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RELATIVE EFFICIENCIES OF TRITIUM ATOMS AND IONIC SPECIES IN PEPTIDE LABELING

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SUMMARY

Glycylglycyl-L-leucine and glycyl-L-leucine dispersed on silica-alumina and supported Ni catalyst, were labeled with tritium by microwave discharge activation of tritium gas, as a model to study the optimum condition for labeling peptides and proteins. In the reaction, decreasing the tritium pressure and increasing the microwave power promote the formation of tritium ions and result in an increase in specific activity of the labeled product. The highest specific activity was achieved with the adsorbate molecules as a monolayer on support. Under similar conditions, amino acids glycine and L-leucine were less efficiently labeled than the peptides. Tritium atoms generated by mercury photosensitization with a uv light were less effective for labeling than tritium ions. Impurities in tritium gas can adversely affect the degree of tritium incorporation in peptides.

Key Words: peptide labeling, tritium labeling, tritium ions, microwave discharge activation, [^{3}H]glycylglycyl-L-leucine, [^{3}H]glycyl-L-leucine

INTRODUCTION

Microwave discharge activation (MDA) of tritium gas is a potentially useful method for labeling peptides and proteins with tritium. Because of the complexity of the reaction mechanisms involved, slight variations in reaction conditions can cause mixed results in protein labeling. Hembree et al. [1] and Tscheche et al. [2] have reported successes and Wessels et al. [3] reported failures.

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The chemistry of tritium gas in microwave discharge is complex [4,5]. The plasma is composed of a mixture of excited tritium molecules, atoms and ions (T_2, T_2^*, T, T^+, T^-, T_2^+, T_3^+ and fast and thermal electrons) [5]; these activated species may differ greatly in their ability to effect tritium incorporation in organics and biomolecules. Tritium ions T_3^+ and T_2^+ when accelerated as an ion beam can tritiate peptides and proteins [6], but the T_3^+ ions are an order of magnitude more efficient than the T_2^+ ions [7]. Theoretical calculations based on ab initio SCF potential surfaces show that ionic species of tritium interact attractively with glycine and glycine zwitter ion, whereas the neutral species do not [8]. These observations clearly point out the importance of T_3^+ ions in peptide and protein labeling.

Ehrenkaufer et al. [9] observed that amino acid bound in polypeptides can be labeled to specific activities 10-1,000 fold greater than those obtained by labeling the free amino acid, implying that a very complex mechanism of labeling macromolecules with tritium may be involved. The composition of the microwave plasma changes with tritium pressure, microwave power, surface characteristics of the apparatus and purity of tritium gas; our study of the labeling mechanism was undertaken to optimize these parameters to achieve high specific activities of proteins and peptides by MDA of tritium gas, using model compounds.

RESULTS AND DISCUSSION

We studied the effects of microwave power, tritium pressure, nature of the support for substrate dispersion and the amount of substrate on radiochemical yield and specific activity of the labeled peptide. The model compounds selected were: the tripeptide glycylglycyl-L-leucine (GGL), the dipeptide glycyl-L-leucine (GL) and amino acids glycine (G) and L-leucine (L). The results are given in Figure 1 and Tables 1-4.

Figure 1 shows the effects of microwave power input on the specific activity of the labeled substrate GGL at constant tritium pressure. Varying the microwave input from 10 to 100 watts to increase the tritium plasma density leads to an increase in the specific activity of the labeled product, and the effect is more pronounced in the 80 to 100 watts region. The percent of tritium

