A NOVEL SURFACE FOR HIGH SPECIFIC ACTIVITY TRITIUM LABELLING USING MICROWAVE DISCHARGE ACTIVATION OF TRITIUM GAS

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SUMMARY

A 10 to 300 fold increase in specific activity has been realized in the microwave discharge activation (MDA) of tritium gas labeling of a variety of compounds, up to the Ci/mmol range by the use of a cellulose Millipore filter (CMF) as the labeling surface. New reaction systems have also been developed to accommodate these and other large samples as well as multiple samples.

Key Words: Tritium Labeling, Microwave Discharge, Millipore Filter, Gonadotropin Releasing Hormone (GnRH), Tritium NMR.

INTRODUCTION

We have previously described a method of tritium labeling by microwave discharge activation of tritium gas (1-3) and have demonstrated its usefulness in the labeling of peptides and proteins for biological studies (4). However, to broaden its application, many attempts have been made to increase the specific activity by varying the reaction conditions, physical state of the sample and the nature of the reaction surface without significant success (1,5,6).

We now wish to report 1) the use of a Millipore filter as a labeling surface to attain high specific activity tritium labeled compounds; 2) a modified reaction vessel‡ to accommodate large Millipore filters (47 mm diam.); and 3) development of a teflon holder for the simultaneous labeling of up to

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‡ The increase in the size of the reaction area was from 0.8 cm^2 to 16 cm^2 in the modified reaction system.

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three Millipore adsorbed samples.

a) Materials

GnRH was obtained from NIH (Abbott lot # 19-192 AL). L-Valine, L-proline and L-leucyl-L-tryptophyl-L-leucine were all obtained from Schwarz/Mann. The amino acids were used without purification. The tripeptide was purified by chromatography on Dowex 50W-X2 cation exchange resin with pyridine acetate (pyr/Ac) buffers and lyophilized prior to use. The microporous filters were obtained from Millipore Corporation.

The column materials used in the purification of the tritium labeled samples were carboxymethyl cellulose (CM-22 Fibrous, Whatman) and Bio Gel P-4 (200-400 mesh, Bio Rad) for purification of GnRH. The H-70 and HP-B80 cation exchange resins (7-10μ) used in the purification of the amino acids and the leu-trp-leu tripeptide respectively were purchased from the Hamilton Company.

The aluminum reaction tray was prepared from 0.007" aluminum foil by use of a suitable die. The resulting tray was 45 mm in diameter with 3 mm high edge. The surface was roughened by scratching with an abrasive material.

The glass reaction tray was of similar dimensions as the aluminum and the surface was roughened by sandblasting.

b) Sample Preparation

Samples to be labeled were dissolved in an appropriate amount of water such that 200 μl contained the desired amount of solute. The 200 μl was then applied dropwise to an appropriate Millipore filter and allowed to permeate the filter until it was completely wetted. The water was then evaporated under vacuum in a desicator containing silica gel.

The glass and aluminum tray samples were prepared either by placing the solid sample on the tray as a powder or a water solution containing the appropriate amount of solute and evaporating the water slowly.

c) Labeling System

To accommodate the large Millipore filter (as well as larger samples in general) the reaction area as originally described (1) and modified by the installation of light traps (2,7) was further modified as shown in Figure 1. A

