FACTORS INFLUENCING REACTIVITY OF THERMAL H(T) ATOMS WITH SOLIDS. I. PHYSICAL EFFECTS

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SUMMARY

The effects of surface area and the nature of the surface were investigated for samples labeled by the microwave discharge activation (MDA) - tritium atom technique. When labeling powdered samples of different chemical structure the crude specific activity (prior to purification) was shown to increase linearly with the specific area (m^2/g) although the final specific activity of the purified compound was found to be dependent on the chemical nature of reacting sample. Samples deposited on high surface area, microporous membrane filters, showed large increases in specific activities compared to the labeling of powdered samples. In this case the specific activity was found to be a function of sample thickness (mg/m^2) reaching a maximum when the amount of sample was less than or equal to a monolayer on the filter surface and decreased as the reciprocal of the sample weight when a monolayer was exceeded.

Finally, a survey of the labeling properties of 20 selected filters using L-proline as a model compound was carried out. Cellulosic (hydrophilic) filters were found in general to yield labeled proline of the highest specific activities whereas the hydrophobic (vinyl, fluorocarbon, etc.) yielded the lowest. It was also found that the specific activity of most (15 of 20) of the filters was linearly related to tritium "yield" (% tritium recovered in proline after purification) in the labeled proline.

Key Words: Tritium labeling, microporous membrane filters, surface area, amino acids, L-proline, microwave discharge.

INTRODUCTION

Tritium labeling by microwave discharge activation of tritium gas (1) has been shown to be an effective method of incorporating tritium into biologically important molecules (1-6). In the past we and others have extensively investigated the chemistry of the tritium exchange reaction (5,6,7,8) and now would like to report results of studies on some physical aspects of the labeling process; mainly focusing on the surface characteristics of the compound being labeled. These studies have been divided into three parts, (1) effect of surface area on the labeling of a powdered substance, (2) effect of surface

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area of a substance deposited on a "secondary" or "carrier" surface, and (3) the effect of the chemical nature of the carrier surface on which a substance is deposited.

RESULTS AND DISCUSSION

Surface Area: Powders

The effect of the surface area on the labeling process has been described by several researchers (1-6,9-11). As expected, apparent increases in surface area usually related to amount of sample labeled lead to increases in labeling. However, no attempts were made in these studies to quantitate the surface area - specific activity relationship.

To quantitate this presumed relationship we labeled samples which were chemically different (the amino acids L-val, L-ala, L-phe, and a peptide L-val-L-ala-L-ala-L-phe, composed of those amino acids) but with known surface areas. Comparison of surface areas to both "initial" (crude) specific activities (calculated on the basis total tritium incorporation prior to chromatographic purification) and the "final" specific activities (of the chromatographically pure compound) will enable one to separate the effect of surface area differences as well as chemical differences and their influence on the specific activity. From results shown in Table I, several observations can be made. First, there

TABLE I

SPECIFIC ACTIVITY AS A FUNCTION OF SURFACE AREA

Compound	Specific Surface Area (m^2/g)	Crude Specific Activity ($\mu Ci/mg$)[a]	Purified Specific Activity ($\mu Ci/mg$)[b]
L-valine	0.154	57.2	4.26
L-alanine	0.239	104	3.54
L-phenylalanine	0.571	258	2.37
L-val-L-ala-L-ala-L-phe	7.90	3200	443

[a]Specific activity based on total tritium in crude unpurified sample after tritiation.

[b]Specific activity of purified sample.

is a linear correlation between the measured surface areas and the "initial" specific activities of the amino acids and peptides (correlation coef. 0.99996, slope $\approx$ 400 mCi/m^2). Second, there is no correlation found between the surface areas and the "final" or purified specific activities except that as has been observed before i.e. the peptide had the highest specific activity and was far greater than the sum of its component amino acids (7a,7d,12). These results indicate that the amount of tritium reacting with the sample is directly proportional to the surface area but the fate (ie. reaction pathways leading to observed products including labeled parent compound) is predominantly determined by the chemical nature of the substrate.

