount of unlabeled TCDD increased with time, not because it was being formed but because the tritiated analogues were lost through the intramolecular decay reaction. Moles of

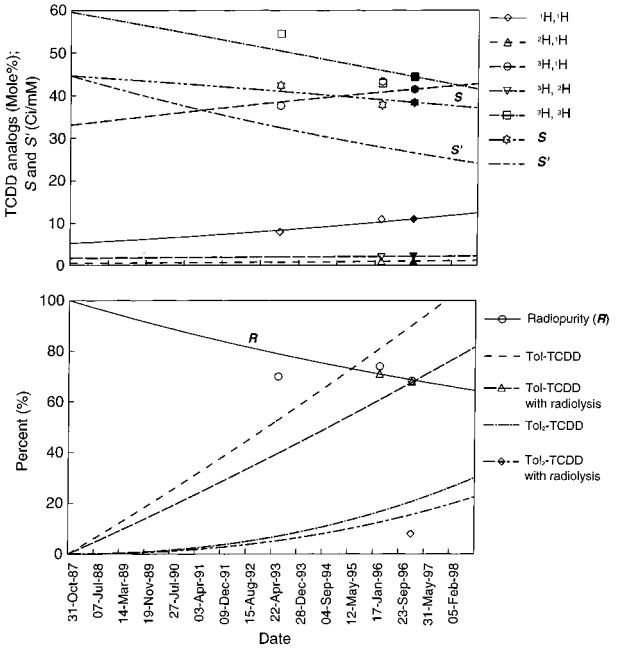

FIGURE 5. (a) Decay model prediction of temporal changes in solution A analogue composition (mol % of TCDD), *S* and *S′*, the specific activity expected as a result of ^{3}H decay without loss of TCDD from decay reactions. Based on analysis of TCDD analogues conducted in Jan 1997 (filled symbols), the initial (Oct 1987) analogue composition and *S* were determined. Model predictions are compared to [^{3}H]TCDD analogue data from GC/MS analysis of solution A in June 1993 and March 1996 (open symbols). (b) Decay model predicted change from 1987 to 1997 (assuming $R \geq$ 97% in 1987) in *R* and the mol % of Tol-TCDD and Tol$_2$-TCDD relative to TCDD. Measured amounts of decay reaction products were closer to predictions based on reactions caused by both decay and radiolysis.

doubly labeled TCDD decreased twice as fast as moles of singly labeled TCDD because the rate of reaction was proportional to the number of ^{3}H labels. Values of ***S*** measured in June 1993 and March 1996 fall within ±3% of the predicted values. This accuracy allowed definition of the errors associated with use of original ***S*** determinations as a constant over time or use of original ***S*** values with corrections for ^{3}H loss through decay with a faulty assumption that the labeled molecule was not transformed by decay of its label. The divergence over time of ***S*** corrected for tritium decay (***S′***) in contrast to true ***S*** for solution A may be seen in Figure 5a.

While the rate of tritium decay may seem slow on the time scale of solution storage and use, Figure 5b illustrates how fast contaminants (especially Tol-TCDDs) produced by the ^{3}H decay reaction accumulated in relation to the remaining [^{3}H]TCDD. The rate of decrease in ***R***, however, was less than the rate of production of contaminants because the ***S*** of Tol-TCDD was only half that of [^{3}H]TCDD and the Tol$_2$-TCDD formed through the decay reaction has no ^{3}H label. Measured amounts of Tol-TCDD and Tol$_2$-TCDD, when corrected for a 25% loss due to radiolysis, are in good agreement with the predicted values (Figure 5b). Fortunately, radiolysis did not change analogue ratios for [^{3}H]TCDD or any of the reaction products that still have ^{3}H labels. The agreement of model predictions with measured analogue ratios of Tol-TCDD confirmed the general applicability of the basic decay reaction mechanism for all tritiated components of the mixture through time.

HPLC analysis of solution A in 1996 indicated that only 60% of the total ^{3}H activity was associated with TCDD although the decay model prediction was 70%. The difference between ^{3}H counts from a pure [^{3}H]TCDD fraction obtained by HPLC and total counts for all fractions provided the most accurate measure of total radioimpurities (40% of total radioactivity), and GC/MS determination of analogue ratios for decay reaction products was the most accurate method of determining the amount of radioimpurities attributable to the ^{3}H decay reactions (30% of total radioactivity). The difference between the amounts of total and decay-related radioimpurities (10%) indicated that 25% of the total radioimpurities were caused by radiolysis of [^{3}H]TCDD.

Solutions B and C were used in toxicity tests (*27*−*31*) after formulation from solution A which had been only partially purified with an activated carbon-silica gel column due to the planar, aromatic nature of the impurities. The determination of TCDD doses for fish relied on GC/MS characterization of the exposure solutions to determine ***S.*** It was assumed that radiation from impurities which were retained in the tissues of test organisms would be an insignificant source of error when LSC measurements were combined with ***S*** to calculate concentrations of TCDD.