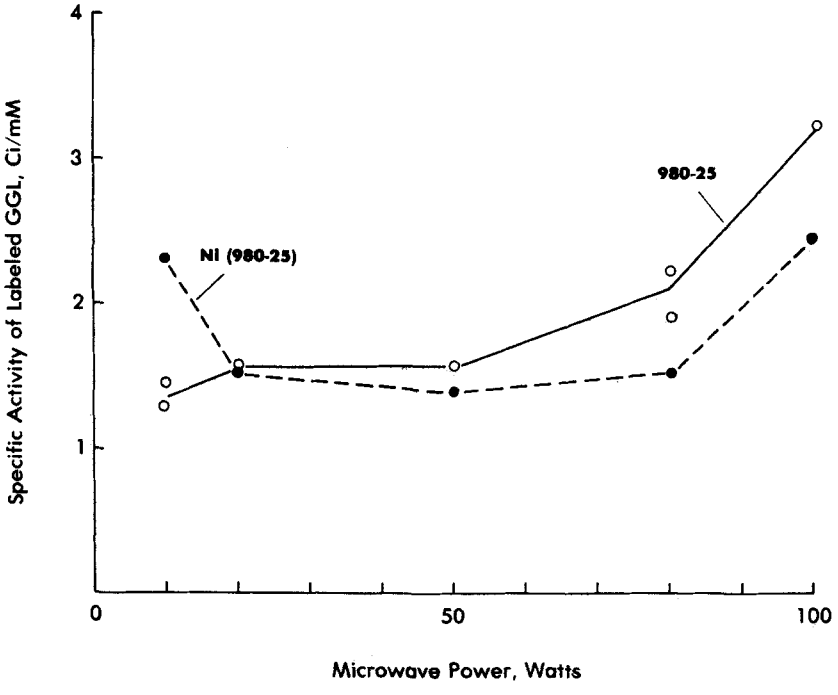

Fig. 1. Specific activity of [^{3}H]GGL vs. microwave power input at 3 torr tritium pressure. Substrate support: o ——— o for (980-25), o - - - o for [Ni(980-25)]. All data were from one batch of tritium gas.

incorporation by the crude product was estimated to be as high as 35% of the activity of the tritium gas in the discharge. The substrate was labeled in dispersed form on silica alumina (980-25) and on Ni catalyst on silica-alumina [Ni(980-25)]. Dispersion increases the contact surface between the solid substrate and gaseous tritium. At 3 torr of tritium pressure and 10 watts of microwave power, GGL labeled on (980-25) yielded low specific activity but when labeled on [Ni(980-25)] gave high specific activity; this is probably caused by additional labeling of the substrate by tritium adsorbed on the Ni catalyst. At intermediate microwave power, the specific activities of the products labeled on (980-25) and [Ni(980-25)] were comparable.

Table 1 gives the specific activities of [^{3}H]GGL labeled in dispersed form on different support surfaces. The glass fiber paper (GFP) is not an efficient

TABLE 1. Dependence of Specific Activity of [^{3}H]GGL on the Nature of Support

Sample No.	Support[a]	T_2 Pressure[b], Torr	Microwave Power, Watt	Crude Activity, mCi	Non-labile Activity, %	Specific Activity, Ci/mMol
1014-4-4b	GFP	5	20	128	14.6	0.66
1023-4-4a	"	5	20	82.4	2.9	0.18
725-5-2	"	3	80	74.8	38.1	0.29
725-5-7	"	0.3	80	43.9	35.2	0.96
1014-4-3	Ni(980-25)	5	20	-	5.5	2.55
328-5-3	"	3	80	90.0	30.7	1.39
409-5-2	980-25	3	80	83.8	35.8	1.86
725-5-6	"	0.3	80	43.9	35.2	0.96
725-5-3	Millipore, RAWP	3	80	86.5	44.3	1.63
725-5-4	"	0.3	80	96.4	31.9	0.95
725-5-1	Pt catalyst(E)	3	80	134.7	1.4	0.052
725-5-5	"	0.3	80	131.1	1.4	0.06

[a]GFP = glass fibre paper, Type A, no binder (Gelman).

Ni(980-25) = 5% Ni on #980-25 prepared and activated according to Ref. [8].

980-25 = silica-alumina catalyst support #980-25 in 1/8" pellets from Davidson Chemical Division, W.R. Grace and Co., Baltimore, MD.

Millipore RAWP = mixed esters of cellulose acetate and nitrate.

Pt catalyst(E) = 0.5% Pt on 1/8" alumina pellets from Engelhard Industries, Newark, NJ.

[b]Digital reading for T_2 pressure varied from 2.900-3.200 Torr.

substrate support for tritium labeling and gives labeled product of lower specific activity than silica-alumina (980-25) support or [Ni(980-25)] catalyst or cellulosic membrane (Millipore RAWP) filter.