Surface Area: Samples deposited on carrier surfaces

Most studies which have investigated the effect of labeling compounds deposited on a carrier surface have had the goal of increasing the specific activity presumably due either to the increased surface area or adsorptive effects. Unlike improvements observed in Wilzbach labeling (13) adsorption of samples on surfaces such as charcoal or glass wool prior to microwave discharge labeling, did not show increases in tritium incorporation (9). In fact, decreases are observed when charcoal platinum-black and aluminum are used which may be attributed to increased tritium atom recombination rates on these surfaces (9,10). In contrast, recent reports have shown that the labeling of samples deposited on microporous membranes (10), or glass fiber filters (6), give rise to large increases (40-500 fold) in specific activity compared to labeling the same samples as powders or films. To investigate the role that surface area may contribute to these observed increases, L-proline in varying amounts (0.1 mg to 10 mg) was deposited on a cellulosic microporous membrane filter. The surface areas were subsequently determined and the filters containing the L-proline were labeled by the microwave discharge method. The specific activities of the purified L-proline samples were determined and compared to one another as well as with the L-proline labeled as a powder (also of known

TABLE II

LABELING OF L-PROLINE AS A FUNCTION OF ITS SPECIFIC SURFACE AREA AND SAMPLE THICKNESS

Amount of L-Proline labeled (mg)	State of L-Proline	Specific Surface Area m^2/g	Sample Thickness mg/m^2 [c]	Sp. Act. mCi/mmol
None (control filter)	--	13.875	--	--
0.10	CMF[a]	12.471	0.092	250
1.00	CMF	12.075	0.940	112
10.00	CMF	8.835	11.68	21.2
5.60	Powder[b]	0.241	--	2.42

[a]Samples were deposited on a cellulose Millipore filter (CMF) in 200 µL water and evaporated in a desiccator. CMF HAWP04700, 47 mm diameter, 0.45 µm pore size.

[b]L-Proline powder dispersed on a glass surface (45 mm diameter).

[c]Sample thickness $(mg/m^2) = \frac{W_s}{W_t \cdot S}$

when:

W_s = Amount of sample (mg)

W_t = Total weight of sample + filter (g)

S = Specific surface area (m^2/g)

(average wt. of an empty filter was determined to be 86.9 mg).

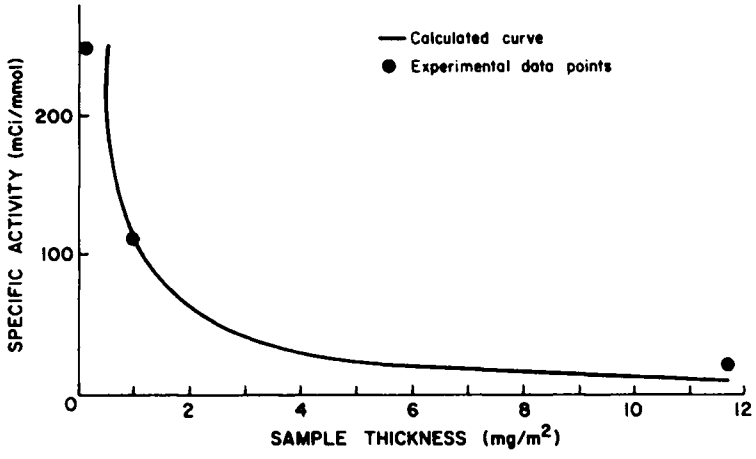
surface area). Results are shown in Table II. Large increases in surface area and specific activity are noted when comparing the microporous filter deposited samples to the powdered sample. Further increases are also noted among the millipore deposited samples as the amount of sample labeled is decreased. These increases are functionally related to the reciprocal of the sample weight (1/x) and can be explained in the following way: At a low loading of sample on a filter surface (ie. one monolayer or less) a maximum (α) labeling occurs. Once a monolayer (β) is exceeded the top layer still gets maximally labeled (α)

but is diluted with unlabeled carrier (x) and the specific activity drops as $\alpha/(\beta+x)$. This continues until a large amount of sample is deposited causing drastic changes (decrease) in surface area at which point, due to clogging of pores, a further large dropoff in specific activity would be observed greater than the $\alpha/(\beta+x)$ prediction. Evidence for this explanation can be seen in Figure 1. The curve plotted is an empirically calculated curve (see appendix) relating a calculated specific activity (using $\alpha/(\beta+x)$ relationship) vs. the sample thickness (mg/m^2). The circled points are experimental points.