Solution B was prepared for toxicity tests with brook trout and invertebrates in June and August 1993, respectively, by addition of native TCDD to [^{3}H]TCDD at a ratio of 10:1. Chemical purity, which was thereby increased 10-fold, changed little (∼1.4%) during a 2 year period in which the tests were conducted. The TCDD mass concentration and the ratio of total ^{3}H radioactivity, measured by LSC, to total moles of TCDD, measured by GC/MS, for the two preparations were 5.87 ng/μL with 4.69 Ci/mmol and 7.97 ng/μL with 4.61 Ci/mmol, respectively. GC/MS analysis of the June 1993 solution B determined ***S*** to be 3.5 Ci/mmol (Table 1). The ***S*** for solution B in toxicity tests was, however, set at 3.80 for June 1993, based on measurement of ***S*** for solution A and the 10:1 dilution factor used to prepare solution B. The direct GC/MS analysis of solution B was not used because [^{3}H]-TCDD analogue ratios were less accurately determined when the preponderance of TCDD was unlabeled, as in solution B. This limitation for the GC/MS determination of ***S*** was exemplified by the value of 1.9 ± 0.5 Ci/mmol measured for solution B in January 1997 in contrast to an expected value of 2.8 Ci/mmol based on the decay model. The ***S*** for solution B was adjusted lower by 16% during the brook trout study to account for loss of labeled TCDD due to decay reactions. The reactions caused a large change in ***S*** over the 2 year period of the study because the ratio of TCDD to ^{3}H was large with the addition of native TCDD. The difference between total ^{3}H radioactivity and that attributable to [^{3}H]-TCDD suggests that ***R*** was 75% for solution B in 1993. In 1996, HPLC analysis indicated that ***R*** of solution B had decreased to 68%.

Solution C was prepared in May 1994 and used in fish embryo exposures during the period of June 1994 to December 1996. GC/MS analysis determined ***S*** to be 39.7 ± 0.4 Ci/mmol which was in good agreement with the model predicted value of 38.4 Ci/mmol. As with solution B, the ***S*** for solution C was adjusted for decay changes from the date of preparation to time of LSC analyses. Solution C was determined to be 72% radiopure by HPLC in 1996 after the fish early life stage toxicity tests had been completed.

Analysis of TCDD and Radioimpurities in Tissues of Aquatic Organisms Exposed to [^{3}H]TCDD. The preferred dosimetric for toxicity studies with persistent bioaccumulative chemicals such as TCDD is the concentration of the chemical in tissues of the test organism. During long-term dietary exposure of brook trout to solution B (*27*), samples of various tissues were analyzed by GC/MS and LSC. The S_t used for LSC analysis of tissues was corrected for changes in [^{3}H]TCDD caused by decay reactions which occurred up to the time of instrumental analysis, as described above. The correlation of GC/MS and LSC results is plotted in Figure 6. Using *t*-tests (*38*) the intercept, 15 ± 13.1, was not significantly different than 0 ($p < 0.05$), but the slope of 0.87 ± 0.02 was

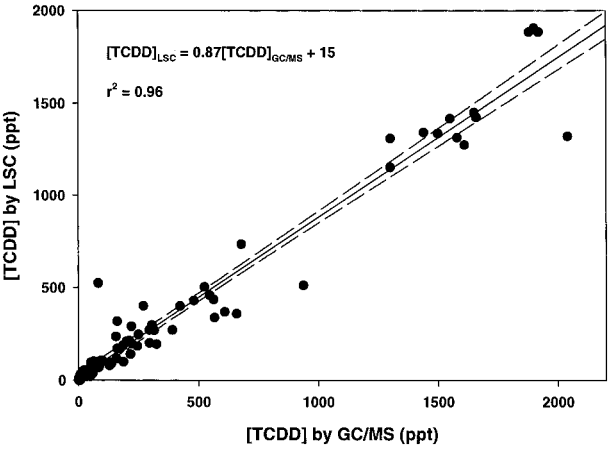

FIGURE 6. Correlation (with 95% confidence range) between TCDD concentrations determined by LSC and GC/MS for tissue samples collected from brook trout over a 6 month exposure to [^{3}H]TCDD in food.

significantly different than 1 ($p > 0.05$). The TCDD concentrations determined by GC/MS were systematically 13% greater than by LSC, despite the presence of trace radioimpurities. The ability to compare the two independent analytical techniques, with both having good precision, allowed detection of the small systematic error. No measurable shifts in the TCDD analogue ratios were noted in tissues. The difference between GC/MS and LSC results could be explained by a combination of TCDD loss during combustion of tissues for LSC and any possible error in concentration of TCDD in the NIST standard used for GC/MS analysis.

