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RADIATION-INDUCED TRITIUM LABELLING AND PRODUCT ANALYSIS

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SUMMARY

By-products formed in radiation-induced tritium labelling are identified by co-chromatography with authentic samples or by structure prediction using a quantitative structure-retention index relationship. The by-products, formed from labelling of steroids, polynuclear aromatic hydrocarbons, 7-membered heterocyclic ring structures, 1,4-benzodiazepines, 1-haloalkanes, etc. with activated tritium and adsorbed tritium, are shown to be specifically labelled and anticipated products from known chemical reactions. From analyses of the by-products, one can conclude that the hydrogen abstraction by tritium atoms and the substitution by tritium ions are the mechanisms of labelling. Classification of the tritium labelling methods, on the basis of the type of tritium reagent, clearly shows the active role played by tritium atoms and ions in radiation-induced methods.

Key Words: Mechanism of tritium labelling, tritium atoms, tritium ions, radiation-induced method, structure-retention index relationship.

INTRODUCTION

Tritium labelling by radiation induced methods can be performed in different ways and under different conditions. As a result, the substrate may be labelled practically radiochemically pure or accompanied by a number of by-products or may undergo conversion to a different chemical form. To understand the mechanism of labelling it is necessary to identify these by-products. Analysis by gas-liquid radiochromatography (GLRC) can identify the original compound by matching its radioactive peak with the mass peak but the labelled by-products are formed in no-carrier-added state with negligible mass. The lack of mass makes the identification by conventional means difficult. Identification of by-products by co-chromatography with authentic samples may or may not be feasible, since these by-products

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are not known and no authentic samples can be provided. Tritium nuclear magnetic resonance spectroscopy can measure the distribution of the tritium label in a compound [1] but cannot establish its identity without the corresponding proton NMR spectrum.

PRODUCT ANALYSIS

We have used a chromatographic method to identify the labeled products. This involves analyzing the sample on non-polar and polar columns by linear temperature programmed gas chromatography and expressing the retention data as retention index according to Kováts convention [2] which uses n-alkanes as calibration standards. According to this convention, the retention index of a n-alkane is the number of carbon atoms in the molecule multiplied by 100. Thus, n-hexane will have a retention index of 600, n-octane 800, etc. The retention of a compound can then be calculated relative to the retention of the adjacent n-alkane standards and expressed in numerical index units. The difference between the retention indices on polar and non-polar columns reflects the polarity of the functional groups and substituents. The fact that the by-products are formed from one known parent compound can often simplify data interpretation and structure prediction. Derivatization, such as silylation, methylation, esterification, bromination, etc., may also be performed to provide additional information for structure prediction.

The theory and the experimental details of the method have been published [3-6]. The method uses a quantitative structure-retention-index relationship (SRIR) to predict retention index from structure or structure from retention index.

According to this method, the retention index (I) of a compound can be shown to be dependent upon (i) the number of the atoms (Z) in the molecule, (ii) the retention index increment for atom addition (A) and (iii) the group retention factor $(GRF)_Z$ for the substituents and functional groups, based on Z. The relationship of these parameters with I can be shown in the following equation:

$$I = A \bullet Z + (GRF)_Z \tag{1}$$

In this equation, A is arbitrarily assigned a value of 100 according to the Kováts convention, Z is obtained directly from the structure of the compound and $(GRF)_Z$ is the chromatographic

contribution of the functional groups and substituents, based on z. The (GRF) values are characteristic for a given column and can be pre-calibrated. This equation constitutes the basis for predicting retention index from structure or structure from retention index. According to this equation, a change of retention index value of 100 signifies a change of one carbon atom in the structure. A change of retention index of about 60 units indicates chain branching. Any small structural change will be reflected by a change in retention index. A difference of 15-20 units in retention may indicate a conformational change in structure.

We used this chromatographic method to analyze products from reactions of many different categories of compounds with tritium. Based on these results, we proposed a general mechanism for tritium labelling by radiation-induced methods. It should be pointed out that the tritium atoms and ions are small and extremely reactive, and their reactions with organic compounds can be affected by the purity of the tritium gas, the substrate, the presence of extraneous impurities, the nature of the support surfaces, the tritium gas pressure, the microwave power, etc. [7].

