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The Preparation of Labelled Organic Compounds by Treatment with Atomic Tritium

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The methods for the atomisation of gaseous tritium by thermal dissociation or under the influence of a high-frequency discharge, the reactions of atomic tritium, and its use for the preparation of labelled organic compounds are considered. Special attention is devoted to labelled biologically active compounds.
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I. INTRODUCTION

The use of isotopic methods has exerted an extremely significant influence on the development of a whole series of branches of science and technology and in chemistry, biology, and fields located at the junction between these two sciences, namely biochemistry, molecular biology, and bio-organic chemistry, the application of isotopes has undoubtedly proved particularly fruitful.

Although compounds labelled by isotopes of organogenic elements are used in the isotopic method in both organic chemistry and in biological disciplines. the requirements which they must meet differ greatly in most cases. In the study of the mechanisms of organic reactions, one is interested primarily in the position of the label in the molecules of the starting materials and products; on the other hand, the concentrations of these substances are as a rule fairly large. For this reason, one uses here specifically labelled compounds (i.e. compounds labelled at a particular atom) whose molar radioactivity is not too great.

In biochemistry, molecular biology, and bio-organic chemistry, the isotopic method has come to be most widely used as a procedure for qualitative and quantitative analysis, its high sensitivity being particularly important because it makes it possible to determine reliably substances at concentrations close to those at which their biological activity is manifested. Since the sensitivity of the method is unambiguously related to the molar radioactivity (a) of the labelled compound, a tendency has clearly arisen recently towards using tritium-containing compounds instead of their analogues labelled with carbon-14 (when a single T atom is introduced into a molecule, $a \cong 30$ Ci mmol^{-1} compared with 0.064 Ci mmol^{-1} for ^{14}C) and also towards the use, together with compounds labelled specifically with tritium, also of multiply labelled compounds (labelled in more than one position in the molecule), of non-specifically labelled compounds (i.e. those in which the exact distribution of T atoms in the molecule is unknown), and generally labelled compounds (those labelled in all possible positions). All these features make it possible to increase a to 100–200 (and sometimes more (Ci mmol^{-1}. Using certain other radioactive isotopes of organogenic elements (^{32}P, ^{33}P, ^{35}S, and ^{125}I), it is possible to achieve even higher values of a; however, in contrast to tritium and ^{14}C, these isotopes are not universal for organic compounds, which naturally narrows the region of their applicability.

Other advantages of tritium over ^{14}C are its weaker β-emission, the possibility of introducing the tritium label by exchange methods, and also the much lower cost of tritium-labelled compounds.

Thus the importance of tritium-containing compounds as research tools is particularly great in biology and related disciplines and the development of methods for the introduction of the tritium label into organic compounds (in the first place into complex molecules of biopolymers) is required very urgently. In order to obtain compounds labelled with tritium, chemical (synthesis), biological (biosynthesis, and enzymic reactions), and exchange (isotope exchange) methods are used.[1,2] However, one should bear in mind that, for a whole series of complex organic compounds, chemical synthesis has so far been either not described at all or its achievement in the isotopically labelled version is virtually impossible. Biosynthesis is also by no means possible in all cases and frequently it does not allow the attainment of the required values of a and involves considerable difficulties in the isolation and purification of the products.

The important advantages of exchange methods are their universality, the presence of only one stage in the process ("high speed"), and the fact that they do not require the presence of the corresponding precursors and permit the introduction of tritium directly into the "finished" molecule. The exchange methods can be divided into catalytic (see Neiman's review[3]) and methods which can be called physical. The latter are understood to involve the substitution of hydrogen by tritium as a result of the interaction of organic compounds with tritium ions or atoms possessing an excess energy (hot tritium atoms, atomic beams, activation of gaseous tritium by its intrinsic β-emission or external factors—ultraviolet light, high-voltage electric discharge, high-frequency microwave discharge, and thermal dissociation).

In all probability the first method of this kind is that of Wilzbach,[4] which consists of the maintenance of the given substance in an atmosphere of gaseous tritium. The exposures required for the appreciable incorporation of the label were fairly long: ranging from several days to months. The Wilzbach method is universal, but its application entails vigorous decomposition reactions of the test substance, the formation of a large amount of labelled side products, and a low coefficient of utilisation of tritium. In subsequent years a whole series of improved versions of the Wilzbach method were proposed, but fundamental improvements—a decrease of

the degree of decomposition of the test substance and an increase of a—were achieved only using activated tritium atoms as the main reactive species.

The present review is devoted precisely to these methods for the activation of gaseous tritium—the thermal dissociation method and the discharge method (particularly the microwave discharge in the high-frequency and ultrahigh frequency ranges), which will be designated below as TAM (the thermal activation method) and HFAM (the high-frequency activation method). Both procedures have been investigated very vigorously and improved during recent years, but the data referring to them are being surveyed for the first time. Their advantages include true rapidity (the duration of exposure is 0.25–60 min), universality, and the possibility in many instances (this applies particularly to the TAM) to reduce the consumption of tritium in a single experiment to several tens of millicuries. The latter factor is extremely important, since it permits experiments in radioisotope laboratories of class III where special protective equipment is not required.

On the other hand, the trend which has arisen in the employment of radioisotopic methods in biological disciplines can be formulated thus: increasing numbers of a wide variety of labelled compounds with an increasingly complex structure are needed; it is required to reduce their amounts as much as possible but to maintain at the same time a high molar radioactivity. For this reason, the commercial manufacture of such compounds, which involves the development of individual methods of purification, storage, and analysis, has become increasingly complex and expensive. The most attractive solution to this problem evidently involves the creation of a cheap, reliable, universal, and rapid laboratory method for the introduction of the label†; we believe that the HFAM and particularly the TAM are suitable candidates as laboratory methods of this kind.

The mechanisms of the processes leading to the incorporation of the label in the compound on treatment with atomic tritium and the methodological problems associated with the practical application of the TAM and the HFAM are examined below. The Wilzbach method and its modifications are not included in this review, because, firstly, they have already been considered in detail (see, for example, the relevant reviews[1,5]) and, secondly, in most cases the involvement of tritium atoms in these reactions has not been reliably demonstrated. However, these procedures as well as the results of experiments with atomic beams[6] and data on the chemistry of hot tritium atoms[1,7] will be employed so far as it is necessary for the comparison of the results and the analysis of the theoretical principles of the TAM and the HFAM.

II. THE PREPARATION OF ATOMIC TRITIUM BY THE HIGH-FREQUENCY ACTIVATION METHOD

The fact that the microwave discharge plasma contains high concentrations of atomic hydrogen in various excited and ionised states has been known for a fairly long time and this is the basis for the employment of discharges in the high-frequency and ultrahigh-frequency ranges as sources of hydrogen atoms and ions in the acceleration technology and as sources of resonance ultraviolet radiation.

The first report[8] of the use of a high-frequency discharge for the preparation of labelled compounds apparently appeared in 1960. It was shown later[9,10] that the introduction of the label is due to the tritium–hydrogen exchange occurring with participation of tritium atoms. Best results are achieved when the discharge is initiated in hydrogen diluted with helium and not in pure hydrogen: the intensity of the molecular bands in the emission spectrum diminishes and the intensity of the atomic hydogen line at 121.6 nm increases and at a frequency of 40 MHz the line becomes the only one present in the spectrum of a mixture containing 99% of helium.[11] The introduction of metallic electrodes into the discharge contaminates the plasma as a consequence of the atomisation of the material of the electrodes and in addition there is an appreciable reduction of the yield of hydrogen atoms owing to their recombination on metallic surfaces. Sources with an electrodeless discharge, i.e. apparatus in which the discharge tube containing the working gas (tritium or its mixture with an inert gas) is placed in a high-frequency field generated by external electrodes, are free from this disadvantage.

Sources of two types are usually employed—with initiation of the discharge in the region of high frequencies (tens and hundreds of MHz) and ultrahigh frequencies (thousands of MHz). The two discharges differ both as regards the conditions for the existence of a stable plasma and the apparatus used. In the high-frequency range the discharge is initiated at relatively low pressures (10^{-2}–10^{-3} mmHg) and its characteristics depend significantly on the frequency of the gases employed and the entire apparatus, particularly the components located in the discharge zone; the intensity of the resulting atomic beam in a high-frequency discharge is usually lower by two–three orders of magnitude than in the ultrahigh-frequency range. A discharge at a frequency of 2450 MHz is initiated at a pressure of the working gas of 1–5 mmHg and the beam obtained has an intensity of $\sim 10^{14}$–10^{15} atoms cm^{-2} s^{-1}; the characteristics of the discharge depend less on the presence of impurities, but the design of the apparatus is in this case more complex. Fig. 1 illustrates schematically the apparatus for the preparation of hydrogen (tritium) atoms with the excitation source in the region of 40 MHz. It has been shown on apparatus of this kind[12] that the yield of the principal labelled product—the parent compound—is almost independent (for an infinitely thick target) of the energy of the incident species in the range 0.5–5.0 keV.

The introduction of the label is possible not only by means of a neutral atomic beam but also with the aid of tritium ions T^+. Here charge transfer takes place in the first stage: $T^+ + RH \rightarrow T^* + RH^*$ (the asterisk denotes the excited state) and the label incorporation process itself takes place with participation of a neutral atom. The formation of excited states is due to the fact that the charge transfer conditions differ from resonance conditions. Processes leading to the thermalisation of a fast ion and to charge transfer are accompanied by the excitation of the molecules of the medium, whose character resembles that occurring under the influence of radiation. This can lead to the degradation of the test substance, which is not observed in the case of neutral species, as demonstrated fairly convincingly by comparing the effects on the target of beams of helium atoms and ions with the same kinetic energies.[12] For the preparation of labelled compounds, the apparatus can be significantly simplified:[13] instead of a system of beam focussing electrodes and the charge transfer chamber, one uses a narrow withdrawing channel at the Earth's potential, which is located at the end-face of the source and is shielded from the effects

†When the label is to be introduced into complex biological substances, it is important to ensure direct contact between the user and an investigator familiar with the procedures involved in experiments with the given specific compounds; this can be readily achieved if the labelled preparation is obtained in the laboratory where it is to be employed.

of the plasma. The form of the withdrawing electrode-channel is chosen empirically so as to ensure the best focussing of the beam; charge transfer involving atomic ions is achieved mainly in the source itself and in the channel of the electrode. The target is located at a short distance from the source and the intensity of the beam of particles is fairly high.