Further GC/MS analysis of adult brook trout tissues indicated that concentrations of radiochemical impurities were present at lower concentrations relative to TCDD than in exposure solution B (Table 2). Since ***R*** was not improved by addition of unlabeled TCDD to prepare solution B, the reduction of impurities in tissues relative to [^{3}H]TCDD, rather than total TCDD, best defines the degree of error possible when using $\boldsymbol{S}_t$ predicted by the model to determine TCDD concentrations in the tissues by LSC. Tol-TCDD and TriCDD concentrations in brook trout whole body samples were reduced 20- and 6-fold, respectively, and Tol-TriCDDs were not detected, probably as a result of metabolism rather than lack of assimilation from food. Since ***S*** values for the impurities (Table 1) were less than half that of [^{3}H]TCDD, less than 1% of the LSC counts from brook trout tissues could be attributed to impurities.

Eggs of fish exposed to [^{3}H]TCDD through water containing solution C with 0.5% acetone appeared to retain more of the impurities than did the brook trout (Table 2). Less capability of embryos to eliminate impurities through metabolism, or perhaps insufficient time for induction of metabolism, may explain the greater retention of impurities in eggs. Egg samples from five species were analyzed by both LSC and GC/MS. Using *t*-tests (*39*) the intercept for the correlation between LSC and GC/MS was not significantly different than 0. The slope of 0.79, although similar to that found for brook trout despite the greater amounts of radioimpurities, was not significantly different than 1 ($p < 0.05$). The contribution of impurities to total LSC counts from fish eggs appears to range from <1 to 8%. Impurities were only measured in eggs sampled early in development. The amounts of impurities measured in eggs, if retained through the larval stage to the ends of the toxicity tests at 30 days post-hatch (*31*), should not have influenced organism responses.

Implications for Other Studies. Despite some limitations in resolving the analogues of [^{3}H]TCDD and impurities, GC/MS proved to be uniquely capable of both accurately determining ***S*** and characterizing previously unknown mechanisms involved in decreases in ***R*** that occur over time following sample purification. The potential for underestimation of partition coefficients as a consequence of small amounts of radiolabeled sorbent impurities has been described (*41*), and one may conclude that obtaining accurate measurements of organic carbon−water equilibrium partition coefficients with ^{3}H-labeled hydrophobic organic chemicals would be difficult. This study has additional implications for other investigations which have used or may use ^{3}H or other β^- emitting radioisotopes with short half-lives for LSC determination of concentrations of radiolabeled chemicals. A common practice for use of [^{3}H]TCDD has been to assume that the TCDD molecule is conserved when the label is lost through decay. Under this assumption, ***S*** is reduced to account for decay loss of ^{3}H over time and ***R*** does not change. The magnitude of error produced by the decay correction depends on the length of time for which the correction is made and the distribution of ^{3}H atoms among molecules of the chemical (analogue ratios). Change in ***S*** cannot be predicted accurately without knowledge of the analogue ratios at some point in time. When solutions of [^{3}H]TCDD are purified effectively by HPLC, ***S*** can be determined from the LSC counts for the pure fraction and the chemical mass measured spectroscopically. Only mass spectrometry can determine the analogue ratios needed to predict how the sample will change in storage or during the course of long-term experiments.

The presence of impurities in samples of [^{3}H]TCDD has been previously reported. Denison et al. (*41*) reported that radioactive contaminants greatly increased the background radioactivity in Sephacryl S-300 profiles obtained by incubation of rat hepatic cytosol with unpurified [^{3}H]TCDD. Tritiated contaminants isolated by HPLC were tentatively identified by comparison of their elution positions to elution positions of polychlorinated dibenzo-*p*-dioxin (PCDD) standards. Only the fractions identified as [^{3}H]tri- and [^{3}H]penta-CDDs bound weakly in the specific receptor region expected for PCDDs. It is possible that the nonresponsive, later eluting fractions contained TCDD-solvent adducts as reported in the current study. Kedderis et al. (*42*) reported that radiopurity of [^{3}H]-TCDD could be determined by measuring rat biliary excretion. Contamination of different samples ranged from 1 to 8% radioimpurity, which was fully eliminated in bile within 24 h of iv dosing. The authors warned that "the presence of undetected radiochemical impurities may have confounded interpretation of many reports in the literature". Of particular concern was the effect of rapidly released contaminants on short-term disposition and metabolism studies.

