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Publication Date

1981-12-01

Lawrence Berkeley Laboratory

UNIVERSITY OF CALIFORNIA

CHEMICAL BIODYNAMICS DIVISION

Submitted to the International Journal
of Applied Radiation and Isotopes

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December 1981

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EXCITATION LABELING WITH TRITIUM. STUDIES OF SIDE REACTIONS WITH MODEL COMPOUNDS.

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SUMMARY

A series of model compounds such as hexane, heptene-2, 3-methylhexane, methylcyclohexane, benzene, toluene, styrene, benzoic acid and phenylalanine have been exposed to a stream of excited tritium atoms produced in a microwave-induced plasma. Product analysis by gas-liquid radiochromatography revealed that the yields of labeled parent compound, based on incorporated non-exchangeable tritium label, ranged from less than 1 per cent for n-hexane to 71 per cent for methylcyclohexane. Other labeled products included high-boiling polymers, degradation products, and saturated analogs formed by tritium addition to double bonds or to phenyl ring. Incorporation of tritium into the molecule appears to be selective, and the presence of a methyne hydrogen favors the labeling. Not only tritium atoms from the plasma but also long-lived radicals containing tritium in the sample caused tritium-labeling. The latter attacks susceptible solvent molecules post-labeling to produce additional labeled contaminants.

INTRODUCTION

In 1960 Ghanem and Westermarck [1] reported a method of enhancing tritium labeling yields and reducing side reactions by exposing a Wilzbach cell to a microwave discharge at 2450 MHz. This frequency dissociates T_2 into tritium atoms which can then react with the substrate. Labeling by tritium atoms causes less radiation degradation than Wilzbach labeling [2] mainly because of the short duration of exposure. Gosztonyi and Walde [3] applied this method to the labeling of biologically important compounds such as amino acids and anesthetics and obtained labeled compounds with specific activity in the millicuries/millimole range. In 1973, Hembree et al. [4] improved the microwave discharge activation method by circulating the tritium gas over the sample and applied the method to the labeling of biomacromolecules such as adrenocortrophin (ACTH). Ehrenkauf et al. [5] later explored the various parameters of this method of excitation labeling to optimize the yield of tritiated parent compound.

The method of excitation labeling has been used to tritiate steroids achieving a specific activity of multicuries per millimole [6]. The ease of sample handling, the short duration of exposure, and the high specific activity that can be obtained make the method ideal for general use in tritium labeling. Since no information relating the chemical structure of the substrate with the yields in tritium labeling, side reactions, and the extent of degradation is available, this study is undertaken with model compounds to determine such a relationship.

MATERIALS AND METHODS

Chemicals

The model compounds selected for labeling by microwave excitation method were benzene, n-hexane, methylcyclohexane, heptene-2, 3-methylhexane styrene, benzoic acid, and phenylalanine. These chemicals were of spectro-quality or of analytical grade and were shown by gas chromatography to be greater than 99% pure. Styrene was purified by preparative gas chromatography on a Carbowax-20M column and stored under nitrogen at -10°C and used within a day.

Solvents for tritiated samples were benzene, isopropylbenzene, and n-decane from Matheson Colman Bell Company and were used without further purification.

Tritium gas was generated from uranium tritide by heating to 350°C. Tritium gas was stored as uranium tritide in two generators which were interconnected to allow separation of ^3He , the decay product, by repeat generation and absorption of tritium alternately between the two generators followed by pumping. Each generator contains 20 g of uranium (depleted) metal and is capable of absorbing about 7 kilocuries of tritium gas. Waste tritium and volatile organics containing tritium were continuously circulated through a heated bed of CuO at 800°C until complete oxidation to tritiated water which was then absorbed in silica gel for waste disposal.