The effect of varying tritium pressure on the specific activity of labeled product is given in both Tables 1 and 2. In general, GGL and GL labeled at low tritium pressure and high microwave power show higher specific activities than those labeled at high tritium pressure and low microwave power. GGL labeled on

Table 2. Effect of Tritium Pressure on Specific Activity[a]

Sample No.	Peptide	Support	T_2 Pressure, Torr	Microwave Power, Watt	Crude Activity, mCi	Non-labile Activity, %	Specific Activity, Ci/mMol
725-5-6	GGL	980-25	0.29	80	43.9	35.2	0.96
419-5-3	GGL	980-25	2.04	80	111.7	47.1	4.93
409-5-2	"	"	3.01	80	83.8	35.8	1.86
419-5-1	"	"	3.02	80	79.3	37.4	2.58
1219-5-6[b]	"	"	3.12	80	128.8	13.77	2.05
410-5-3	"	"	5.5	80	52.7	22.4	0.91
419-5-4	"	"	2.15	100	105.4	44.1	2.96
419-5-2	"	"	3.01	100	118.5	42.3	3.72
1211-5-1[b]	"	"	3.03	100	260.8	7.86	3.11
1219-5-4[b]	"	"	3.14	100	135.1	9.33	1.47
410-5-4	GL	980-25	3.22	80	115	48.7	3.28
419-5-5	"	"	2.07	100	158.6	47.7	5.90
1014-4-3	GGL	Ni(980-25)	5.12	20	-	5.5	2.55
1219-5-5[b]	"	"	3.32	80	177.0	9.62	1.96
1211-5-2[b]	"	"	1.81	100	235.1	8.95	2.61
1219-5-2[b]	"	"	3.04	100	94.6	9.57	1.61
1219-5-3[b]	"	"	2.94	100	141.4	10.35	1.78

[a]See text.

[b]Fresh tritium gas from uranium tritide was used for these experiments, and no distinctions were made for the other samples.

[Ni(980-25)] yielded higher specific activity at high tritium pressure and low microwave power, since the Ni catalyst can adsorb tritium and cause additional labeling of the dispersed substrate. The crude and purified yields of these preparations fluctuate widely; these fluctuations may be attributable to the presence of traces of oxygen and impurities in tritium gas. The tripeptide labeled on a commercially available supported Pt catalyst on 1/8" alumina pellets had a much lower specific activity than expected, this was attributed to

Table 3. Effects of Substrate Mass on Specific Activity of [^{3}H]GGL[a]

Sample No.	Amount of GGL on 908-25, mg	T_2 Pressure, Torr	Microwave Power, Watt	Crude Activity, mCi	Non-labile Activity, %	Specific Activity, Ci/mMol
808-5-1	5	2.96	80	33.5	21.0	0.47
808-5-8	2.5	2.98	80	69.8	22.7	3.22
808-5-2	0.5	2.97	80	42.1	3.78	1.38
808-5-3	0.1	3.01	80	37.5	3.5	2.60

[a]All the samples were run on one batch of tritium gas on the same day.

the presence of reactive sites on the surface of the catalyst that contained adsorbed oxygen and facilitated the formation of tritiated water, effectively removing tritium from the plasma at the expense of exchange labeling. The high crude radioactivity and the low non-labile tritium activity (less than 2% of the crude) obtained with this support as compared to other supports clearly demonstrated the importance of surface activity in tritium labeling. In addition to greater contact with gaseous tritium, dispersion of substrate on surfaces can cause adsorption and chemisorption which may afford the substrate protection from degradation. We have shown earlier that metal-ligand binding and chemisorption can protect compounds containing hydrogen sensitive groups from reaction with tritium [11].

The effect of substrate mass on the specific activity of the labeled peptide is shown in Table 3. Two and one half milligram of GGL appears to be an optimum amount for 6 pellets of the catalyst support which has a weight of about 0.148 g corresponding to a surface of about 48.1 m^2, calculated on the basis that the catalyst support (980-25) has a surface area of about 325 m^2/g [12]. Ehrenkaufer et al. [13] postulated that the highest specific activity is labeled as a monolayer on the surface of the support. The monolayer of GGL required for the surface is estimated from the solvent accessible area of the peptide (439.9 sq. Å) [14] to be approximately 2.2 mg, which is in accord with the value of 2.5 mg of GGL observed for maximum specific activity on the support.