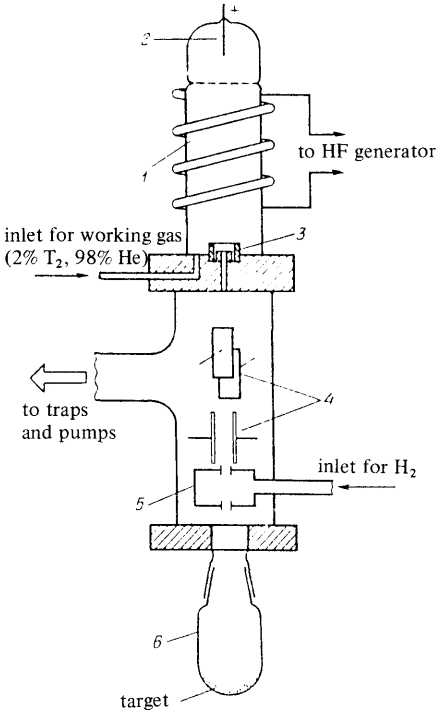


Figure 1. Apparatus for the preparation of fast tritium atoms by charge transfer involving atomic ions: 1) ion source with an electrodeless ring discharge (40 MHz); 2) accelerating electrode; 3) focussing electrode; 4) deflecting plates; 5) charge transfer chamber; 6) removable vessel for the target.

A source of atomic hydrogen with ultrahigh-frequency excitation has been used to synthesise tritium-labelled preparations.[10] The high pressure in the system and the large distance between the source and the target resulted in a low energy of the interacting atoms, which corresponded to the temperature of the walls. The apparatus is shown schematically in Fig. 2: a standard 2450 MHz generator and a standard microwave cell were used. The apparatus was connected to a pump which ensured continuous circulation of the working gas through the discharge and target zones at a controlled rate. The power evolved in the discharge is fairly high and the discharge is accompanied by an intense emission in the visible and ultraviolet regions, which may cause the photochemical decomposition of the test substance; for the protection from the latter, the target was placed outside the field of "vision" of the source. In apparatus with a high-frequency discharge, the intensity of the emission in the visible region is low and in addition only a small fraction of the emission can reach the target after passing through the narrow aperture (1–2 mm in diameter) of the withdrawing electrode. As already mentioned, in order to ensure the stability of an ultrahigh-frequency discharge, a fairly high gas pressure (~4 mmHg) is necessary, which corresponds to 1 Ci of tritium for apparatus whose volume is 500 cm^3.[10] An advantage of the high-frequency discharge is the low pressure (10^{-2}–10^{-3} mmHg), which corresponds to the consumption of 1–10 mc for the same volume. The high coefficient of the incorporation of tritium in the target makes it possible to employ the apparatus with a high-frequency discharge under conditions where the working gas flows through the discharge zone and beyond the pump it is sufficient to place a device for the oxidation of tritium and a trap. On the other hand, in apparatus with a UHF discharge, it is necessary to employ repeated gas circulation as a consequence of the low coefficient of the incorporation of tritium in the target due to the low energy of the atoms, which requires the inclusion of an additional pump and a more effective geometry of the apparatus. Furthermore, as the reaction proceeds, the working gas is enriched in protium.

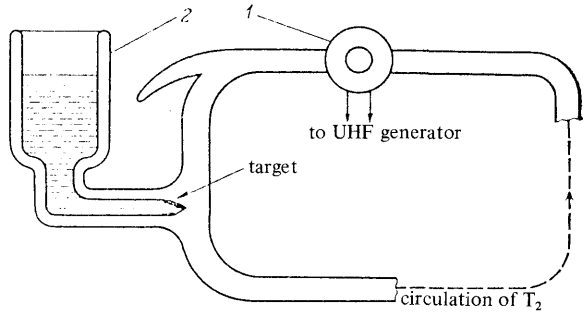


Figure 2. Schematic illustration of the apparatus for the introduction of the label with the aid of tritium atoms generated in an ultrahigh-frequency discharge (2450 MHz): 1) microwave cell; 2) trap with liquid nitrogen for the cooling of the target. The pump for the circulation of tritium and the system of pressure sensors are not shown in the diagram.

As regards the influence of various parameters on a, it has been found that the value of a for the labelled product increases with decrease of the thickness of the target or with increase of the duration of exposure to 5 min and is independent of the power of the discharge.[10] An increase of the distance from the source to the target reduces the yield of the labelled product. Low energies of tritium atoms are responsible for the dependence of the degree of incorporation of the label on the target temperature—on passing from 77 to 300 K, the activity a increases by a factor of ~5, but the degree of degradation of the test substance increases at the same time.

III. THE PREPARATION OF TRITIUM ATOMS BY THE THERMAL DISSOCIATION OF T_2

High temperatures in the range ~2000–3000 K are necessary in order to dissociate molecular hydrogen into atoms at an appreciable rate. On the other hand, the so-called furnace

sources employed in the molecular beam technique make it possible to heat the gas only to several hundreds of degrees and are naturally unsuitable for the preparation of atomic hydrogen. A novel and extremely simple technical solution has been found on the basis of the phenomenon of the catalytic dissociation of hydrogen on heated metallic filaments, which had been observed a long time ago by Langmuir.[14] The sources based on this principle have been used fairly extensively in studies of the reactions of atomic hydrogen with solid organic substances.[15,16] In order to improve the sensitivity, a number of workers have used a radioactive isotope (tritium) in their experiments.

The results of these studies are the basis of the method of synthesis of labelled preparations. The first experiments were performed with saccharides,[17] but the high yield of degradation products cast doubt on the practical value of the method. The main cause of the first failures was the use of thick targets which prevented the removal of heat from the reaction zone. The surface of the target was heated significantly both as a result of the absorption of the light emission from the filament and heat transfer through the molecular gas and as a result of the reactions of tritium atoms with the test substance. If effective removal of heat is ensured, the degree to which the test substance is preserved reaches 100% in some cases.[18]

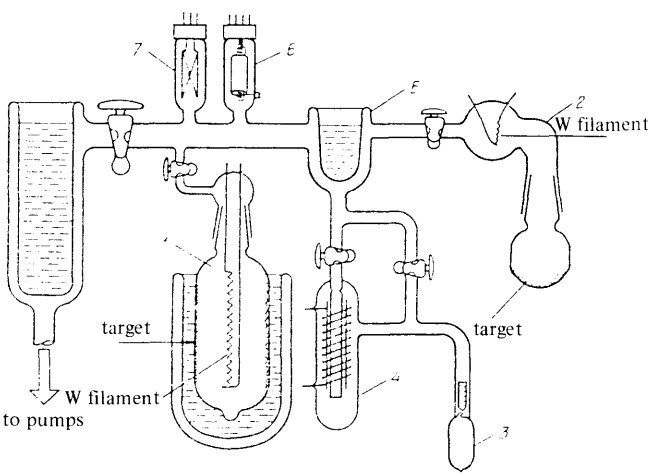


Figure 3. Apparatus for the preparation of labelled compounds with the aid of thermally activated tritium atoms: 1) vessel for the target used when the label is introduced under the conditions of direct light; 2) ditto for the introduction of the label into substances sensitive to light; 3) bulb with tritium opened by means of a metallic striker; 4) palladium leak valve for admitting measured amounts of tritium; 5) liquid nitrogen trap for freezing out condensable impurities; 6) and 7) pressure measuring sensors (thermocouple and ionisation sensors).

The apparatus used in such experiments is extremely simple (Fig. 3) and consists of a glass cylinder with electrodes sealed into it between which is stretched a metallic (tungsten) filament heated electrically. A high vacuum is created in the cylinder, after which it is filled with tritium to the required pressure. The target substance is deposited on the inner walls of the cylinder cooled with liquid nitrogen or other cooling agents, which is necessary to prevent the access of the vapour of the test substance to the filament. The target film can be obtained by evaporating solutions or, what is more convenient for biopolymers, by lyophilisation. It is also possible to obtain frozen films of solutions, but it is then necessary to take into account the possible decomposition of the specimen as a consequence of the crystallisation of water during freezing. The undesirable effects can be avoided by employing rapid cooling of the target (for this purpose, it is best to use cooling agents other than liquid nitrogen) or by atomising the solution onto precooled walls. Satisfactory results are obtained when porous supports (filter or chromatographic paper, etc.) are used. The formation of a homogeneous film is then greatly simplified and the chemical and radiochemical yields are improved.[19]

In most cases reaction vessels with a comparatively small volume are used, since the establishment of the required pressure (10^{-2}–10^{-3} mmHg) then requires only a small amount of tritium. In such vessels the distance between the filament and the target is 3–4 cm. In larger vessels experiments with solid targets can be performed at room temperature. To prevent the incidence of direct light onto the specimen, the filament and the target are placed at an angle relative to one another.

Tritium is admitted into the reactor through capillaries, fine control high-vacuum valves, etc. It is best to use the so-called palladium leak valves in which molecular hydrogen diffuses through a palladium foil at an elevated temperature. This type of valve is extremely convenient for metering specified amounts and, furthermore, permits additional purification of the tritium. In the presence of a buffer reservoir it is possible to do without a leak valve altogether.

The conditions used in the introduction of the label differ little for different substances. The optimum results obtained when the temperature of the tungsten filament is 2000 K and heating is switched on for short periods (~15 s) with intervening cooling intervals (20–30 s). The tritium pressure is chosen to ensure that the atoms move from the filament to the target without colliding with molecules. This is associated with the dependence of the rate of the substitution reaction on the energy of the interacting particles. At a filament temperature of 2000 K, about 25% of the atoms formed have sufficient energy to react. The free path conditions for a distance between the filament and the target of ~3 cm are fulfilled at a pressure of ~5×10^{-3} mmHg. In the collisions between tritium atoms and target molecules unaccompanied by reaction the tritium atoms are also "cooled", as a result of which the label is localised in a thin surface layer whose thickness is ~5 Å,[20] and the attainment of high values of a requires the use of thin films of substance or the process must be frequently repeated, preparing anew a film of the substance already treated.