Microwave Discharge Activation Assembly

The system used for tritium labeling by microwave excitation of tritium gas, consisted of a 100-W microwave generator (Model MPC-G-4109, Keva

Instrument Corporation, Rockville, Maryland), an Evenson microwave cavity (Opthos Instrument Co., Rockville, Maryland), and a glass-quartz tritiating apparatus. The latter was modeled after that described by Hembree et al. [4] but with some modification (see Fig. 1), so that liquid samples could be introduced onto the sample stage in an inert atmosphere.

Gas-Liquid Radiochromatography (GLRC)

Sample analyses were performed with a Varian 3700 gas chromatograph connected in series with a heated proportional flow counter. Details of the system has already been described [7].

In analyzing the labeled samples, the effluent from the gas chromatographic column (He: 50 ml/min.) was mixed with propane (100 ml/min.) before passing through the proportional flow counter. This composition of gases gave a good counting plateau in the region of 2500 volts. A multichannel analyzer (Tracor Northern Model 1705) operating in the multiscaling mode was used to record the counts. The dwell time per channel was 6 seconds; thus the 1024-channel analyzer was able to record a radiogas chromatogram for a period of 102.4 minutes. Integration of the area, i.e., the counts, for the entire chromatogram and for each peak was carried out in the integration mode of the multichannel analyzer with the cursor. Mass and radioactivity peaks were matched by simultaneous recording with a dual pen strip-chart recorder using [^{14}C]toluene as the standard.

Counting efficiency of the heated proportional flow counter was calibrated daily with the injection of a sample of tritiated toluene (~50,000 CPM) and was found to be about 35% at a constant flow of the above gas mixture and

with a background of about 70-80 counts per minute. Increase in background due to contamination could be removed by heating the counter to 330°C with O₂ flowing through it overnight. Samples that were low in radioactivity ($<10^3$ dpm) were collected for assay by liquid scintillation counting. Sample collection was from an outlet to which the effluent of the gas chromatograph was directed via a heated switching Valco valve (Valco Instruments Co., Houston).

Labeling and Analytical Procedures

a. For volatile liquids

The apparatus for tritium labeling (see Fig. 1) was evacuated to a vacuum of less than 0.13 Pa and was re-pressured to 1 atmosphere with dry N₂ gas. The volatile liquid sample was introduced with a hypodermic syringe through a long needle via the septum onto the sample stage (cold finger). After introduction of the sample, the apparatus was cooled with liquid nitrogen and re-evacuated to 0.13 Pa before admitting tritium to a pressure of 677 Pa. A tritium discharge was initiated with a Telsa coil and was maintained at a microwave power of 18-20 watts for 5 minutes. During this time the tritium gas was continuously circulated over the sample by means of an all glass pump. The discharge was terminated after 5 minutes and the system was evacuated again to less than 0.13 Pa before flushing with H₂ followed by evacuation. This operation was repeated several times to ensure complete removal of residual tritium gas. The system was finally pressured to 1 atmosphere and opened. The labeled sample on the cold finger was washed off with solvent. Carriers for any anticipated labeled side products, if available, were added to the solution prior to purification.

An aliquot of the labeled sample was purified by equilibration with water five times to remove any exchangeable tritium. The purified sample was analyzed by GLRC on a 3.05-m Carbowax-20M column (6.35×10^{-3} m diameter) with temperature programming from 70°C (initial, 10 min.) to 225°C (final, 28 min.) at a rate of 8°C/min. and again on a 3.05-m SE-30 column (6.35×10^{-3} m diameter), similarly temperature programmed from an initial temperature of 50°C to determine the correspondence between the radioactivity peak and the mass peak. The Carbowax-20M column separates alkanes, cyclo-alkanes, olefins, and aromatics according to their partition coefficients, while the SE-30 column separates these compounds according to their boiling points.