FIGURE 1. MODIFIED TRITIUM LABELING REACTION AREA FOR LARGE SAMPLES

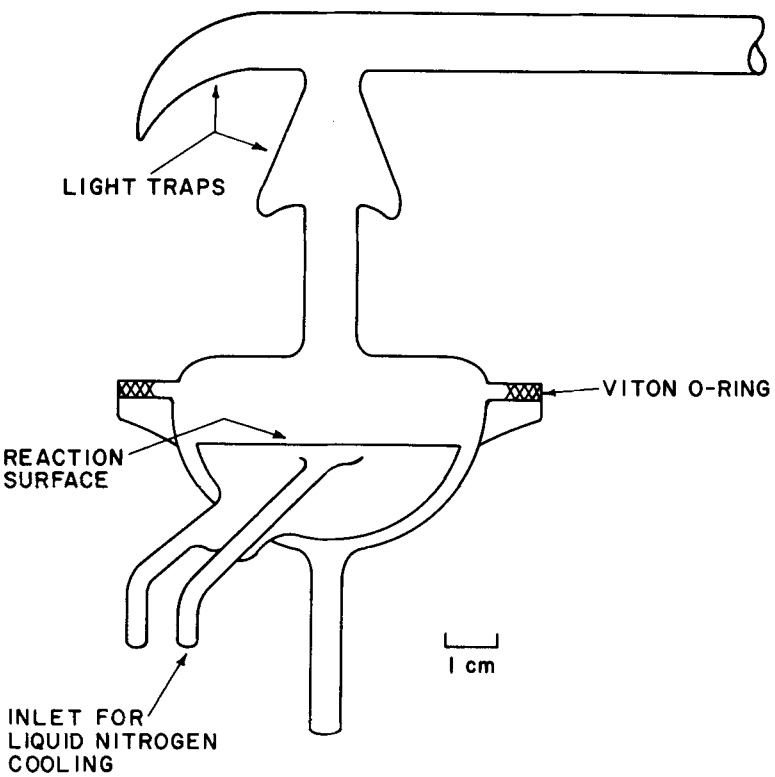
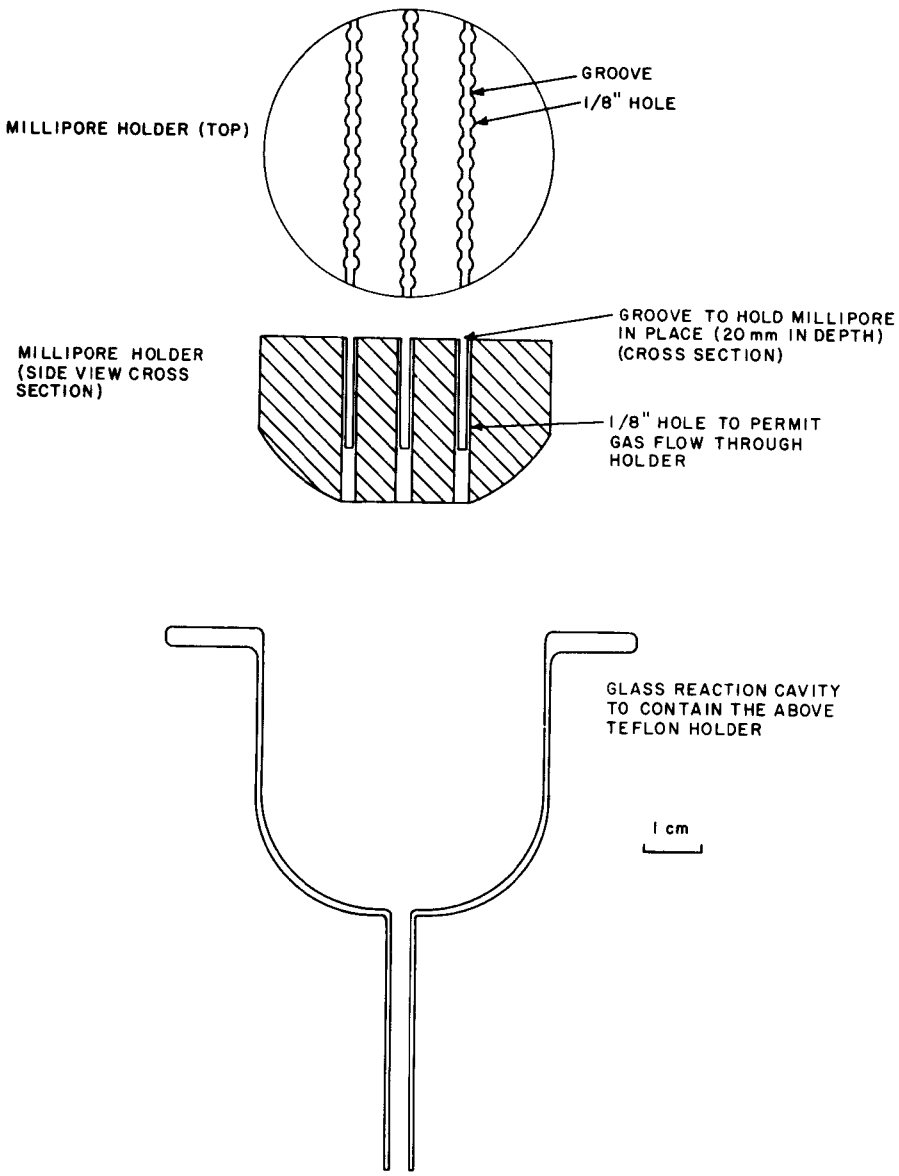


FIGURE 2. MILLIPORE FILTER HOLDER FOR MULTIPLE SAMPLES



sample up to 50 mm in diameter could now be exposed. Cooling is effected by the spraying of liquid nitrogen onto the bottom of the reaction surface under a pressure (4 psi) of nitrogen gas (see Figure 1).