The amino acids glycine (G) and L-leucine (L), when labeled under similar

Table 4. Chemical Nature of the Labeled Substrates and Specific Activity[a]

Sample No.	Substrate[b]	T_2 Pressure, Torr	Microwave, Power, Watt	Crude Activity, mCi	Non-labile Activity, %	Specific Activity, Ci/mMol
521-5-1	Glycine	2	80	168.5	8.7	0.074
529-5-3	" (HCl)	3	80	52.7	3.3	0.088
521-5-2	Leucine(Na)	2	80	60.5	17.7	0.20
529-5-2	" (HCl)	3	80	51.8	29.1	0.43
409-5-4	GL (Na)	3	80	115	48.7	3.28
808-5-5	" (Na)	3	80	82.9	33.0	1.61
808-5-6	" (Na)	0.3	80	106.8	30.5	0.78
419-5-5	" (Na)	2	100	158.6	47.7	4.06
328-5-4	GGL "	3.2	Hg-UV*[c]	24	- [d]	- [d]

[a]All samples were labeled on (980-25). Amount = 5 mg dissolved in a few microliters of solvent to impregnate the support.

[b]Dissolved in equimolar amounts of hydrochloric acid or sodium hydroxide solution.

[c]Sample irradiated with a 250 W Hg-UV lamp for 30 minutes. Energy input estimated to be comparable with the microwave discharge activation.

[d]Incorporated tritium activity was too low to be measured.

conditions as GL and GGL, yielded significantly lower specific activities than the peptides, as shown in Table 4. The specific activities of the amino acids responded less than those of the peptides to changes in plasma density brought about by either lowering the tritium gas pressure or increasing the microwave power.

To distinguish between the reactions of tritium atoms and tritium ions a 30-minutes photolytic irradiation of the sample was performed in the presence of 5 torr of tritium gas, using a mercury-ultraviolet light source with rating of 250 W; the irradiation did not produce enough labeled amino acid or peptide for separation by HPLC or sufficient non-labile radioactivity for measurement by liquid scintillation counting. Mercury photosensitized activation of tritium gas generates only tritium atoms by the reaction [15]:

$$Hg^*(^3P_1) + T_2 \longrightarrow Hg(^1S_o) + 2T \ .$$

The failure of this mode of labeling to achieve adequate activity strongly suggests that the tritium atoms are less efficient than tritium ions in peptide labeling. In non-local thermodynamic equilibrium plasmas, ion formation is favorably influenced by high microwave power and low gas pressure, and a slight change in power and pressure can cause a significant change in plasma density [16,17]. By using GGL, GL, G, and L as a model for tritium labeling, our studies show that peptides and proteins exchange more efficiently with tritium ions than with tritium atoms; the maximum specific activity of the peptides that can be obtained by this method is in the range of curies per millimole when using **pure** tritium for the reaction. The phenomenon that bound amino acids in peptide can be labeled to a specific activity 10-1,000-fold higher than free amino acid may be explained on the basis that peptide molecules are highly polarizable and can attract considerably more electrophilic tritium ions per molecule than the free amino acid. This phenomenon cannot be adequately explained if only tritium atoms are considered to be the labeling entity in the microwave plasma, since neutral atoms react statistically and may not prefer a bound amino acid to a free amino acid.

The purity of tritium gas can exert a profound influence on labeling by MDA of tritium gas. Tritium plasmas generated from tritium gas stored in stainless steel containers for a few weeks gave rise to labeled peptides with significantly lower specific activities than those reported in Tables 1-4. Likely impurities are CT_4 and N_2 which are known to adversely affect tritium-deuterium exchange reactions [5].

MATERIALS AND METHODS

Amino acids glycine and L-leucine and peptides glycyl-L-leucine and glycylglycyl-L-leucine were used as received from the suppliers. The amino acid or peptide (5 mg or less) was dissolved in 0.1 ml of water, dilute hydrochloric acid or sodium hydroxide, and the solution was applied uniformly to 6 pellets of silica-alumina catalyst support (#980-25, Davison Chemical Division, W.R. Grace & Co., Baltimore) or supported nickel (5%) catalyst, prepared according to Cao and Peng [10]. The impregnated pellets were dried in vacuo on a high vacuum

manifold overnight.