Further identification of some of the mass peaks was carried out by hydrogenation of the sample in a microhydrogenation apparatus using 50-100 μ l of the sample (or sample plus solvent) with 2-3 mg of Pd (30%) on charcoal catalyst [8]. The hydrogenated product after purification was rechromatographed to determine the shift of the mass and radioactivity peaks of the unsaturates relative to those of the saturates.

b. For solid samples

A glass fiber paper (1 cm x 1 cm square) was impregnated with a methanolic solution containing 20-25 mg of benzoic acid or phenylalanine, dried, and placed on the cold finger of the tritiating apparatus [6]. The apparatus was evacuated and operated in the same manner as that described for the volatile samples. After labeling, the compound was dissolved in methanol containing a small amount of the saturated analog cyclohexylalanine or cyclohexane carboxylic acid as carrier, and the solution was lyophilized. The dried residue was reconstituted

with methanol and lyophilized. The process was repeated three times to ensure complete removal of tritiated water and the exchangeable tritium. The cyclohexylalanine was prepared by the catalytic reduction of phenylalanine in 3N HCl solvent in a Parr hydrogenator using Adams catalyst with 50 psi of H_2 at ambient temperature for about 16 hours.

The carboxylic acids were silylated with Regisil (bis-trimethyl silyl trifluoroacetamide containing 10% pyridine) for analysis. GLRC of the silylated acids was performed on a 3.05-m packed column (6.35×10^{-3} m diameter) of OVID-17 at a temperature of 184°C for benzoic acid and 208°C for phenylalanine to resolve the aryl and cyclohexyl analogs of each acid. GC/MS using a capillary column showed that derivatized samples of phenylalanine and cyclohexyl alanine contained a mixture of mono- and di-silylated derivatives. Packed columns could not resolve this silyated mixture, but did the phenyl- and cyclohexyl analogues.

RESULTS

Figure 2 shows the radiochromatogram of a tritiated styrene sample on a Carbowax-20M column. With but one exception, all the early emerging radioactivity peaks up to and including ethylbenzene disappeared on hydrogenation forming ethylcyclohexane indicating that these peaks were unsaturated precursors such as ethyl or vinyl cyclohexenes. One of the early emerging radioactivity peaks was not affected by catalytic hydrogenation and was tentatively identified as a C_7 or C_8 alkane by its retention time. Radioactivity peaks emerging above 250°C were attributed to labeled oligomers of

styrene formed by polymerization under the conditions of the experiment, but no co-chromatography with known standards was carried out. The yield of various tritiated products from a tritiated styrene sample labeled by microwave excitation is given in Table 1.

Figure 3 shows the radiochromatograms of tritiated benzene and toluene samples. In addition to low yields of labeled benzene and toluene, tritiated cyclohexane and methylcyclohexane were, respectively, the major products of tritiation, resulting from ring hydrogenation under the conditions of the experiment [9]. Other unidentified tritiated products with early retention times were also present in small amounts.

Figure 4 shows the radiochromatograms of tritiated products formed from benzoic acid and phenylalanine. The major tritiated products in the samples were the saturated analogs, i.e., cyclohexane carboxylic acid from benzoic acid and cyclohexylalanine from phenylalanine. Only insignificant amounts of labeled benzoic acid and labeled phenylalanine were detected. The absence of tritiated parent compounds in these samples is at variance with an early report by Ehrenkaufer *et al.* [10], who found significant tritium labeling of the parent amino acid by this method. It is likely that the addition of tritium to $-C=C-$ bond is affected by many factors, and one of them may be the vacuum uv light from the tritium discharge which activates the phenyl ring thereby resulting in the photo-addition of tritium. For verification we used an umbrella made of pyrex glass to shield the sample from the uv light while permitting the tritium plasma free access to it and obtained essentially the same results with respect to ring saturation without

the sample being shielded, showing that the vacuum uv light had no effect on ring saturation by tritium atoms.

Table 2 gives the percent yield of tritiated products from aromatic compounds by category as identified. These data show that microwave excitation labeling of benzene, toluene, benzoic acid, and phenylalanine gave rise to tritiated products mainly by ring saturation. Labeled parent compounds were formed by tritium substitution to the extent of 1-2% of the yield. Tritiated products formed from the scission of carbon-carbon bonds were more abundant in benzene and toluene than in benzoic acid and phenylalanine; whereas the latter yielded more labeled polymeric products which were retained on the chromatographic column.