MECHANISM

In a microwave generated tritium plasma the active tritium species that are present in high concentrations, are tritium atoms and tritium ions [7]. These species can independently initiate reactions, thus:

Abstraction: $R\text{-}H + T\bullet \longrightarrow R\bullet + HT$ (2)

$R\bullet + T\bullet \rightarrow R\text{-}T$ (Recombination)

$R\bullet \rightarrow$ Decomposition

Substitution: $R\text{-}H + T^+ \text{ (or } T_3^+) \longrightarrow R\text{-}T + H^+$ (3)

Reaction (2) shows that the tritium atom will abstract a hydrogen atom from the substrate, to be followed by radical recombination to form the labelled product. If the substrate radical R• is formed hot, it will break down into smaller radicals before combining with tritium atoms. The radical reaction can be complex; it may propagate and terminate by forming radioimpurities. Both tritium ions and atoms can react with the substrate, and we have evidence to show that tritium ions can label substrates without the formation of by-products [8,9], as shown in Reaction (3).

MODES OF TRITIUM LABELLING

Tritium labelling can be performed in different ways and under different conditions. Some examples are given below:

a. Tritium Plasma

(i) Steroids

Steroids labelled with tritium plasma were analyzed before and after purification to determine if products from random disruption of the C-C bonds were present. Such disruption would signify radiation degradation. Since the steroid nucleus is unique, detection of any ring fission product would be a direct proof that the radioimpurity is from the decomposition of the substrate and not from extraneous sources. Of more than 45 derivatives of androstane, estrane, pregnane, and cholestane we had studied [10], there was no evidence of ring fission. Steroids containing three hydroxyl groups were not labelled.

Many of the steroids were labelled with a radiochemical yield of better than 80%. The by-products were formed from three general reactions: (i) reduction of the phenyl ring and the isolated carbon-carbon double bond (ii) interconversion of the keto-enol functions and (iii) interchange of the a-b configurations at the 5(H). The formation of by-products can be attributed to hydrogen abstraction and tritium atom addition. The phenyl ring, the isolated double bond, and the keto group are reduced by tritium atom addition. The conversion between enol and keto group and between a and b configuration are initiated by hydrogen abstraction. Identification of the by-products was by co-chromatography with known compounds.

It should be pointed out that the support for dispersing the substrate plays an important role in tritium labelling. For example, progesterone supported on plain silica-alumina is labelled at the 2a position with tritium, and progesterone on 5% Ru on silica-alumina is labelled at 2b position [7].

(ii) Small molecules

Excited tritium species from microwave plasma can cause the reduction of the phenyl ring in small molecules. The reduction occurs in benzene, toluene, benzoic acid, phenylalanine, etc., and does not occur in N,N-dimethylaniline, etc. When benzoic acid was labelled as sodium

salt and phenylalanine as dipeptide phenylalanyl-tyrosine or tyrosyl-phenylalanine, the ring saturation was reduced and a substantial fraction of the substrate was labeled unchanged [11]. These results indicate that the resonance effect (±M) of the substituent group can influence the incorporation of the tritium atoms into the ring. In this respect, the active tritium species react selectively as chemical agents and not indiscriminately as high energy particles.

b. Adsorbed Tritium

Catalytic surfaces, when exposed to microwave-generated tritium plasma, can adsorb activated tritium and label compounds in the absence of tritium gas [8]. These surfaces do not adsorb ordinary tritium gas. Unlike the activated species from tritium plasma, the adsorbed tritium does not cause phenyl ring reduction.