An increase of the temperature of the tungsten filament increases the fraction of the reactive species in the atomic beam. It can be shown that

$$n_{\varepsilon>\varepsilon_0} = \frac{2n\sqrt{\varepsilon_0}}{\sqrt{\pi(kT)^3}} \int_{\varepsilon_0}^{\infty} e^{-\varepsilon/kT} d\varepsilon = \frac{2n\sqrt{\varepsilon_0}}{\sqrt{\pi kT}} e^{-\varepsilon_0/kT}$$

where $n_{\varepsilon>\varepsilon_0}$ is the number of atoms in the beam with an energy greater than the specified limited ε_0 (activation energy) and n is the total number of atoms in the beam.

On the other hand, the rate of formation of tritium atoms itself increases ($T_2 \rightarrow 2T$); the activation energy for this process has been determined (see, for example, Brennan and Fletcher[21]) as 51.8 ± 1.0 kcal mol^{-1}, i.e. half the bond dissociation energy in the hydrogen molecule. For practical

purposes, one can employ the expression for the rate of atomisation

$$v_{at} = A\,(p)^{1/2} \exp(-\varepsilon/RT)$$

where $A = (1.5 \pm 0.5) \times 10^{2.5}$ atom cm^2 s^{-1} (mmHg)$^{-1/2}$ for tungsten, $\varepsilon = 51.8$ kcal mol^{-1}, and p is the hydrogen (tritium) pressure (mmHg).

For more rigorous calculations, the roughness coefficient, which according to most estimates is 1.4 for tungsten,[22] must be applied to the area of the filament calculated from its geometrical dimensions. Under the conditions described above at $T_{fil} \cong 2000$ K and a pressure of 5×10^{-3} mmHg, the quantity $v_{at} = 2.9 \times 10^{19}$ atom cm^{-2} s^{-1} and the beam of atoms onto the target is determined by the corresponding solid angle.

In apparatus of the type described it is usually possible to obtain, as a result of a single admission of tritium, preparations with an overall activity of 5–10 mCi; i.e. with $a = 10^3$–10^4 mCi g^{-1} for milligram amounts of the test substance. For biopolymers, for example, for proteins, this is an extremely high value (~10 Ci mmol^{-1}), which satisfies the majority of requirements. A great advantage of the method is the small consumption of tritium, which makes it possible to carry out experiments in radioisotope laboratories of any class.

IV. REACTIONS OF ATOMIC HYDROGEN

The main chemical reaction occurring when hydrogen atoms interact with saturated hydrocarbons is the abstraction of a hydrogen atom with formation of a free radical:

$$H + RH \rightarrow \dot{R} + H_2 \quad . \qquad (1)$$

The activation energy for this process is 6–8 kcal mol^{-1}.[23] For unsaturated compounds, hydrogen atoms should add to the multiple bond with a high efficiency, which also leads to the formation of a radical:

$$H + R{-}CH{=}CH{-}R' \rightarrow R{-}\dot{C}H{-}CH_2{-}R'. \qquad (2)$$

The activation energy for this reaction is 1–2 kcal mol^{-1}. Since the reaction results in the evolution of an amount of energy of ~40 kcal mol^{-1}, the radical formed is excited and can undergo various transformations, for example, can isomerise or decompose with dissociation of C–C or C–H bonds. In the case of functional derivatives of hydrocarbons, the spectrum of possible reactions is significantly broadened.

Not only primary processes but also subsequent reactions, leading to the formation of a labelled product, are evidently important for the synthesis of labelled compounds. The fate of the radicals formed via reactions (1) and (2) depends to a large extent on the experimental conditions: the phase state of the test substance, the temperature, and the chemical nature of the molecules of the medium. Thus the radical can enter into addition reactions with unsaturated compounds:

$$\dot{R} + R'{-}CH{=}CH_2 \rightarrow R'{-}CHR{-}\dot{C}H_2 \; . \qquad (3)$$

In chain reactions of this type, high-molecular-weight products are formed. When this is allowed by energy relations, the radical can abstract hydrogen or other atoms and groups of atoms from the molecules of the medium.

All the above reactions take place in both gas and liquid phases, but in the latter case account must be taken of the so-called cage effect, which limits the interaction of the radicals with one another and increases the probability of the stabilisation of the excited radicals but has little effect on the rates of reactions involving light hydrogen atoms.

In the reactions in the solid phase the differences become fundamental: the radicals formed are incorporated in the matrix and their subsequent fate depends on the properties of the latter. In a "soft" matrix, the diffusion of the radicals frequently limits the kinetic parameters of the entire reaction. In a "hard" matrix the translational movements and in many instances also the rotational movements of the radicals are hindered. This determines not only the quantitative characteristics of the rates of the processes but also the spectrum of the possible solid-phase reactions. Thus in a "hard" matrix there is relatively high probability of the stabilisation of the excited species as a result of the scattering of the energy, the intramolecular rearrangements of the type of *cis–trans*-isomerisation are hindered, etc. The lifetime of the radicals and hence their stationary concentration in a "hard" matrix are as a rule higher by several orders of magnitude than in the liquid and gas phases.

At the low temperatures usually employed for the synthesis of labelled preparations, the secondary reactions of the stable radicals with hydrogen atoms, whose mobility is fairly high even at ~100 K, assume an important role. As in the interaction of the radicals with one another, two processes are possible in this case:

$$RCH_2\dot{C}H_2 + H \rightarrow RCH_2CH_3 \text{ (recombination);} \qquad (4)$$

$$RCH_2\dot{C}H_2 + H \rightarrow RCH{=}CH_2 + H_2 \text{ (disproportionation).} \qquad (5)$$

However, the probabilities of these processes differ from those observed for the quadratic destruction of radicals—in many cases the rate of disproportionation is equal to or greater than the rate of recombination.[24]

In experiments involving the introduction of the label into organic substances, the target is usually thermostatted and the temperature (energy) of the tritium atoms is determined by the conditions of their generation. As a rule, the energy spectrum of the beam incident on the target corresponds to relatively high temperatures (ranging from room temperature to several thousands or tens of thousands of degrees) and this is responsible for their high reactivity. A decrease of temperature from 300 to 77 K should reduce the rate of reaction by a factor of ~10^{18}, i.e. almost to zero. However, it has been found that, even at temperatures close to 4 K, the rate of reaction is extremely high.[25] A detailed study showed that there are two mechanisms of the substitution of hydrogen by tritium, one of which obtains at relatively high temperatures (100–300 K) and is characterised by an activation energy of ~8 kcal mol^{-1}. At low temperatures (<100 K) the reaction with zero (0.3 kcal mol^{-1}) activation energy predominates, proceeding in all probability via a tunnel mechanism,[26] i.e. there is a possibility of completely different features of this reaction and other products may be formed. However, in the usual experiments the contribution of the tunnel reactions is small and can be disregarded.

In the region of high (≥1 eV) energies, direct substitution of hydrogen, first observed for recoil tritium atoms[27] and subsequently studied in detail,[6,28,29] proceeds together with reactions of types (1) and (2):

$$H^h + RH \rightarrow RH^h + H, \qquad (6)$$

where H^h is a hot atom. The process cross-section increases rapidly with increase of the energy and reaches a maximum at 10 eV. The use of reaction (6) for the preparation of labelled compounds is prevented by the lack of sources of hydrogen atoms with a suitable energy spectrum and intensity. Atoms capable of substituting hydrogen via mechanism (6) can be obtained by the photolysis of certain hydrogen-containing compounds (for example, HI and H_2S) or by exposure to radiation. It is also possible to use high-energy (~10^6 eV)

recoil atoms obtained as a result of nuclear transformations [for example, $^{6}Li(n\alpha)T$, $^{3}He(np)T$] and also special apparatus for the generation of beams of accelerated atoms and ions.

However, all these methods are in essence inapplicable to the introduction of a label into biologically active substances: exposure to radiation and recoil atoms is known to entail a considerable degradation of the initial compound, while apparatus with beams is extremely complex and the actual beam intensities are insufficient for the attainment of a high activity. Thus experimenters have at their disposal mainly sources of atoms with moderate energies, where the main primary process is reaction (1) and (or) (2).

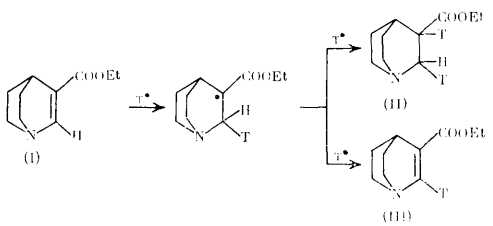
The reactions of hydrogen atoms with organic compounds have been described in detail in reviews.[30,31] Here we shall confine ourselves to the process, which plays an important role in the preparation of labelled compounds and which leads to the localisation of tritium in exchangeable groups of the type COOH, NH, NH_2, OH, SH, etc. Most of the tritium (90–99%) exists in this form and this fraction is independent of the chemical nature of the compound. Naturally such preparations cannot be used in practice without preliminary frequently fairly laborious purification. The mechanism of the reactions leading to the stabilisation of tritium atoms in the exchangeable groups is at present obscure. It may be that, as a result of stabilisation on the surface of a solid, the hydrogen atoms are polarised and interact in the form of a polar species predominantly with polar groups in the molecules of the medium. Such polarisation of hydrogen atoms has been demonstrated on the basis of EPR spectra.[31] The proposed mechanism is supported also by the finding that the coefficient of the introduction of the label into the polar groups depends little on the energy of the atoms.

V. PREPARATIVE SYNTHESIS OF LABELLED COMPOUNDS BY METHODS INVOLVING THE THERMAL AND HIGH-FREQUENCY ACTIVATION OF GASEOUS TRITIUM

As already stated, TAM and HFAM are universal methods for the introduction of the tritium label and are used to prepare labelled compounds belonging to a wide variety of classes. However, proteins (and also their fragments—peptides and aminoacids) have mainly attracted the attention of investigators and most of the studies in this field are devoted to such substances.