Figure 5 shows the radiochromatogram of a tritiated benzene sample using isopropyl benzene as the post-irradiation solvent. The chromatogram has a late emerging, broad radioactivity peak just prior to the mass peak of isopropyl benzene. This radioactivity peak is probably due to the reaction between a long-lived tritium-containing radical and the solvent molecule. Its occurrence was avoided by using n-decane instead of isopropyl benzene as solvent or by adding 0.2% of iodine to the benzene sample. Thawing the frozen benzene sample completely immediately after tritiation before it was taken up in isopropyl benzene failed to prevent the appearance of the observed radioactivity peak [9].

Table 3 shows the percent distribution of tritium radioactivity in samples of some alkanes and cyclo-alkanes. With the saturated compounds, labeling of the parent compound occurred by tritium substitution, while with the unsaturates, addition of tritium to $-C=C-$ bond is preferred to tritium substitution. The high yield of tritiated heptene-2 is an exception. Substitution of methyne hydrogens in methylcyclohexane and 3-methylhexane is particularly noteworthy because of the high labeling yields and the absence of volatile by-products.

DISCUSSION

Our study of tritium labeling of model compounds by microwave excitation has revealed that tritiation of benzene, toluene, benzoic acid, and phenylalanine yielded mostly ring saturated products with approximately 1-2% of the parent compound tritiated. The same labeling technique yielded about 8% tritiated styrene from styrene. Ring saturation of phenylalanine under these conditions has not been previously reported. Considering the ubiquity of phenylalanine in proteins and peptides, its ready saturation by tritium atoms would make microwave excitation for tritium labeling a precarious choice, unless some unknown factors mitigate the undesirable tritium addition reaction.

In tritiated benzene, toluene, benzoic acid, and phenylalanine samples the absence of any labeled intermediates in the hydrogenation of the benzene ring to cyclohexane ring, indicate that tritium addition to the ring is extremely fast [cf.9]. A study of the tritium isotope effect affecting the retention time of tritiated versus the unlabeled compound showed that the tritiated cyclohexyl ring probably contained six tritium atoms [11]. The

mechanism of such addition is not known. The addition reaction is unaffected by the vacuum uv light or by the presence of iodine in the sample [9]. Since iodine is known to scavenge thermal radicals, its inability to prevent tritium addition to the ring implies that hot tritium atoms generated in the microwave plasma are the main agents in the tritium labeling. Ring saturation may be modified by substituent groups [9]. The radiochromatogram of the tritiated styrene sample showed the presence of tritiated styrene in about 8% yield. Apparently, the presence of the vinyl group in the styrene molecule can modify the addition reactions of tritium atoms to the ring.

Tritiation of alkanes and cyclo-alkanes such as 3-methylhexane, cyclohexane, methylcyclohexane, etc. yielded mainly tritiated parent compounds. In alkenes such as cyclohexene, tritium incorporation was by addition to the $-C=C-$ bond with significant tritium substitution. With heptene-2, our data show that tritium substitution and tritium addition reactions were equally important, resulting in a 24.5% tritium incorporation in the parent molecule. The presence of the methyne hydrogen in methylcyclohexane and 3-methylhexane favored tritium substitution, leading to a higher labeling yield of the parent molecule. Methyne hydrogen is apparently also susceptible to tritium substitution by long-lived radicals containing tritium as observed in the reaction of isopropyl benzene when it is used as a solvent for the tritiated benzene sample.

In conclusion, the results of our study reveal that tritium labeling by microwave excitation may involve hot tritium atoms, not scavenged by iodine, and long-lived radicals containing tritium, scavenged by iodine. The former

adds to the benzene ring and $-C=C-$ bonds to yield saturated analogs and is mainly responsible for tritium labeling by addition and substitution, while the latter is long-lived and reacts with susceptible solvent molecules containing methyne groups producing additional labeled contaminant after the irradiation has been completed.