It has been estimated (see appendix) that a monolayer of L-proline on such a filter is approximately 0.45 mg (or 0.42 mg/m^2). We also have experimental evidence that lends support to this estimate. Labeling experiments using 1 mg of L-proline on the filter result in a specific activity of 3 Ci/mmol a level at which statistically one proline out of 10 would be labeled. Proton decoupled tritium NMR's of these samples, however, has clearly shown tritium-tritium coupling within the same molecule (10,12). This indicates that even at 1 mg level some multiple surface labeling events in a molecule have taken place. The lowering of the specific activity to 3 Ci/mmol is therefore caused primarily by dilution with unlabeled subsurface layer L-proline. From these data we can set an upper limit for a monolayer of 1 mg of L-proline.

We have also been able to set a lower limit of 10 mg of proline per filter for the level at which the filter pores may become clogged. The evidence for this is three fold. 1) The filter surface area changes only slightly with increasing amounts of L-proline, decreasing only 36% (compared to the empty filter) with the addition of 10 mg of proline (Table II). 2) The labeling specific activity), even at the 10 mg level, is nicely predicted from the sample thickness (Figure I). This indicates that the fall off is predominantly attributable to dilution with unlabeled subsurface layer proline and that no precipitous fall off has occurred due to other factors such as pore clogging.

FIGURE 1. SPECIFIC ACTIVITY of L-PROLINE as a FUNCTION of SAMPLE THICKNESS



3) Scanning electron microscope pictures of the proline containing filters show little change in surface characteristics, or indication of pore clogging, as compared with the empty filter, even with 10 mg of proline.

Nature of the Secondary Surface - Microporous Filter Survey

From the data so far presented (see also ref. 6,7,9,10,11) it is clear that although surface area plays a major role in the labeling, the nature (structure and chemical composition) of the labeling surface is also important. Realizing this, and the fact that labeling on microporous membrane filters leads to large increases in specific activity, we investigated the labeling of L-proline (as a model compound) on 20 of these filters with varying composition and from various suppliers. The results (see Table III) show the specific activities and the percent tritium recovery as labeled proline (see note g Table III) along with the chemical compositions of the filters. Several observations can be made from this table; (1) in all cases the specific activity was equal to or greater than that of the powdered sample; (2) both the specific activities and the percent recovery as tritiated-L-proline show wide variations