It was already stated above that, when organic compounds interact with tritium atoms generated under TAM and HFAM conditions, various reactions, regarded as side processes,[34–36] are observed together with the substitution of hydrogen by tritium (an analogous phenomenon has been noted also for other versions of the activation of gaseous tritium[3,32,33]). The relative contributions of the reaction involving the substitution of hydrogen by tritium and the side reactions depend greatly not only on the method of activation of T_2 (for example, the degree of addition to a double bond when the label is introduced by the Wilzbach method can be ten times greater than in the case where HFAM is employed[37]), but also on smaller changes in conditions within the framework of the same method. Thus the yield of addition products in TAM is influenced by the T_2 pressure, the temperature of the incandescent filament, the duration of exposure,[38,39] and even the character of the solvent used in the deposition of the specimen.[24] Structure factors are even more important: under identical conditions in the application of the HFAM, the ratio of the contributions of the reactions involving addition to the double bond and substitution of H by T is 2.6 for oleic acid, approximately 12 for unsaturated aminoacids, and only 0.02 for morphine.[37] This ratio can vary markedly even within the limits of a single series of compounds,[16,35] but, for simple alkenes and cycloalkenes, the saturation of the double bond as a rule proceeds more readily than the substitution of H by T.[16,35,36,39] Under TAM conditions, the substitution of H by T takes place with greater difficulty than the substitution of halogen atoms by T for both halogenoalkanes[40] and halogenobenzenes,[41] the ease of substitution of the halogen increasing in the sequence Cl << Br < I.

In a number of instances "side" reactions such as the saturation of double bonds and dehalogenation can be used for preparative purposes. Indeed, 3-ethoxycarbonyl-Δ^2-dehydroquinuclidine (I) has been converted by the TAM into the corresponding labelled saturated compound (II) and the unsaturated compound (III) in yields of 72 and 5.5% respectively:[42]



Other examples are the synthesis of labelled dihydrotestosterones from testosterone by the HFAM [43] and also the use of a high-frequency discharge (combined with heterogeneous catalysis) for the preparation of tritiated nucleoside monophosphates from the corresponding halides.[44]

The introduction of the tritium label into various classes of organic compounds is examined below.

1. Aliphatic Hydrocarbons

The study of the interaction of atomic tritium with aliphatic hydrocarbons was begun more than 20 years ago, but attention has been concentrated on the theoretical study of the reactions involved and not on the preparative synthesis of labelled compounds. In the early studies it was already shown that for alkanes (pentane and hexane) the degree of dissociation of the carbon–carbon bonds under the influence of tritium atoms, generated under both TAM [45] and HFAM [12] conditions, is much smaller than under the conditions of the Wilzbach method or in reactions with hot tritium atoms.[46] The main process is the substitution of hydrogen by tritium and in the TAM (temperature of W filaments 1750 °C)[45] hexane-*t* and pentane-*t* are obtained in yields greater than 80%. The mechanism of the substitution reaction evidently involves the abstraction of a hydrogen atom from the alkane ($RH + T^{\cdot} \rightarrow R^{\cdot} + HT$) and recombination ($R^{\cdot} + T^{\cdot} \rightarrow RT$). An alternative version is also possible—disproportionation of the alkyl radicals into an alkane and an alkene with subsequent addition of a T atom to the alkene, but the contribution of this mechanism at low temperatures is apparently small, which has also been confirmed by the low yields (5–10%) of high-molecular-weight products.[45] However, when the temperature of the specimen is increased to 90 K, the contribution of the disproportionation reaction increases sharply.[47] The first stage, namely the abstraction of a hydrogen atom, proceeds more readily at a tertiary carbon atom than at secondary and primary carbon atoms.[48]

In the case of cycloalkanes the addition of tritium, leading to the rupture of the carbon ring and the formation of a labelled alkyl radical, competes with the hydrogen abstraction reaction. The degree of this reaction is determined primarily

by the ring strain: under identical TAM conditions, the yield of cyclohexane-*t* from cyclohexane was ~80%,[45] while cyclopropane[49] was converted into cyclopropane-*t* only in 37% yield and the remaining labelled products consisted of acyclic hydrocarbons formed from the $TCH_2CH_2\dot{C}H_2$ radical. The reaction of cyclohexane with T_2 under HFAM conditions also leads to the formation of cyclohexane-*t* as the main labelled product.[12] The energy of the tritium atom plays a very significant role:[49] at a temperature of the tungsten filament of 1180 °C, only ethane-*t* and propane-*t* are formed from cyclopropane in proportions of 73 : 27, their contents among other products being reduced at 1520 °C to 37 and 50% respectively (the content of cyclopropane-*t* is ~14%) and cyclopropane-*t* becomes the main product (37%) only at 1750 °C, but the content of propane-*t* also rises to 23%.

In the interaction of cyclohexane with tritium atoms under TAM conditions at 1800–2250 K, the main labelled products, apart from cyclohexane-*t*, are also cyclohexene-*t* and bicyclohexyl-*t*.[50] Thus the cyclohexyl radicals obtained as a result of the abstraction of hydrogen from cyclohexane (activation energy $E_a \cong 7$ kcal mol^{-1}) enter into all possible reactions—recombination, disproportionation, and dimerisation.

As already noted, the addition of tritium atoms to the double bond in alkenes and cycloalkenes proceeds more readily than the substitution of H by T. For example, E_a for addition to cyclohexene is lower by a factor of 2–4 than for the substitution reaction[36] and the main product (~70%) of the reaction of cyclohexene with T_2 under TAM conditions is cyclohexane-*t*.[36,45,51] Analogously, hex-1-ene is converted in ~70% yield into hexane-*t*.[45] It has been suggested[15] that the saturation of the double bond takes place in two stages—tritium adds in succession initially to the alkene and then to the radical formed.

Considerable differences in the ease of saturation of double bonds have been noted for different alkenes:[15,16,35] propene reacts particularly rapidly, but-1-ene, isobutene, and methylbut-1-enes react more slowly, while the reactions of buta-1,3-diene, pent-1-ene, and finally hex-1-ene are slower still. These differences evidently reflect the differences between the values of E_a at low temperatures.[15] The substitution of hydrogen, whose contribution is ~25% for cyclohexane and hex-1-ene,[45] and the migration of the double bond,[24,34,35] compete with the alkene saturation reaction. It has been suggested[16,24,34,50] that tritium initially adds on to form a labelled alkyl radical, which is then converted into a labelled alkene as a result of the loss of H on interaction either with a T atom or (which is less likely) with another alkyl radical. This mechanism also explains the isomerisation of alkenes under the influence of tritium atoms observed for but-1-ene[34,35] and cyclohexylcyclohexane:[24]

$$RCH{=}CHCH_2R' \xrightarrow{T} RCHT{-}\dot{C}HCH_2R' \xrightarrow{T} RCHTCH{=}CHR' .$$

The direction of isomerisation is determined by thermodynamic factors: but-2-ene does not isomerise to but-1-ene under these conditions.

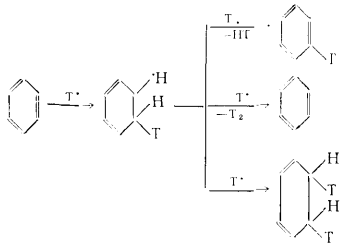
The data for alkynes are restricted to those for acetylene,[12] which is labelled by tritium under HFAM conditions without the saturation of the triple bond. When the TAM (tungsten filament, 2000 °C) is used, labelled saturation products—ethane and ethylene (25–30 and 18% respectively)—predominate and the yield of HC≡CT falls to 18%.

The reactions of atomic tritium with functional derivatives of aliphatic hydrocarbons have been very little studied. Apart from the halogenopentanes already mentioned as well as aliphatic acids, carbohydrates, and aminoacids (dealt with in separate sections—see below), there are only a few examples. Triethyloxonium tetrafluoroborate $Et_3O^+BF_4^-$ readily exchanges its hydrogen atoms for tritium under TAM conditions (2200 °C, T_2 pressure ~0.01 mmHg).[19,52] The labelled preparation had a high value of a (~5 Ci mmol^{-1}), contained no labile tritium, and did not require special purification, because all the theoretically possible cleavage or hydrolysis products are volatile substances and are removed under the experimental conditions.

Tritium-labelled sodium dodecyl sulphate ($n\text{-}C_{12}H_{25}OSO_3Na$) with $a = 0.014$ Ci g^{-1} has also been obtained by the TAM, a film of a frozen aqueous solution being bombarded.[53] A label has been introduced into dicyclohexylurea ($a = 0.178$ Ci mmol^{-1}) with the aid of the HFAM (pulse discharge, 800 kHz, T_2 corresponding to 25 c) but only ~20% of the tritium introduced was contained in the cyclohexane rings.[54]

2. Aromatic Compounds

The powerful activating influence of the discharge on the incorporation of tritium into aromatic compounds placed in T_2 was already noted comparatively long ago[55–57] and appreciable decomposition of the test substances was not observed. Aromatic compounds also do not decompose under TAM conditions (temperature of tungsten filament 1460 °C, pressure 0.001–0.005 mmHg)[58] but in many instances the saturation of the aromatic ring takes place vigorously. For example, labelled benzene, cyclohexene, and cyclohexane have been obtained from benzene (temperature of tungsten filament 1750 °C, pressure ~0.01 mmHg, their radioactivities being distributed in proportions of 21 : 35 : 39.[45] Evidently labelled cyclohexadienyl radicals (IV) are formed initially:



(7) (8) (9)

(IV)

Their subsequent interaction with tritium atoms can lead to the formation of labelled benzene, unlabelled benzene, or labelled cyclohexadiene, which is converted into labelled cyclohexenes and cyclohexane on combining with more tritium. The formation of cyclohexadienyl radicals on addition of excited H(T) atoms to aromatic compounds has been confirmed by EPR.[48,59,60] The relative contribution of reaction (7) is small and the radiochemical yield of benzene-*t* under these conditions does not exceed 5%.[58] In the reactions involving alkylbenzenes the substitution of H in the side chain competes with the saturation of the aromatic ring. Although this reaction, in the first stage of which H is abstracted with formation of the corresponding benzyl radicals, proceeds much more slowly than the addition to the ring,[61] it is the main process leading to the formation of labelled alkylbenzenes. With increase of the number of alkyl groups, the ability of this reaction to compete increases (i.e. in the sequence benzene < toluene < *o*-xylene < mesitylene).[58]

Thus in labelled alkylbenzenes tritium is localised mainly in the side chain (55–67% T for toluene-*t* when the HFAM is used).[62] An analogous phenomenon has been observed[63,64] for labelled aromatic aminoacids (see below). It is of interest that, in other versions of isotope exchange involving alkylbenzenes (the Wilzbach method, reaction with hot atoms,

exchange initiated by ultraviolet light or γ-radiation, and acid catalysis in solution), tritium is localised mainly in the aromatic ring.[62,65]

In addition substituents hinder (under the influence of both electronic and steric factors) the addition of tritium to the ring.[48,58] The H(T) atom never enters the position in the ring where a substituent is present and attacks only the *ortho*- or *para*-positions.[48] On the other hand, the addition to the *meta*-position is difficult, which evidently explains the greater tendency towards the addition of H(T) to 1,3-disubstituted benzenes compared with the 1,2- and 1,4-disubstituted derivatives.[48] Probably, for the same reason the aromatic ring is not involved when the tritium label is introduced into the alkaloid morphine by the HFAM.[37]

Thus when the HFAM and TAM are used, the introduction of the tritium label into aromatic compounds proceeds with greater difficulty than in the case of aliphatic hydrocarbons and it is as a rule impossible to attain high values of a. This is confirmed by the data in Table 1, which includes, together with aromatic hydrocarbons, also aromatic heterocycles—acridine and xanthine. It is significant that, under the conditions presented in Table 1, values of a greater by a factor of 50–100000 (for the HFAM) and 10–50 (for the TAM) had been obtained for certain labelled compounds of other classes under the conditions presented in Table 1.[10,19] Although the TAM is more suitable for the preparative synthesis of labelled aromatic compounds, rigorous comparative studies have not been carried out.