ACKNOWLEDGMENT

The authors gratefully acknowledge Ray Aune for manipulating the tritium generating facility and Craig Hodges for preparing the cyclohexylalanine. This work was supported by the Division of Biomedical and Environmental Research of the U.S. Department of Energy under Contract No. W-7405-ENG-48.

TABLE I

Distribution of Tritium Activity in a Sample of Labeled Styrene

<u>Labeled Product</u>	<u>Tritium Yield, %</u>
(Ethylcyclohexane, cyclohexenes, cyclohexadienes) ^a	27
Ethylbenzene	24
Styrene	7
Oligomers ^b	27
Heavy Polymers ^c	15

a) Tentatively identified by their retention times, not cochromatographed with known samples. See Figure 2.

b) Radioactivity peaks following styrene in the radiochromatogram.

c) Activity retained on the chromatographic column. % activity obtained by difference between the total tritium activity and the eluted activity.

TABLE II

Tritium Yield in the Labeling of Aromatic Compounds

<u>Substrate</u>	<u>Tritium Yield, %^a</u>			
	Cyclohexyl analog	Parent compounds	Degradation products ^b	Polymer ^c
Benzene	60.5	1.9	7.3	6
Toluene	66.6	1.5	18.5	<1
Benzoic acid	14.5	2.3	5.8	69
Phenylalanine	23.1	2.0	13.3	57

a) % of tritium activity injected for analysis by GLRC

b) Based on the assumption that these compounds have shorter retention times than the corresponding cyclohexyl analog.

c) Represents the difference between the injected and the eluted radioactivity.

TABLE III

Tritium Yield in the Labeling of Aliphatic Hydrocarbons

<u>Substrate</u>	<u>Tritium Yield, %^a</u>			
	<u>Saturated analog</u>	<u>Parent compound</u>	<u>Degradation products</u>	<u>Polymers^b</u>
Cyclohexane	-	66.5	23.5	9.5
Cyclohexene	42.7	0.8	6.5	46.2
Heptene-2	43.4	24.5	1.9	30
Methylcyclohexane	-	71	ND ^c	~30
3-methylhexane	-	54	ND	30
			1.3 ^d (before)	
n-Hexane	-	<1	19 (after)	~80

a) % of activity introduced for analysis by GLRC.

b) Represents the difference between the injected and the eluted radioactivity.

c) Not detectable.

d) For radioactive products emerging before or after n-hexane on a SE-30 column.

LEGENDS

- Figure 1. Diagrammatic sketch of the tritiation apparatus.
- Figure 2. Radiochromatogram of a tritiated styrene sample on a Carbowax-20M column.
- Figure 3. Radiochromatogram of tritiated benzene (a) and toluene (b) samples.
- Figure 4. Radiochromatograms of tritiated products formed from benzoic acid unshielded (a) and shielded (b) from the uv light from the tritium discharge.
- Figure 5. Radiochromatogram of a tritiated benzene sample with isopropyl benzene as the post-irradiation solvent.

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XBL 793-4676A

Figure 1.
Diagrammatic
sketch of the
initiation apparatus

XBL755-5230

Figure 2.
Radiochromatogram
of a tritiated styrene
sample on Carbowax-
20M column

(a)

(b)

XBL 8011-2622

Figure 3.
Radioschromatogram
of a tritiated benzene
(a) and toluene (b)
samples

XBL 8010-4464

Figure 4.
Radiochromatograms of
irradiated products formed
from benzene acid unshielded (a) and shielded (b)
from the UV light from
the tritium discharge.

XBL 8011-2624

Figure 5.
Radiochromatogram of
tritiated benzene sample
with isopropyl benzene
as post-irradiation
solvent.

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