(i) Small molecules

Adsorbed tritium can label single phenyl ring compounds, such as benzene, toluene, m-iodotoluene, nitrocyclohexane, nitrobenzene, etc. without ring saturation. The substrate is usually labelled radiochemically pure and specific. Analysis by tritium NMR spectroscopy shows that the *ortho* and *para* positions of toluene are labeled; the C2, C4 and C6 positions in 3-iodotoluene are labelled but the C5 position is not labelled [11]. This implies that the adsorbed tritium acts as an electrophile, in the form of tritium ions. In nitrocyclohexane the label is at the C1 position; in nitrobenzene the label is at the *ortho* position. This is expected from the electron density distribution in the molecule [12]. Adsorbed tritium on different supported metal catalysts can react with the same substrate to form different labelled products. Cyclohexene reacts with adsorbed tritium on Ni-silica-alumina, yielding labelled cyclohexane and a cyclohexane dimer ([^{3}H]tricyclo[6,4.0.0^{2,7}]dodec-3-ene) [13], but adsorbed tritium on Pd on alumina can label cyclohexene with a minimum formation of by-products. These examples show again the importance of the supported metal catalysts on tritium labelling.

(ii) Polynuclear aromatic hydrocarbons

Adsorbed tritium can label polynuclear aromatic hydrocarbons, such as a- and ß-naphthalenes, biphenyl, p-terphenyl, phenanthrene, fluorene, fluoranthene, triphenylene, 9-phenylanthracene, benzanthracene, pyrene, etc. at high temperature [14]. The labelled products were analyzed by gas-liquid radiochromatography and found to be radiochemically pure.

Analysis by tritium NMR spectroscopy shows that in naphthalene and biphenyl the tritium labels are in the *ortho* and *para* positions. But in fused ring systems, all the peripheral positions can be counted as *ortho* and *para* positions from the bridge heads and as a result, these positions are labelled but not equally. Details of the methods and data on tritium distribution will be published [14].

(iii) Seven-membered ring compounds and benzodiazepines

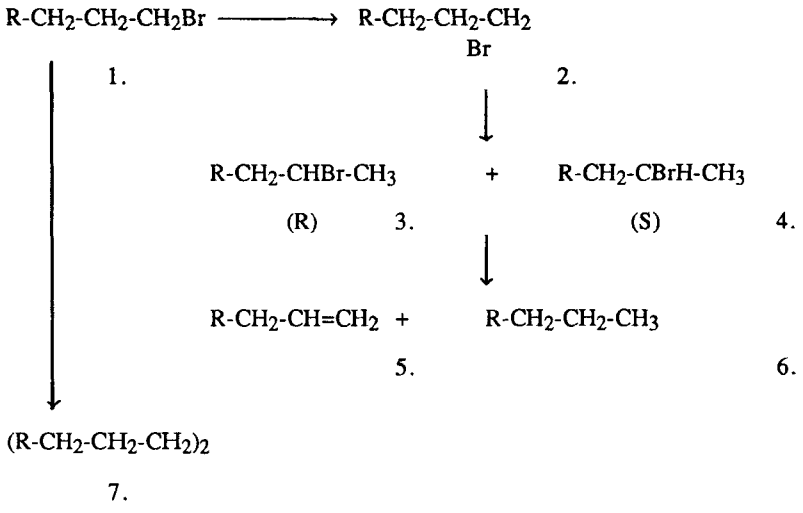
Adsorbed tritium can also label 7-membered ring structures and tricyclics with a minimum formation of by-products. The 7-membered heterocyclic diazepine ring is more stable towards adsorbed tritium than towards activated tritium from microwave plasma. The compounds studied included: 1,3,5-cyloheptatriene, 2-oxohexamethyleneimine (caprolactam), 1-benzosuberone, 1,8-diazo-[5,4,0]undec-7-ene, 5H-dibenzo[b,f]azepine, clozepine, etc. [9] and derivatives of 1,4- and 2,3-benzodiazepines, carbolines and others [15]. The labelled products were analyzed by reversed-phase HPLC. Samples found radiochemically pure were analyzed by tritium NMR spectroscopy to determine tritium distribution. Our results show that the 7-membered ring structures, including benzodiazepines, are labelled in specific positions. The specific activity of the labelled products can be in the range of curies per millimole which may vary with experimental conditions, the type of supported metal catalysts used, etc.