To accommodate multiple Millipore adsorbed samples a new sample holder was also designed (Figure 2) which consists of a teflon hemisphere (50 mm diam.) with vertical grooves (1 cm apart) to hold up to three filters. Holes (1/8" in diam., 1/16" apart) were then drilled along the grooves to facilitate the flow of tritium gas around the filters. A glass cavity was designed to contain the teflon holder, with the dimensions such that both the large cooling reaction system (Figure 1) and the multiple Millipore system (Figure 2) are interchangeable in the present labeling system. Sample cooling cannot be effected in the multiple lableing system.

d) Labeling Conditions

Samples were labeled under conditions similar to those previously reported (1) i.e. 30 watts of microwave power, 2Ci $^{3}H_{2}$ gas at 4 mm Hg pressure and the cycling pump pulsing at 190 cycles/min. Reaction times were as follows: GnRH 15 min, valine and leu-trp-leu 5 min, and proline exposure varied from 5-20 minutes.

e) Purification

GnRH was purified by three separate chromatographic steps. 1) Stepwise elution from CM-22 (10 cc pipette) initially with 0.01 M NH_4Ac pH4.5 to remove labile tritium and other components that are not strongly retained, followed by 0.0461 M NH_4Ac pH 4.5 to remove the GnRH.

2) The above recovered GnRH was then chromatographed on a 10 cc pipette column of CM-22 with a gradient consisting of 150 ml each 0.002 M NH_4Ac and 0.1 M NH_4Ac both at pH 4.5. The GnRH peak would elute approximately half way through the gradient at a conductance equal to a solution of 0.0461 M NH_4Ac (3500 μ mohs). Specific activities were taken in a serial fashion across the peak, and the region of constant or near constant specific activity was pooled for further purification.

3) Final purification was carried out by chromatography on a Bio Gel P-4

column (1.2 x 75 cm) in 0.1 N HCl. Using ^{14}C-glycine as a marker GnRH was shown to elute beyond the total volume of the column.

Leu-trp-leu was purified by two step ion exchange chromatography. The peptide was chromatographed first on a 10 cc pipette column with AG50W-X2 in a stepwise fashion with first 0.1 M pyridine acetate (pH 3.15) then with 2.0 M pyr/Ac (pH 5.0) to remove the peptide. This was followed by high pressure liquid chromatography (HPLC) on a HP-B80 cation exchange resin (0.9 x 23 cm) with a gradient consisting of 200 ml each of the above 0.1 M, 2.0 M buffers at 50°C and a flow rate of 90 ml/hr. The amino acids (valine and proline) were similarly chromotographed on a 10 cc pipette column of AG 50W-X8 with 0.1 M pyr/Ac (pH 3.15) followed by HPLC on a 55 x 0.9 cm H-70 cation exchange resin in 0.1 M pyr/Ac buffer at 50°C and a flow rate of 90 ml/hr.

RESULTS AND DISCUSSION

Comparative results of labeling on Millipores vs glass and aluminum surfaces are shown in Table 1. Samples labeled on aluminum showed little labeling of leu-trp-leu and no detectable labeling of GnRH. The glass surface proved moderately successful especially in the labeling of GnRH at the 100 µg level. The Millipore labeling, however, produced a 40 to 300 fold increase in specific activity compared to the glass (or aluminum) reaction surfaces resulting in specific activities up to the Ci/mmol range for both peptides and in the tenths of Ci/mmol range for the amino acids.

Several parameters of the Millipore labeling have been investigated, i.e. effect of varying the type of Millipore, pore size and amount of sample.

GnRH was labeled on both cellulose and Solvinert (fluorocarbon) Millipore filters. The cellulose filter was found to be superior to the Solivinert filter, possibly due to hydrophobic interactions in the fluorocarbon filter. (Samples #5,7).

The effect of pore-size of the Millipore filter was investigated in the labeling of valine on the 47 mm diameter CMF with 1.2, 0.45, 0.22 µm pore sizes. As expected the large pore-size (1.2 µm) gave the lowest specific activity (sample #16) of the three Millipores, presumably due to the decreased surface area.