Table 1. Labelled aromatic compounds.

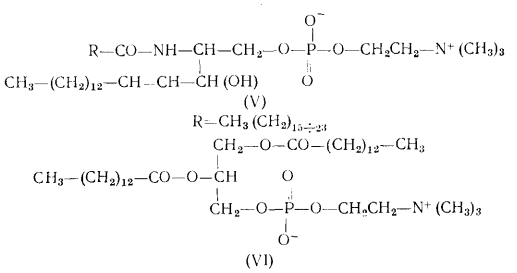
Compound	a, m Ci mmol^{-1}	Refs.
Acridine	0.045	[10]
p-Aminobenzoic acid (Na salt)*	35	[66]
Ethyl *p*-amino-benzoate*	135	[19, 66]
p-Aminosalicylic acid	0.045	[57]
Anthracene	0.515	[10]
Benzoic acid	3.8	[10]
Xanthene	0.423	[10]
9-Methylanthracene	0.04	[10]
Naphthalene	0.01	[10]

*The substance was deposited on a paper support; method of preparation—TAM; in the remaining cases the HFAM was used.

In conclusion we shall point out the likely usefulness of the synthesis of tritium-containing aromatic compounds from halogeno-derivatives. The tritium atoms generated under the TAM conditions substitute the halogen atom in halogenobenzenes; the yields of the substitution products for iodobenzene and bromobenzene are respectively 2.8 and 1.5 times greater than for chlorobenzene,[41] which agrees with the C–Hal bond dissociation energies in the above series.

3. Lipids and Aliphatic Acids

The question of the introduction of the tritium label into lipids is a matter of considerable urgency but the attempts at the direct introduction of tritium by the TAM and HFAM have so far not met with notable success. This can apparently be explained by the extensive cleavage of lipids under the conditions of their interaction with excited tritium atoms (for example, the complete decomposition of egg lecithin and dimyristoylphosphocholine has been observed[67]) and also by the presence in certain lipid molecules of residues of unsaturated aliphatic acids. Nevertheless, labelled sphingomyelin (V) (a = 0.2 c mmol^{-1}) and 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (VI) (a = 0.02 c mmol^{-1}) have been obtained[67] by the TAM. In the case of sphingomyelin the presence of an admixture of labelled dihydrosphingomyelin, which does not preclude further use of the preparation, was not specially tested for. On the other hand, despite the low value of a, the experiment with lecithin (VI) may lead to new prospects for preparative synthesis: although compound (VI) decomposed fully under the influence of tritium atoms after being deposited on the surface of a glass vessel,[67] after the deposition on a paper support the yield of the labelled preparation proved to be almost quantitative:[19]



(V)

(VI)

Another method of synthesis of labelled lipids consists in the employment of labelled aliphatic acids, which makes it possible, in particular, to obtain phospho-lipids containing simultaneously the radioactive label and photoreactive groups,[67,68] The results of the use of the TAM and the HFAM for the introduction of tritium into aliphatic acids are presented in Table 2.

Table 2. Labelled aliphatic acids.

Compound	Method	a, m Ci mmol^{-1}	Refs.
11-Aminoundecanoic acid	TAM	300	[67, 68]
Isocaproic acid (Na salt)	HFAM	19.4	[10]
Methyl 12-hydroxystearate	TAM	15	[67]
Oleic acid	HFAM	0.832	[10]
Stearic acid	ED	0.026	[69]

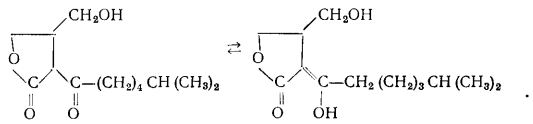
Note. In the TAM the temperature of the tungsten filament was 2000 °C and the walls of the vessel were cooled with liquid nitrogen; the parameters of the HFAM were 2450 MHz and 20 W; ED—excitation with an electric discharge (pressure 100 mmHg).

Appreciable decomposition of carboxylic acids under the conditions of their interaction with tritium atoms has been noted[67,70] and is apparently associated with the abstraction of hydrogen from the carboxy-group: the acyloxy-radicals RCOO$^{\cdot}$ formed are known to be readily cleaved into CO_2 and R$^{\cdot}$. The replacement of the free acids by their salts or esters sharply reduces the degree of decomposition: the yield of the labelled methyl ester of 12-hydroxystearic acid is 75% under conditions where the acid itself is fully

decomposed.[67] As can be seen from Table 2 for 11-aminoundecanoic acid, which is an inner salt, the binding of the carboxy-group also makes it possible to achieve higher values of a.

The mechanism of the incorporation of tritium into the hydrocarbon chain of saturated aliphatic acids is the same as for alkanes. The formation of radicals as a result of the abstraction of H from the carbon atoms in malonic and methylmalonic acid under the HFAM conditions has been confirmed by EPR.[71] As for alkenes, for unsaturated aliphatic acids the addition reaction predominates over the substitution reaction. When the HFAM is applied to oleic acid, labelled stearic and oleic acids are formed in proportions of 2.6 : 1, but the ratio is much greater when the Wilzbach method is used (24.4 : 1).[37]

The so-called A-factor (VII)—the substance which regulates the biosynthesis of the antibiotic streptomycin—should also be included among aliphatic acid derivatives. The labelled A-factor has been obtained[19] in ~30% yield with a = 0.26 c mmol^{-1} (TAM, paper support) and saturation products which might have been formed from the enolic forms of the A-factor were not detected:



(VII)

4. Low-Molecular-Weight Bioregulators

The tritium label has been introduced into low-molecular-weight bioregulators (steroids, alkaloids, antibiotics, etc.) only with the aid of the HFAM. The labelled A-factor, the synthesis of which was described above, can be regarded as the only exception#.

For steroids (corticosterone and progesterone), the classical version of the HFAM (2450 MHz, 0.7 mmHg, T_2 corresponding to 80 mCi, 5 min) yielded low results: although the chemical yield was 60–65%, the radiochemical yield did not exceed 1% and a = 0.0025–0.003 Ci mmol^{-1}.[75] When the HFAM was used in the case of aflatoxin,[76] similar values of a were achieved, but these increased markedly with increase of the surface area (with the use of a glass paper support), with decrease of the size of the test sample, and with increase of the T_2 pressure. Thus labelled 6-azido-6-dehydrodexamethasone and 21-diazo-21-deoxycorticosterone (0.15 mg, deposition on glass paper, pressure 9 atm) have been obtained by means of the HFAM with $a \cong 1$ Ci mmol^{-1}.[43] It is extremely important that the highly reactive $-N_2$ and $-N_3$ groups are not involved under HFAM conditions. On the other hand, under the same conditions in the case of testosterone the isotope exchange is accompanied by the addition of T_2 to the $\Delta^{4,5}$-bond with formation of labelled 5α- and 5β-dihydrotestosterones.[43]

On the other hand, the double bond in the alkaloid morphine is hardly saturated under HFAM conditions and morphine-t is formed with a = 0.0027 Ci mmol^{-1} (for a sample of 1 mg).[37,77] Better results were achieved for the alkaloid l-cocaine ($a \cong 0.03$ Ci mmol^{-1}), while the local anaesthetics which are its analogues (carbocaine, xylocaine, citanest, tetracaine, and procaine) are labelled less successfully.[78,79]

5. Carbohydrates

The few attempts at the preparative synthesis of labelled carbohydrates by exposure to atomic tritium cannot be recognised as entirely successful, since the values of a for the labelled compounds obtained proved to be low; however, the results achieved are no worse than those obtained when the Wilzbach method is used. Glucose, sucrose, glucose-1-phosphate (a = 0.006–0.008 Ci g^{-1}) as well as starch grains[80] (both amylose and aminopectins proved to be labelled) have been labelled with the aid of the TAM;[17] the HFAM has been used to introduce tritium into methyl-β-D-glucoside [a 99 : 1 mixture of He and T_2 and a electrodeless ring discharge (40 mHz) were used], with a radiochemical yield of ~2% and a = 0.006 c mmol^{-1},[9] and into dextran (tritium corresponding to 0.5–2 Ci, 1–4 mmHg, 2450 MHz, a = 0.2 Ci mmol^{-1}).[76] Under identical conditions, peptides and proteins are labelled to a much greater extent than carbohydrates.[9,76]

Table 3. Labelled nucleic bases, nucleotides, and nucleic acids.

Compound	m, mg	Consumption of tritium, c	Conditions	a, Ci mmol^{-1}	Refs.
Adenine	110	0.08	2450 MHz, 150 W, 0.7 mmHg, 5 min	0.021	[75]
Thymine	80	0.08	ditto	0.0048	[75]
ATP	100	25	800 kHz (pulse conditions), 200 mmHg	0.03	[81]
Guanosine monophosphate	—	—	2000 K, 0.01 mmHg, 2–5 min	10–20	[18]
Deoxycytidine tri-phosphate	—	—	99 : 1 He–T_2 mixture, 80 W, 40 MHz, 0.01 mmHg, 1 h	0.5	[82]
p-Nitrophenyl ester of AMP	—	—	ditto	0.001	[82]
ATP-3′-OCH$_3$	—	—	"	1	[82]
λ phage DNA	—	—	"	0.08	[82]
Total tRNA	—	—	"	0.01	[82]
tRNA from *E. coli*	1	30	2200 K, 0.01 mmHg, 0.5 min	0.3	[19]

Remarks. Labelled guanosine monophosphate and tRNA from *E. coli* were prepared by the TAM, while in the remaining cases the HFAM was used; the chemical yields of adenine and thymine were 85 and 80% respectively, while in the remaining cases the yield was 50% (not indicated for guanosine monophosphate); the radiochemical yields were 21% for adenine, 0.02% for tRNA for *E. coli*, and 3.6% for thymine; in the remaining cases the radiochemical yield was not indicated.