(iv) 1-Haloalkanes

Three series of 1-chloro-, 1-bromo-, and 1-iodo-alkanes were labelled with adsorbed tritium [16]. The carbon-halogen bond, especially the C-I bond, is of great interest in tritium labelling, since tritium is generally incorporated into organic compounds by way of tritio-deiodination. The aromatic C-I bond in phenyl ring is stable towards adsorbed tritium but the aliphatic C-hal bonds in 1-haloalkanes are more reactive and can participate in side reactions. For example, tritium adsorbed on 1.0% Ni on silica-alumina support can convert 1-bromodecane (**1**) to [^{3}H](±)-2-bromodecane (**3,4**) which is formed in no-carrier-added state and in high yield. The use of Pd or Ru catalyst instead of the Ni catalyst can increase the formation of [^{3}H]1-bromdecane, the intended product, but the major reaction product is still [^{3}H](±)-2-bromodecane.

The shift of the Br atom from position 1 to position 2 in the alkyl chain may involve a cyclic intermediate (**2**) with the bromine atom bridged between the two carbon atoms. The identity of the labelled 2-bromodecane was confirmed by co-chromatography

with a known sample. Debromination may occur and will yield labelled 1-decene (**5**) and n-decane (**6**). Various catalyst supports may promote different side reactions. For example, alumina but not silica-alumina can cause the formation of labelled eicosane (**7**) from 1-bromodecane, a product of the Wurtz type of reaction. The ability to analyze and identify these products demonstrates the usefulness of the quantitative SRIR in structure prediction. A mechanism for labelling 1-haloalkanes by adsorbed tritium, using 1-bromo-octane as example, is proposed as follows:



where R=CH_3-$(CH_2)_4$-, and (R) and (S) are chirality descriptors.

(v) Ethers and n-alkanes

Dialkyl ethers and n-alkanes contain long alkyl chains which are known to be difficult to label with tritium. Adsorbed tritium can label n-alkanes but depending upon the supported metal catalysts the product may contain a substantial amount of labelled 1-alkenes. Other by-products from chain scission and chain lengthening may or may not be present; these may be specifically promoted by a particular metal catalyst. In dioctyl ether the presence of an oxygen atom makes the molecule more reactive than a hydrocarbon; it causes the alkyl groups to rearrange and undergo chain scission and chain lengthening. As a result, oxygenated compounds with long alkyl chains are difficult to label. In contrast, adsorbed tritium can label diphenyl ether in high radiochemical purity and with no by-product formation [16].

(vi) Liquid and solid tritium

Phenylalanine, cyclosporin, etc. can be labeled with liquid and solid tritium at temperatures near absolute zero. The use of extremely low temperatures is to minimize degradation, which will be prevented by the greatly reduced rate of diffusion at temperatures near absolute zero. Our study shows that at this low temperature tritium labelling occurs by tunnelling, but the function of the supported metal catalysts and plain catalyst supports remains unchanged, relative to the function at normal temperature. In addition, the phenyl ring in phenylalanine is not saturated by solid tritium, and analysis by tritium NMR spectroscopy revealed that only the phenyl ring contains the tritium label. Bulk tritium is not necessary for labelling; a few monolayers of solid tritium over the substrate can be equally effective. The specific activity of the labelled substrate increases with time of exposure at the low temperature [17].

DISCUSSION

Hydrogen binds singly to oxygen, nitrogen and carbon atoms in organic compounds. Hydrogen atoms bound to oxygen and nitrogen atoms are labile and can undergo ready exchange. Exchange between two hydrogen atoms is not detectable but when one of the hydrogens is tritium, the exchange becomes detectable and measurable. Hydrogen atoms bound to carbon atoms are stable but those bound to activated carbon atoms (a-methylene groups to carbonyl, carboxyl, nitrogen, etc.) are labile. Owing to the difference in mass, the C-T bond has a lower zero point energy than the C-H bond and should be more stable.