Table 1. Comparative Tritium Labeling Using Millipore Filters vs. Glass and Aluminum Reaction Surfaces

Sample #	Compound Labeled	Amount Labeled (mg)	Reaction Surface	Specific Activity mCi/mmol	Relative Specific Activity
1	GnRH[a]	1.32	aluminum Rx tray (45 mm)	unlabeled	0.0
2		0.42	glass fiber filter (25 mm diam)	13.7	1.0
3		1.00	glass Rx tray (45 mm)	17.0	1.2
4		0.10	glass Rx tray (45 mm)	255	19
5		0.42	Solvinert Millipore (0.22 μm x 25 mm)[b]	483	35
6		1.04	cellulose Millipore (0.22 μm x 25 mm)	647	47
7		0.42	cellulose Millipore 0.22 μm x 25 mm	2163	158
8		0.10	cellulose Millipore 0.22 μm x 25 mm	3182	232
9		0.43	cellulose Millipore 0.45 μm x 47 mm	3500	255
10	L-Leu-L-Trp-L-Leu	0.96	aluminum Rx tray (45 mm)	27	1.0
11		1.61	glass Rx tray (45 mm)	91	3.4
12		0.70	glass Rx tray (45 mm)	174	6.4
13		0.80	cellulose Millipore 0.22 μm 25 mm	1088	40
14	L-Valine	5.33	glass Rx tray (45 mm)	0.296	1.0
15		0.83	cellulose Millipore (0.22 μm x 25 mm)	12.7	43
16		0.80	cellulose Millipore (1.2 μm x 47 mm)	14.7	50
17		0.80	cellulose Millipore (0.22 μm x 47 mm)	59.7	202
18		0.80	cellulose Millipore (0.45 μm x 47 mm)	87.4	296
19	L-Proline	5.56	glass Rx tray (45 mm)	2.4	1.0
20		0.95	cellulose Millipore (0.22 μm x 47 mm)[c]	64[d]	27
21		0.50	cellulose Millipore (0.45 μm x 47 mm)[c]	304[d]	127
22		0.50	cellulose Millipore (0.45 μm x 47 mm)[c]	443[d]	185

[a] GnRH is the decapeptide pyroGlu-His-Trp-Ser-Tyr-Glu-Leu-Arg-Pro-GlyNH_2, gonadotropin releasing hormone.
[b] 0.22 μm is the pore-size, 25 mm the diameter of the filter.
[c] 3 samples combined in Teflon cellulose Millipore holder. The specific activity distribution of the 3 individual cellulose Millipores in the holder was determined. The outer samples were equivalent to each other and the central cellulose Millipore was approximately 50% greater than either of the other samples.
[d] Tritium distribution studies were carried out on these samples by means of ^3H NMR.

Increases compared to the 1.2 μm filter were observed on the smaller pore-size filters. However, unexpectedly, valine labeled on 0.45 μm CMF had nearly a 50% greater specific activity than on 0.22 μm filter (samples #17, 18). This is probably due to the fact that the surface area of the Millipore filter is not only related to pore-size but the number of pores in the filter as well.

The specific activity dependence on the amount of sample labeled was determined with GnRH. The specific activity was found to increase linearly as progressively smaller amounts of sample were labeled. (samples #6-8). This is also attributed to an increase in effective surface area of the sample when going to lower amounts of sample, and has been observed on glass reaction surfaces as well (samples #3, 4; see also ref 1).

Many samples labled by this novel technique have not only been of higher specific activity than previously attainable, but also were considerably easier to purify than when labeled on other surfaces (i.e. glass and aluminum), indicating the formation of greatly reduced amounts of tritiated impurities on the Millipore filters.*

CONCLUSION

First, a general method of high specific activity labeling of peptides and proteins as well as small molecules (i.e. drugs etc. which cannot be labeled by common synthetic or exchange techniques) now appears to be accessible by the use of Millipore filters as the labeling surface in the MDA labeling method.+ Second, samples labeled by this method should be of sufficiently high specific activity to perform labeling distribution studies by the use of tritium nuclear magnetic resonance (^{3}H NMR). Studies currently in progress in collaboration with Dr. Lawrence J. Altman at the State University of New York at

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*It is also possible that the labeled by-products may be physically or chemically trapped or bound to the cellulose surface.

+It is realized that proline and valine are readily available commercially in high sp. act. We have, however, successfully labeled other small molecules (i.e. Pyrazofurin) currently being used in biological studies, of other peptides and proteins (i.e. gastrin and lysozyme, respectively has also been accomplished.

Stony Brook[‡] on the tritium distribution of proline (samples #21,22) labeled by this method have supplied us with structural information on labeled proline. The use of ^{3}H NMR appears to be useful analytical tool for structural analysis of MDA labeled materials. These results will be published elsewhere.

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‡These experiments are being conducted on a Varian XL-100-12 pulsed NMR spectrometer at a frequency of 106.7 MHz.