Long-term disposition and metabolism studies with [^{3}H]-TCDD, regardless of initial purity, should also be a concern because of the potential for the confounding appearance of hydroxylated TCDD as a result of ^{3}H decay, formation of $TCDD^+$, and reaction with water in vivo. The reaction of [$^3H_{0,1}$]$TCDD^+$ with water would produce a hydroxylated-[$^3H_{0,1}$]-TCDD, whereas hydroxylated-[$^3H_{0,1,2}$]TCDD could be produced by metabolism of [^{3}H]TCDD. The amount of hydroxylated-[$^3H_2$]TCDD relative to hydroxylated-[$^3H_{0,1}$]TCDD eliminated by an organism could provide a measure of the TCDD metabolism rate relative to the ^{3}H decay rate. A good example of this decay/metabolism conundrum is provided by the study of [^{3}H]TCDD elimination in feces of a human volunteer (*3, 44*). The $t_{1/2}$ for elimination of TCDD was calculated to be 8.9 years based on measurements of radioactivity in feces collected for 6 years following a single ingestion exposure. It appears that a correction for decay of ^{3}H was applied, and [$^3H_2$]TCDD was 37% of the TCDD at exposure (*44*). The use of a decay correction to reduce ***S*** would result in a 20−25% overestimation of the amount of TCDD eliminated. More

TABLE 2. Radiopurity Analysis of Fish Tissue Samples

sample (wet weight in g)	TCDD concn (pg/g)	% of TCDD concn[a]						
		TetraCDD			TriCDD	PentaCDD		
		peak 1	peak 2	peak 3		peak 1	peak 2	peak 3
Adult Brook Trout Exposure								
exposure solution B		1.1	2.2	1.7	0.5	na	na	na
whole body (17.8)	2300	0.04	0.07	0.04	0.06	<0.1	<0.1	<0.1
whole body (21.5)	2300	0.07	0.05	0.05	0.08	<0.1	<0.1	<0.1
gonad (3.1)	1200	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
Embryo Exposures								
exposure solution C		0.3	8.0	9.5	2.1	1.1	0.38	1.6
channel catfish (4.2)	920	<0.2	0.5	0.6	<0.4	<0.2	<0.2	<0.2
Lake Herring (2.2)	1700	<0.4	<0.4	<0.4	<0.5	<0.2	<0.2	<0.2
fathead minnow (0.37)	5200	<0.3	5.8	8.8	0.4	<1	<1	0.8
Medaka (0.86)	3200	<0.5	1.2	1.3	<0.4	<0.5	<0.5	<0.5
Zebrafish (0.22)	3900	<3	<3	<3	<1	<2	<2	<2
White Sucker (0.89)	3200	<2	5.3	5.2	0.6	<0.1	<0.1	<0.1
Northern Pike (2.8)	2600	0.02	4.0	4.3	0.5	<0.2	<0.2	0.4

[a] Not analyzed = na; < = not detected at minimum level of detection.

importantly, the assumption that all radioactivity in feces represented either TCDD or its metabolites was probably a greater source of overestimation of the rate of elimination. At least 50% of the radioactivity in the feces was attributed to metabolites of TCDD (*20*); a significant finding since there do not appear to be any other studies in which human metabolism of TCDD has been measured. Our study indicates that the ^{3}H decay reaction of [$^3H_2$]TCDD, at twice the rate of ^{3}H decay, probably produced hydroxylated-[$^3H_1$]TCDD, or other polar adducts of [$^3H_1$]TCDD, in the human subject by reaction of the [$^3H_1$]TCDD carbocation with water or other abundant nucleophilic material in cells. Such compounds would be rapidly eliminated and thus counted with metabolites in feces. Given the amount of [$^3H_2$]TCDD in the exposure and the apparent rate of TCDD elimination in comparison to the predicted rate of hydroxylated-[$^3H_1$]TCDD formation, most of the radioactivity attributed to TCDD metabolites was probably due to hydroxylated-[$^3H_1$]TCDD from the ^{3}H decay reaction. Thus, reinterpretation of the human feces data using the findings and model provided in this study indicates that the rate of metabolism of TCDD in humans is probably much less and the $t_{1/2}$ for elimination of TCDD greater than reported.

While the current study has identified some complications and limitations, experimental advantages remain for use of tritium as a radiolabel, including use of [^{3}H]TCDD. The decay-related reactions, analytical methods, and kinetic model reported here can be used to better plan experiments and interpret LSC data. In addition, we strongly recommend that future reports of studies which rely on ^{3}H labeling include more information about factors which define the quality of the LSC data. Specifically, quality assurance of data should require a clear statement of when and how the solution was purified, solvent(s) used and concentration of ^{3}H in the solution, how and when ***S*** was determined and adjusted for temporal changes, the duration of the experiment, and the time for which samples were stored prior to LSC analysis. Beyond these basic requirements for use of ^{3}H, the vulnerability of any study design to errors associated with the radioactive decay and radiolysis phenomena reported here should be considered.

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