Tritium incorporation can be achieved by synthetic and non-synthetic methods. In synthetic methods tritium is incorporated into the precursor by catalytic dehalogenation and catalytic reduction to yield the desired labelled product. In non-synthetic methods tritium is incorporated directly at a lower specific activity by catalytic exchange and sometimes by radiation-induced exchange. Figure 1 shows the conventional classification of tritium labelling according to the methods of preparation, with tritium gas as the isotope source (18). Tritium gas has a high specific activity and is easier to handle than other tritiated reagents.

From a mechanistic point of view, catalytic dehalogenation, catalytic hydrogenation and catalytic exchange are heterogenous reactions. The heterogeneous reaction requires the tritium gas to diffuse successively from the gas phase across the gas-liquid and liquid-solid

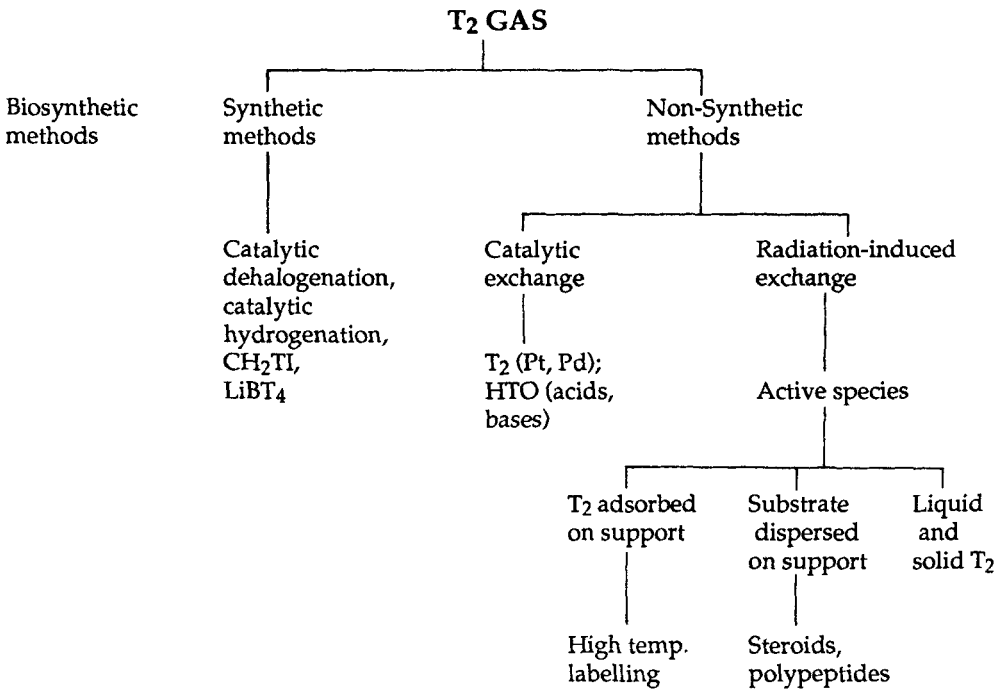


Figure 1. Classification of tritium labelling according to the methods of preparation.

interfaces onto the surface of the catalyst where it will be sorbed and activated to react with the substrate. In a homogeneous catalytic system, diffusion of the tritium gas across the liquid-solid interphase is not required. Tritium gas activated by the catalyst forms a metal tritide radical (M-T•) which can selectively react with the C-I, C=C, C=O, or C-H bond to bring about tritium substitution, addition or isotopic exchange. Different metal tritide radicals (M-T•) may possess different reactivity and selectivity when compared with the tritium atoms (T•). Tritium atoms and ions in the gas phase are the smallest and most reactive chemical entities.