6. Nucleic Bases, Nucleotides, and Nucleic Acids

The direct introduction of tritium into nucleic acids and nucleotides is extremely important in view of the role which these compounds play in biological processes and the large number of studies in which they are employed. It has been shown[75] that the HFAM is much more convenient for this purpose than the Wilzbach method and subsequently the introduction of tritium into certain nucleic bases, nucleoside monophosphates, and nucleic acids with the aid of both the

#There are also reports[72–74] of the use of the TAM for the preparation of the tritium-labelled alkaloid aconitine and tetrodotoxin (the venom from the Fugu fish) with $a \cong 0.5$ c mmol^{-1}, but no experimental data are presented in these communications.

HFAM and the TAM was investigated; the results are presented in Table 3. The extent to which compounds of this class remain undamaged under HFAM and TAM conditions is high but polymeric molecules can be modified without cleavage. In particular, there is a possibility of the addition of tritium to the unsaturated bonds of the heterocyclic DNA and RNA nuclei because solid thymine and uracil form free radicals by combining with hydrogen atoms in a discharge in gaseous hydrogen.[83]

It is also noteworthy that a high-frequency discharge has been used[44,81] for the activation of T_2 in the synthesis of labelled nucleoside monophosphate and uracil by solid-phase reductive dehalogenation. Under HFAM conditions, the pyrophosphate bonds in adenosine triphosphate are ruptured with formation of adenosine diphosphate and adenosine monophosphate (in yields of 22 and 7% respectively), the values of a of which exceed that of labelled adenosine triphosphate by factors of ~1.5 and ~2 respectively.[81]

7. Aminoacids, Peptides, and Proteins

It was already stated above that the reactions with atomic tritium are of special interest as a method for the introduction of the label into proteins. A high degree of radioactivity can be introduced into the protein molecule also with the aid of ^{125}I or ^{131}I, but, firstly, the iodinated proteins are chemically (and frequently biologically) no longer identical with the native proteins, and secondly, the protein iodination methods used until recently involve oxidative reactions.

The synthesis of tritium-containing proteins is also difficult. The biosynthesis of the required product in any kind of satisfactory yield is possible only in a few instances and requires the employment of very high radioactivities of the HTO water and isolation and purification by extremely laborious and carefully elaborated methods. The scope of the chemical synthesis of proteins labelled with tritium is also limited. The greatest successes have been achieved[3] in the case of peptide hormones and toxins, containing up to several tens of aminoacid residues, into the molecules of which tritium had been introduced by the combination of peptide synthesis (from halogenoaminoacids) and catalytic dehalogenation. On the other hand, the exchange methods, particularly the thermal and high frequency activation of gaseous tritium, whose advantages have already been considered in the Introduction, are the most promising and universal for the synthesis of tritium-containing proteins.

Since the interaction of atomic tritium and proteins can involve various processes which depend on the primary, secondary, and tertiary structure of the protein molecules, the study of the general characteristics of this reaction was begun with model compounds—aminoacids and short peptides.[18,78,84] Table 4 shows that, with the aid of the TAM and the HFAM, it is possible to label both aliphatic and aromatic aminoacids. According to the results for alanine and phenylalanine, the TAM makes it possible to achieve higher values of a, but the comparative advantages of the TAM and HFAM for the preparative synthesis of labelled aminoacids have not been specially studied.

Apart from the reaction involving the direct substitution of H by T (Table 4), the reduction with atomic tritium of unsaturated C=C bonds and certain functional groups has also been used for preparative purposes. Thus, under HFAM conditions (20 W, 2450 MHz, T_2 equivalent to 1 Ci, 5 min), 3,4-dehydro-DL-proline undergoes mainly an addition reaction with formation of labelled DL-proline,[37] while DL-alanine-t (a = 0.0004 Ci mmol^{-1}) was the only labelled product of the interaction of α-hydroxyiminopropionic acid with HT (8000 W, 40 mmHg, 10 min).[89] The synthesis (HFAM, 1.5 keV, working gas—hydrogen with an admixture of 0.0001% of tritium, pressure 5×10^{-4} mmHg, 30 min) of labelled phenylthiohydantoin derivatives of aminoacids $RCH(NH_2).CON(Ph)C{=}S$ [13] in 1–10% yield and with $a \cong 0.001$ Ci g^{-1} should also be mentioned. The intramolecular distribution of tritium between the aminoacid residue and the phenyl group was not determined.

Table 4. Labelled aminoacids.

Aminoacid	Method	τ, min	φ, %	η, %	a, Ci mmol^{-1}	Refs.
Alanine	A	6	83	2.2	0.0016	[75, 78]
Alanine	B	—	55	—	0.05	[81]
Alanine	C	—	—	4	0.2	[9]
Alanine	D	60.17	—	—	0.65	[70, 85, 86]
Arginine	B	—	42	—	0.055	[81]
Aspartic acid	B	—	—	—	0.1	[81]
Valine	B	—	—	—	0.043	[81]
Valine	E	15	—	—	0.087	[87]
Histidine	B	—	15	—	2.32	[81]
Glycine	A	6	60	1	0.0006	[75, 78]
Glutamic acid	B	—	—	—	0.14	[81]
Isoleucine	B	y—	47	—	0.075	[81]
Leucine	A	12	72	10.2	0.0107	[75, 78]
Leucine	B	—	—	—	0.085	[81]
Leucine	E	—	—	—	0.13	[76]
Methionine	B	—	—	—	0.23	[81]
Proline	B	—	20	—	0.38	[81]
Proline	E	15	—	—	0.25	[87]
Serine	B	—	—	—	0.035	[81]
Tyrosine	B	—	—	—	0.35	[81]
Tyrosine	D	—	—	—	0.315	[88]
Tryptophan	A	0.5	64	8.9	0.0145	[75, 78]
Tryptophan	B	—	45	—	0.062	[81]
Phenylalanine	A	6	76	9	0.0119	[75, 78]
Phenylalanine	B	—	—	—	0.165	[81]
Phenylalanine	D	—	—	—	0.3	[88]
Cystine	B	—	30*	—	0.03	[81]

Remarks. Methods: A—microwave discharge (150 W, 2450 MHz), T_2 equivalent to 0.08 Ci, pressure 0.7 mmHg, DL-aminoacid sample 100 mg; B—microwave discharge under pulse conditions, T_2 equivalent to 25 Ci, pressure 200 mmHg, DL-aminoacid sample 100–150 mg; C—electrodeless ring discharge (80 W, 40 MHz), 99:1 He–T_2 mixture (pressure in the region of target 10^{-4} mmHg), sample 5 mg; D—TAM, temperature of tungsten spiral 2000 K, pressure 10^{-2} mmHg, sample 0.2–1.0 mg; E—microwave discharge (30 W, 2450 MHz), circulation conditions (190 circulations per minute), T_2 equivalent to 0.5–2.0 Ci, pressure 1–4 mmHg. In the case of proline and valine (the L-aminoacid samples were 0.1–0.4 mg each) the test substance was deposited on a paper membrane.

Notation: τ is the duration of exposure and ϕ and η are respectively the chemical and radiochemical yields.

*Cysteine (a = 0.316 Ci mmol^{-1}, yield 50%) was obtained together with cystine.

As regards the influence of the experimental conditions, it has been established for the TAM (Table 4, procedure D)[86] that a increases by a factor of approximately 7 with increase of the temperature of the film of the deposited substance from 77 to 300 K (in relation to tyrosine). For the HFAM (procedure A), a change in sample size (from 13 to 100 mg) is only slightly reflected in the value of a but leads to an approximately fourfold increase of the radiochemical yield,[78] while in the case of labelled L-proline (TAM, procedure D) an increase of the sample size from 0.1 to 1 and 10 mg entails a decrease of a by factors of ~2 and ~10 respectively.[87] In

procedure A, the variation of the power of the discharge (within the limits 50–200 W) and of its duration (in the range 3–12 min) has little influence on a and on the radiochemical yield, but both these parameters decrease by a factor of almost 2 with decrease of the time of exposure to 1 min.[78] A twofold increase of the pressure makes it possible to increase a by a factor of 2 but a subsequent increase of the pressure is less effective and for 0.96 c/T_2 the value of a is only slightly greater than for 0.08 c, while the radiochemical yield falls by a factor of ~10. The amount of labile tritium in the labelled aminoacids depends greatly on the reaction conditions. Thus alanine-t, obtained by methods C [9] and D,[86] contained respectively 60 and 90–95% of labile tritium.

The mechanism of the reaction of aminoacids with atomic tritium has been studied almost exclusively under TAM conditions. According to general ideas (see above), in the first stage attack on the molecule of the aminoacid $RCH_2CH(NH_2).COOH$ by the tritium atom results in the abstraction of hydrogen and the formation of HT and the radical $RCH_2\dot{C}.(NH_2)COOH$ (VIII) or $RCHCH(NH_2)COOH$ (IX), which are converted into labelled aminoacids in the second stage on recombination with the tritium atom. The first stage is rate-limiting and the relative contributions of the reactions involving the formation of radicals (VIII) and (IX) can be inferred from the intramolecular distribution of tritium between the α- and β-positions in the labelled aminoacids. Data for such intramolecular distribution in the case of the TAM are presented in Table 5. In all cases the formation of radicals of type (IX) is energetically more favourable, which agrees well with calculated data,[63,64,90] which indicate a greater stability of compound (IX) (greater degree of delocalisation of the unpaired electron) and also a greater mobility of the hydrogen atoms in the β-position (in all cases except alanine; the limiting electron density was used as the criterion). These results also agree with the known behaviour in the formation of free radicals: the abstraction of H from the CH_3 group is more difficult than from the substituted CH_2 group (cf. data for alanine). The influence of substituents in the introduction of tritium into aminoacids by the TAM (see Table 4) is on the whole the same as in the reaction of aminoacids with hydrogen atoms in aqueous solution.[91]

Table 5. Intramolecular distribution of tritium (%) in the labelled aminoacids $R\overset{\beta}{C}H_2\overset{\alpha}{C}H(NH_2)COOH$ obtained with the aid of the TAM.