The dried pellets were labeled in the following manner. The dry pellets were placed on a cold finger in the tritiation apparatus described earlier [18]. The apparatus was evacuated to less than 3 millitorr before admitting tritium gas. Tritium gas was generated by heating uranium tritide to 350-400°C and in early days was stored in a reservoir from which the quantity needed for each run was metered; this practice was later abandoned in favor of fresh tritium gas generated as needed from the uranium tritide because the results were found to be more consistent with fresh tritium. Depending on the sample, the tritium pressure was varied from 0.3 to 5.5 torr, and the microwave power input from 10 to 100 watts. A distance of 10 cm intervened between the cold finger and the center of the Evenson microwave cavity. The duration of plasma labeling was chosen to be 5 minutes after which excess tritium gas was removed by evacuation and the system flushed twice with helium before opening to the atmosphere to retrieve the labeled pellets.

The labeled peptide was eluted off the catalyst support with 2 ml of water plus 0.1 ml of ethanol. The eluate was lyophilized to dryness to remove the labile tritium in the form of tritiated water and other volatiles.

Analysis of the labeled underivatized peptide was by reverse-phase high performance liquid chromatography on a Radial-Pak μBondpak C18 cartridge (Waters) using methanol: 0.1% phosphoric acid in water (30:70, v/v) as solvent for isocratic elution at a flowrate of 1.5 ml/min., with monitoring wavelength at 205 nm and sensitivity at 0.05 AUFS [19]. Some of the labeled amino acid and peptide samples were also precolumn-derivatized with o-phthalaldehyde (OPA) [20] and analyzed by gradient elution at 340 nm and at 0.05 AUFS. The solvents used for linear gradient elution were acetonitrile:phosphate buffer 0.02M KH_2PO_4 (pH 6.8) at a flowrate of 1.4 ml/min beginning at (5:95, v/v) to (50:50, v/v) in 45 minutes in a Waters gradient elution system consisting of two Model 510 pumps and a Model 680 gradient elution controller. The eluate was collected with a Pharmacia FRAC-100 fraction collector in 0.56 ml fractions in plastic pico-vials which were counted directly in a Beckman LS9000 liquid scintillation counter after addition of 4 ml of an emulsion-type scintillator. Recovery from HPLC of

the radioactivity peak of the labeled peptide was generally in the range of 50 to 80% of the injected sample. Specific activity of the labeled peptide was determined from the mass peak using a standard calibration curve and the radioactivity of the collected fractions corresponding to the labeled peptide mass peak.

The proton-decoupled triton NMR spectrum and the proton NMR spectrum of [^{3}H]glycylglycyl-L-leucine in perdeuterated dimethylsulfoxide containing TMS

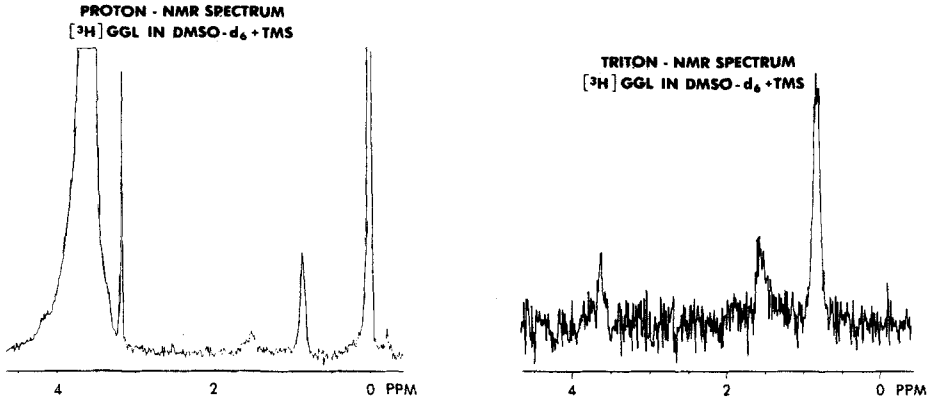

Fig. 2. Proton-decoupled ^{3}H NMR and proton NMR spectra of [^{3}H]glycylglycyl-L-leucine

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