In radiation-induced labeling, all bonds and groups in an organic molecule are open to gas phase tritium atoms and ions. This will lead to the formation of numerous labelled products. The labelled products can be detected and identified by co-chromatography with known compounds or by structure prediction using a quantitative SRIR. This method can yield sufficient information about all the products formed in a reaction to elucidate the labelling mechanism. Suggestions from the literature emphasize the randomness of tritium in the products from radiation-induced methods of labelling but recent product analysis and tritium

NMR spectrometric measurement have proven this to be incorrect. To accommodate these findings we suggest a reclassification of the methods for tritium labelling on the basis of the type of tritium reagent used, such as metal tritide radicals, tritium ions, etc., as shown in Figure 2. This classification is more informative and will better explain the complex data from tritium labeling from a new viewpoint. The literature contains extensive data on the reactions of hydrogen (protium) atoms with small organic molecules, but yields scanty information about

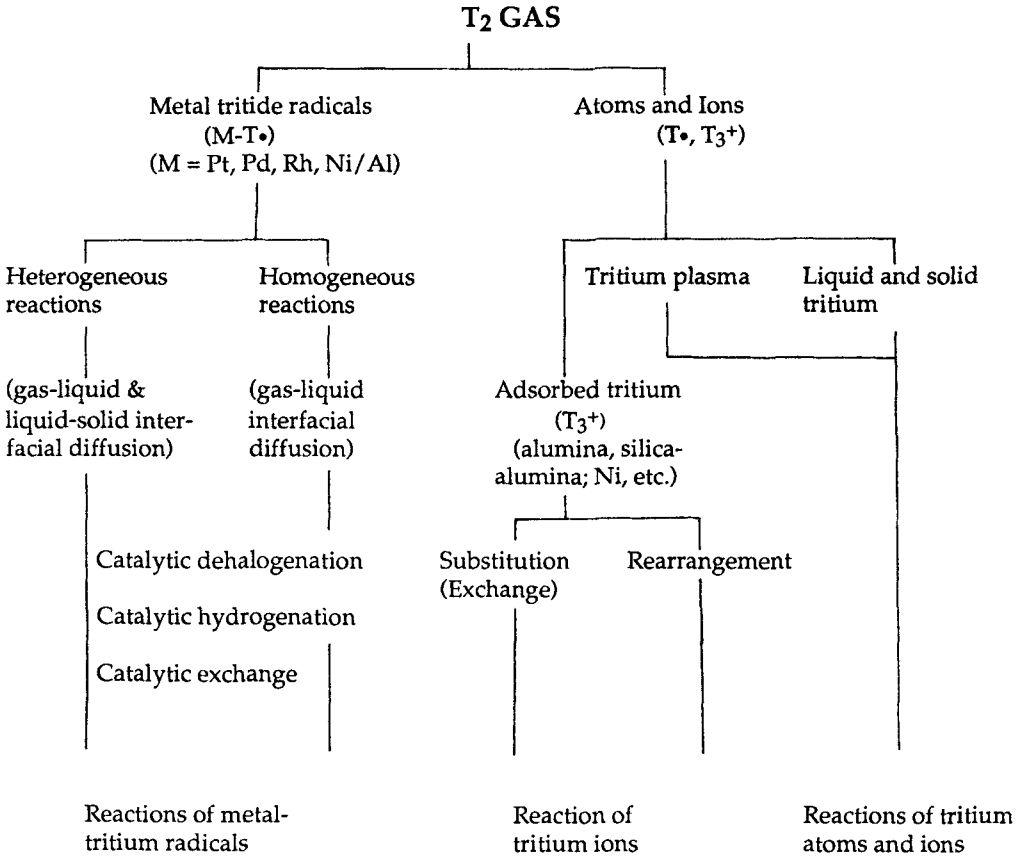


Figure 2. Classification of tritium labelling according to the type of tritium reagent used.

reactions between hydrogen atoms and large molecules. In our opinion, the reaction of tritium atoms and ions with organic molecules is an extension of the data for hydrogen (protium) atoms, and tritium has the advantage of being readily detected by radioactive counting. With the chromatographic method for structure prediction and tritium NMR spectroscopy for determining tritium location, analysis of product mixtures from radiation-induced labelling becomes less formidable than before. We need to know the behavior of the hydrogen atoms around large molecules, peptides, etc. to utilize this information for labelling biomacromolecules with tritium. Direct reactions with activated tritium species may be the only means to achieve such a goal.

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