Aminoacid	R	α-Position	β-Position		Aromatic ring	Refs.
			per C–H bond	total		
Alanine	H	43	19	57	—	[63, 73]
Serine	OH	18	41	82	—	[63, 73]
Cysteine	SH	11	45	89	—	[63, 73]
Aspartic Acid	COOH	30	35	70	—	[63, 73]
Phenylalanine	Ph	5	19	38	57	[90]
Tyrosine	p-HOC_6H_4	8	42	84	8	[64, 88]

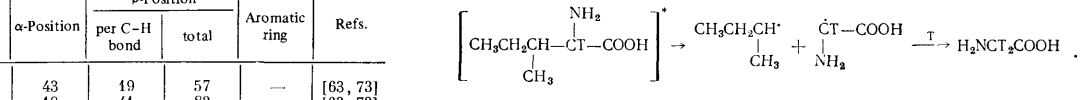
The preferential localisation of tritium in the β-position has been observed[92,93] also for labelled L-isoleucine, L-alloisoleucine, and L-valine obtained with the aid of the HFAM. In the case of valine tritium was distributed among the α-, β-, and γ-positions in proportions of 7 : 33 : 60 (i.e. 10% for each γ-C–H bond).

As regards the intramolecular distribution of tritium in labelled aromatic aminoacids—phenylalanine and tryosine (Table 5), the much greater radioactivity of the aromatic ring in phenylalanine can be readily accounted for, because, as already mentioned above, the ring substituent (in the given instance OH in tryosine) greatly hinders the addition of tritium (the rate-limiting stage).

More detailed ideas about the mechanism of the interaction of atomic tritium with aminoacids are based on the fact that the kinetics of the reactions of tritium atoms (formed by the TAM) with phenylalanine and tyrosine at a temperature <200 K deviate from the Arrhenius equation,[88,94] while at a temperature ≤60 K, the rate of reaction is altogether independent of temperature, although even at 30 K these reactions proceed at an appreciable rate.[72,95] The low-temperature kinetic limit indicates a tunnel mechanism (see above). It has been suggested[72,95] that the tunnel effect predominates at temperatures ≤200 K. This is confirmed by the dependence of the intramolecular distribution of tritium in labelled aromatic aminoacids on temperature.[88,95] For tyrosine at 300–400 K and for phenylalanine in the range 77–300 K, a change in the distribution of tritium between the aromatic ring and the side chain is also observed when the duration of exposure is altered,[88,94] which is associated with the losses of tritium from the ring as a result of disproportionation.

The side processes in the interaction of atomic tritium with aminoacids involve the cleavage of the C–C bonds with formation of labelled molecules having a lower molecular weight, the substitution of functional groups by tritium (decarboxylation, deamination, deoxygenation, and desulphurisation), stereoisomerisation, and also the addition of tritium to the aromatic ring and the dissociation of sulphide bridges. The extent of these processes depends both on the reaction conditions and on the structure of the aminoacids.

The cleavage of the carbon skeleton has been observed in many instances. Thus, under the TAM conditions, labelled propane and ethane[86] are formed from alanine, while labelled benzene, toluene, as well as glycine and alanine[94,96] are formed from phenylalanine, the last two acids being obtained also from tyrosine under the same conditions.[94] In many instances the cleavage is apparently caused not by tritium but by the emission from the incandescent tungsten spiral.[94] However, the shortening of the aminoacid chain leading to the formation of lower homologues is a true side reaction and its character is general. Under HFAM conditions, the formation of labelled glycine from L-valine[93] and L-alloisoleucine[92] has been observed; labelled glycine, containing ~20% of the total radioactivity has been obtained from L-isoleucine; it was formed as a result of the cleavage of the excited L-[2-T]isoleucine molecule:[92]



The substitution of functional groups by tritium atoms is also a general process for all aminoacids. As a result of the substitution of the carboxy-groups under TAM conditions, labelled phenylethylamine and tyramine, containing up to 10% of the total radioactivity, are obtained from phenylalanine and tyrosine respectively.[94,96] Deamination under the same conditions leads to the formation of labelled aliphatic acids: propionic acid from alanine,[85] phenylpropionic acid from phenylalanine and tyrosine (in the latter case the phenolic hydroxyl is also replaced by tritium),[94,96,97] and γ-aminobutyric acid from α,γ-diaminobutyric acid,[73] for which deamination is difficult in an acid medium. This agrees with the mechanism[95,97] according to which the tritium atom initially adds to the amino-group and then NH_2T is split off. The

substitution of the hydroxy-group in tryosine by tritium (TAM) leads to the formation of labelled phenylalanine[94] and in the case of cystine (HFAM), the sulphide bridge is readily cleaved with formation of labelled cysteine whose yield and radioactivity (Table 4) exceed the corresponding values for the labelled cystine obtained simultaneously.[81]

The addition of thermal hydrogen atoms to the aromatic rings of L-phenylalanine, L-tyrosine, and DL-tryptophan at 77 K has been detected by EPR.[60] When phenylalanine is tritiated by the Wilzbach method, labelled β-cyclohexylalanine is the main side product,[98] but under TAM conditions the benzene ring of phenylalanine or tyrosine is not saturated.[94]

The question of the racemisation of optically active aminoacids in the course of the introduction of tritium is particularly important, because it determines the suitability of the method for the preparation of tritium-containing proteins with retention of their biological activity. In general, in the reactions of tritium with solid L- or D-aminoacids, the degree of formation of the labelled optical isomer is low. In the Wilzbach method it varies from 1 to ~20%[98,99] and in the HFAM it is even smaller (for example, it amounts to 1.5% for L-valine compared with 4.0% in the Wilzbach method),[93] while the inversion of the configuration at the α-C-atom in L-isoleucine and L-alloisoleucine takes place to the extent of 1.0 and 0.3% respectively.[92] This can be explained by the fact that the α-positions in aminoacid molecules are attacked by tritium atoms only to an insignificant extent—1% for L-alloisoleucine and <8% for L-isoleucine. The predominant attack on the β-position is, however, associated also with the greater degree of epimerisation at the asymmetric β-C-atom. Thus, under HFAM conditions, labelled L- and D-isoleucines and L- and D-alloisoleucines are formed from L-isoleucine in proportions of 72 : 1 : 23 : 4 (in terms of radioactivity), while the same stereoisomers are formed from L-iso-leucine in proportions of 29 : 0.3 : 67 6 : 3.[92]

The racemisation of optically active aminoacids under TAM conditions has been investigated only in relation to L-alanine,[100] which formed a mixture of labelled L- and D-alanines (~70 : 30) at a target temperature of 77 K, while after the introduction of the label by the HFAM (under the conditions of charge transfer involving atomic ions accelerated to an energy of 0.6 keV) the aminoacid did not racemise although it contained ~25% of the added tritium in the α-position. From this, the authors[100] assume that tritium atoms with an energy >1.5 eV interact with aminoacids as a result of direct synchronous substitution of H by T and not via a radical mechanism.

It is noteworthy that the products of the side reactions are dominant overall. For example, the yield (as a percentage of the total radioactivity) of labelled phenylalanine (labelled by the TAM) is ~25% for short exposures (10–30 s), while the main side reactions involve cleavage to glycine (yield 8%) and alanine (yield 10–15%), decarboxylation to phenylethylamine (yield 10–20%), and deamination to phenylpropionic acid (yield 10–20%). For longer exposures (1–20 min), the yields of phenylalanine and tyrosine decreased to ~15 and ~25% respectively.[94]

Since the single free carboxyl and the single free amino-group (which determine the occurrence of both side reactions and the amount of labile tritium) in peptides correspond to not one but several or even several tens of aminoacids residues, one can assume that peptides are more favourable (compared with aminoacids) objects for reaction with atomic tritium. Indeed, the peptide linkage in alanylalanine is not ruptured under TAM conditions,[101] while the fraction of labile tritium in labelled peptides is smaller by a factor of 3–5 than in the labelled aminoacids obtained under identical conditions[9] and the molar radioactivity of peptides is 100–1000 times greater than that of aminoacids.[92] Thus, under identical HFAM conditions, the labelled valine and the peptides containing it, namely Ile–Ile–Val and Phe–Ala–Ala–Val, have been obtained with radioactivities of 1.1, 0.2, and 2 c mmol^{-1} respectively. The data for labelled peptides obtained by treatment with atomic tritium are compiled in Table 6.

Table 6.

Peptide	N	Method	a, Ci mmol^{-1}	φ, %	η, %	Refs.
Leu–Tyr–Leu	3	A	0.1	—	—	[10]
Ile–Ile–T–Val	3	A	0.2	—	—	[10]
Val–Ala–Ala–Phe	4	A	2.0	—	—	[10]
T-4 for a mixture obtained on hydrolysis of tobacco mosaic virus	7	B	0.2	—	—	[101]
Asp^1–Val^4–Angiotensin-II	8	C	0.3	40	—	[102]
Bradykinin	9	D	0.1	—	10	[9]
Angiotensin-I	10	E	0.4	32	2	[49, 66]
Gonadotropin releasing factor	10	F	2–3	—	—	[87]
Leuteinizing hormone-releasing factor (luliberin)	10	A	10.6	40	—	[103]
α-Amanitin		A	0.02	—	—	[76]
Somatostatin	14	A	0.75	—	—	[76]
T-8 from the products of hydrolysis of tobacco mosaic virus	20	B	2.8	—	—	[101]
Adrenocorticotropic hormone (ACTH)	39	A	10*	—	—	[76]
Antibiotic A-128	10	B	1–10*	—	—	[18]

Remarks. Method: A—HFAM (20 W, 2450 MHz, T_2 equivalent to ~1 Ci under circulation conditions, pressure 4 mmHg, 5 min), sample ≤10 mg; B—TAM (pressure ~0.01 mmHg, temperature of tungsten filament 2000 K, 2–5 min). The substance was deposited on the walls of a glass vessel cooled with liquid nitrogen; C—electric arc discharge, T_2 equivalent to ~6 Ci, pressure 10 mmHg, 30 min, sample 15 mg; D—HFAM (80 W, 40 MHz), 99 : 1 He–T_2 mixture, pressure 0.01 mmHg, sample 15 mg, E—TAM (T_2 equivalent to 0.03 Ci, pressure 0.01 mmHg, temperature of tungsten filament 2000 K, 0.5 min). The test substance was deposited on paper; F—HFAM (30 W, 2450 MHz, T_2 equivalent to 2 Ci under circulation conditions, pressure 4 mmHg, 15 min). The test substance (0.1–0.5 mg) was deposited on filter paper.

Notation: N is the number of aminoacid residues and φ and η are respectively the chemical and radiochemical yields.

*The biological activity was fully retained.

The aminoacid residues in peptides are labelled to different extents, which should be determined to a first approximation by the chemical structure of these residues. According to the results of model experiments with mixtures of 17 aminoacids, the probability of the introduction of tritium into non-labile positions in different aminoacids under TAM conditions (see Table 7)[20] decreases in the sequence aspartic acid ≥ serine > phenylalanine > tyrosine > cysteine > alanine, which is in general consistent with data for the reactions of atomic hydrogen with aminoacids in solution.[91] However, according to other data,[63,73] the sequence based on the decreasing reactivity of aminoacids under TAM conditions is different: alanine > cysteine > serine > aspartic acid. All these data apparently require revision.

The non-uniform incorporation of tritium into the aminoacid residues of peptides cannot apparently be explained solely with the aid of coefficients similar to those presented in Table 7. There is no doubt that the attack on the aminoacid

residue in the peptide is influenced both by the chemical nature of the neighbouring residues and by the steric structure of the peptide, which is indicated, in particular, by the different tritium contents in the like aminoacid residues in the T-4 and T-8 peptides from the hydrolytic reaction mixture obtained from the tobacco mosaic virus[20,101,104] (Table 6), while in the 20-membered T-8 peptide the deviation from the equi-probable distribution of tritium is significantly greater than in the shorter seven-membered T-4 peptide. Furthermore, the use of a mixture of aminoacids as a model is illegitimate, because the roles of the free amino- and carboxy-groups in the reactions of atomic tritium with both aminoacids and peptides are not comparable.

Table 7. The probability of the introduction of the label into various aminoacids (TAM conditions) per molecule (K) and per C–H bond (K') relative to alanine.

Aminoacid	K	K'	Amonoacid	K	K'
Alanine	1.00	0.33	Lysine	1.60	0.80
Arginine	1.19	0.60	Methionine	1.35	0.67
Aspartic acid	2.75	1.37	Isoleucine	1.48	1.48
Valine	1.72	1.72	Leucine	1.20	0.60
Histidine	1.76	0.88	Proline	1.39	0.70
Glycine	1.81	—	Serine	1.78	0.90
Gutamic acid	1.83	0.91	Tyrosine	1.08	0.54
Cysteine	1.04	0.52	Threonine	2.51	1.51
			Phenylalanine	1.20	0.60

Naturally in proteins the influence of steric factors should be manifested to an even greater extent and it has been suggested[72,105,106] that the very non-uniformity of the intermolecular distribution of tritium in the labelled proteins obtained on treatment with atomic tritium be used for the determination of the tertiary structure. In the case of the labelled sperm whale myoglobin, for example, the radioactivities of the valine-1 and valine-10 residues differed by a factor of 20,[101] and the residues with polar groups together with the N-terminal aminoacid proved most susceptible to attack by tritium atoms; on the other hand, the hydrophobic residues were labelled to a significantly smaller extent.[106] This is consistent with the generally accepted ideas concerning the preferential localisation of the polar aminoacid residues in the surface layer of the protein globule. The suitability, in principle, of the proposed method has been confirmed by X-ray diffraction data for different aminoacid residues in myoglobin although significant deviations have been observed for several residues.

Another application of labelled proteins obtained by treatment with atomic tritium is the determination of their primary structure.[76,101,105] Contrary to the previous case, here it is necessary that all the aminoacid residues should be labelled, i.e. that the label should be "total". It has therefore been suggested[101] that the proteins be denatured, before introducing tritium, by disrupting the order in their structure. Subsequent stepwise Edman degradation of the labelled protein leads to labelled aminoacid derivatives which can be determined in amounts of 0.1–10 nanomoles (depending on the value of a for the initial labelled protein).

Despite the interesting prospects for the investigation of the structure and functions of proteins with the aid of "totally" labelled compounds, hitherto the label has been introduced by treatment with atomic tritium into less than 20 proteins (Table 8); in a number of instances fairly high values of a have been achieved. For comparison, we may point out that labelled proteins with an almost total retention of their enzymic activity but with a only 0.001–0.015 Ci mmol^{-1} can be obtained by a modification of the Wilzbach method proposed recently (pressure ~0.1–35 mmHg, duration of exposure 1–60 h).[113]

Table 8. Labelled proteins.

Protein	Method	a, mCi mg^{-1}	a, m mmol^{-1}	Refs.
Bovine serum albumin	A	0.24	—	[72, 73, 107]
Ditto	B	0.005	—	[8]
"	C	0.1	—	[55]
Tobacco mosaic virus protein	A	—	1 000	[20]
γ-Globulin	D	—	—	[108]
Insulin	D	0.003–0.05	—	[109]
Collagen	A	—	540	[73]
β_2-Lactoglobulin	A	—	270	[73]
Lysozyme	D	1.0	—	[76]
Leuteinizing hormone	D	50	—	[10]
β_2-Microglobulin	D	0.5–5.0	—	[76]
Sperm whale myoglobin	A	—	4 000	[101, 106]
Ditto	D	0.5–1.0	—	[76]
Bovine pancreatic ribonuclease	A	—	500	[72, 73, 110]
Ditto	E	—	100	[9]
"	F	1000	—	[111]
Folic mulating hormone	D	50	—	[10]
α-Chymotrypsin	A	—	24 300	[72, 73]
Cyclo-AMP-dependent histone kinase	A	—	5 000	[112]

Remarks. Method: A—TAM (temperature of tungsten filament 2000 K, pressure 0.01 mmHg, 10–90 s), sample 2–5 mg; B—HFAM (T_2 equivalent to 0.05 Ci, 110 W, 90 s), sample ~20 mg; C—electric discharge (pressure 0.03–0.06 mmHg, 100 μA, 500 W, sample 0.5 g); D—HFAM (T_2 equivalent to 0.5–2 Ci, pressure 2–6 mmHg, 2450 MHz, 78 K, 5–20 min), sample 3–20 mg; E—HFAM (99:1 He–T_2 mixture, 40 MHz, 80 W, pressure 0.001 mmHg), sample ~5 mg; F—HFAM (T_2 equivalent to 10 Ci, pressure ~10 mmHg, 100 μA, 60 kW, 5 min (sample 50 mg).

Apparently further development of research involving labelled proteins, obtained by treatment with atomic tritium, is held back primarily by fears concerning side reactions which might take place during the introduction of the label and damage the structure and functions of the protein molecules. Such side reactions were already considered above for aminoacids and peptides; they are naturally possible also in the interaction of tritium atoms with proteins. Thus, when powdered aromatic aminoacid polymers as well as insulin, carboxypeptidase A, or papain were treated with hydrogen atoms generated under HFAM conditions, the radicals formed as a result of the addition of hydrogen atoms to the aromatic rings,[60,114] which can lead to the saturation of the latter, were identified (from the EPR spectra). The possible rupture of the sulphide bridges is a much greater risk for proteins than for peptides. The cleavage of proteins on application of the TAM has been observed in relation to bovine serum albumin.[107] However, in a number of other cases,[20,101,105,106] the bridges in polypeptides are not ruptured under TAM conditions.

The extent of all the side reactions indicated cannot be predicted *a priori* and can vary greatly for different proteins. On the other hand, the influence of the conditions used in the introduction of tritium on the retention of the native structure of the protein molecules has so far been investigated quite inadequately. All this shows how important it is to separate carefully the labelled native proteins from the products of side reactions. A general consideration of methods for the purification of proteins is outside the scope of the present review but affinity chromatography and, in particular, its variety, namely immunosorption, which has been used successfully in the purification of labelled ribonuclease[110] and serum albumin,[107] appear to be the most promising. The main advantage of these methods is their high specificity; furthermore, they make it possible to determine immediately the degree of retention of biological activity. We may note that, when the labelled protein does not lose its enzyme activity, the modification of some of the aminoacid residues in the course of the introduction of tritium does not prevent its employment in biological experiments, but such preparations are unsuitable for the study of the primary structure.

The difficulty of eliminating the products of side reactions makes another possibility of introducing the tritium label into proteins, namely with the aid of labelled modifying agents, extremely attractive. Many of these agents can be undoubtedly obtained successfully by treatment with atomic tritium. A good example is the labelled triethyloxonium tetrafluoroborate, mentioned above, whose radioactivity, when it is prepared with the aid of the TAM, can reach 5–6 Ci $mmol^{-1}$.[52,66] The use of this preparation for the esterification of terminal carboxyls makes it possible to obtain labelled modified proteins with a high radioactivity.

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Thus one may conclude that the thermal activation method is at present the most promising for the preparation of tritium-labelled compounds. Its universality, simplicity of application, low-consumption of tritium, and also the high levels of specific activities make it the most convenient for use in scientific organisations where radioisotope labels are employed in research. It is important to note in this connection that, using a simple apparatus, the investigator can himself introduce the label into the required preparation, including preparations of very complex structure, and obtain as a result a labelled product which is commercially unavailable. The method can be evidently easily converted into one of industrial importance and it may then find extensive applications in the isotope industry manufacturing tritium-labelled organic compounds.

From our point of view it is important to note yet another feature of the thermal activation method whereby it can be converted into a direct instrument for the study of the structures of micro-objects and macro-objects. Here we are dealing with the preferential localisation of the label in thin (3 – 10 Å) surface layers of the target due to the short free path of the reactive atoms. As a consequence, in analysing the distribution of tritium between the molecules constituting the given object or its intramolecular distribution, one can obtain a fairly clear and exact picture of the relative spatial dispostion of the fragments of the object. When applied to biological objects, this implies the possibility of investigating the topography of proteins in membranes and involved complexes (for example, nucleosomes), the steric organisation of biological macromolecules, etc. This application of the thermal activation method has already been shown to be promising and will undoubtedly develop.

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