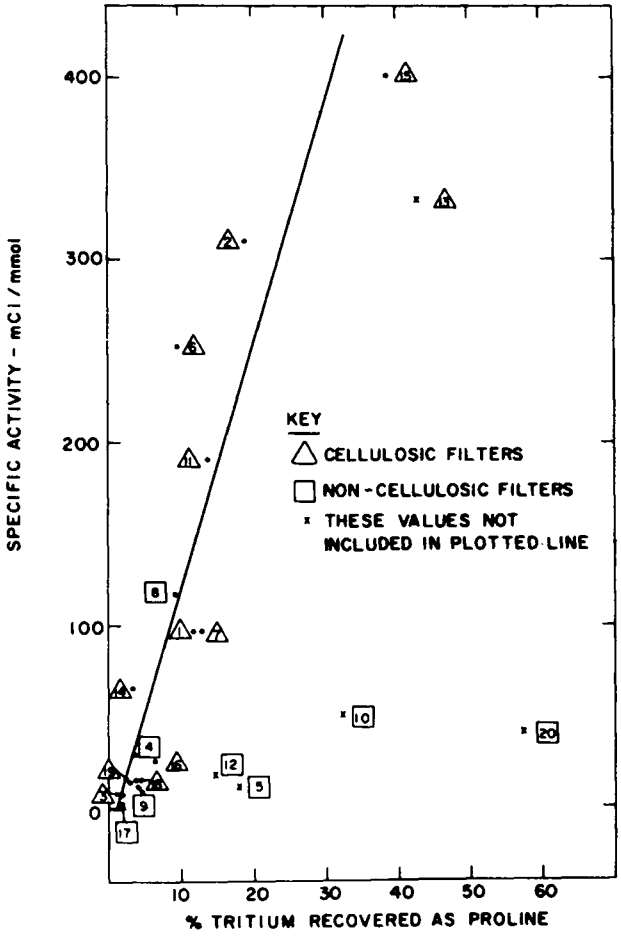
TABLE III

LABELING CHARACTERISTICS OF L-PROLINE DEPOSITED ON SELECTED MICROPOROUS MEMBRANE FILTERS

#	Filter Material	Catalogue #	Company	Pore Size μm	Sample Application	Specific Activity mCi/mmol	% Recovery as H-Pro	Relative Specific Activity
0	Standard-L-Proline Powder deposited on a glass reaction tray			N.A.	N.A.	2.4	4.5	1.00
1	Cellulose Acetate	SM-11106	S	0.45	c	87.4	11.8	36.40
2	Cellulose Nitrate	SM-11306	S	0.45	c	311.0	19.0	130.00
3	Regenerated Cellulose	SM-11606	S	0.45	d	8.0	1.8	3.33
4	Polyamide	SM-11906	S	0.45	c	29.0	3.6	12.10
5	Polyvinylchloride(PVC)	SM-12806	S	0.45	e	12.3	17.8	5.13
6	Cellulose Triacetate	Metricel GA-6	G	0.45	c	253.0	9.7	105.00
7	Cellulose-Mixed Esters	Metricel GN-6	G	0.45	c	86.9	13.0	36.20
8	Acrylonitrile-PVC co-polymer on Nylon	Acropor AN-450	G	0.45	c	128.0	9.3	53.30
9	Acrylonitrile-PVC co-polymer	Metricel DM-450	G	0.45	c	12.6	4.0	5.25
10	Vinyl (PVC)	Metricel VM-1	G	5.00	e	59.5	32.3	24.80
11	Regenerated Cellulose	Metricel Alpha-6	G	0.45	f	191.0	13.9	79.60
12	Polyaromatic	HT-450	G	0.45	e	18.0	14.8	7.50
13	Cellulose Triacetate	Metricel TCM-200	G	0.20	e	332.0	43.0	138.00
14	Cellulosic	Amicon	A	0.45	c	66.1	3.6	27.50
15	Nitrocellulose	BA-85	S&S	0.45	c	401.0	33.8	167.00
16	Cellulose Acetate	OE-67	S&S	0.45	c	26.3	6.5	10.90
17	Teflon on Polypropylene	TE-36	S&S	0.50	c	2.5	2.0	1.05
18	Cellulose-Mixed Esters	MF-Millipore HAWP 04700	M	0.45	c	15.5	3.8	6.46
19	Cellulose Acetate	Celotate EHWP 04700	M	0.50	c	14.6	2.9	6.08
20	PTFE on Polyethylene	Fluoropore FHLP 04700	M	0.50	d	20.2	57.2	8.42

[a]Catalogue description -- all filters were 47 mm diameter
[b]S = Sartorius; G = Gelman; A = Amicon; M = Millipore Corp.; S&S = Schleicher & Schuell
[c]Samples (1.5 mg L-Pro) were applied in 200 μL aqueous solution unless otherwise noted.
[d]Sample applied in 200 μL MeOH
eDue to wetting problem filter was sprayed with a mist of EtOH prior to sample application in aqueous solution.
[f]Sample applied in 200 μL EtOH.
[g]% Recovery = [(Specific Activity of purified ^{3}H-Pro)/(Specific Activity of crude proline eluted from filter)]X100.

FIGURE2. SPECIFIC ACTIVITY vs. TRITIUM RECOVERY of L-PROLINE LABELED on MICROPOROUS FILTERS



among the different filter types, and (3) labeling on the cellulosic types of filters generally resulted in the highest specific activities.

Further examination of the data reveals a linear relationship between the specific activity of a sample and the % recovery as proline (see Fig. II). This appears to hold true for 15 of the 20 filters examined. These data correlate well for the hydrophilic filters (except #13) with the major deviations being observed for the hydrophobic filters (filter #'s 5,10,12,20).

CONCLUSIONS

The relationship between surface area and specific activity achieved in the MDA labeling technique is qualitatively different when comparing powdered samples with samples deposited onto "secondary" or "carrier" surfaces. In the case of powdered samples the tritium incorporation was shown to be a function of specific surface area (m^2/g) increasing with increasing surface area. Increasing the surface area by deposition on a secondary surface such as a microporous filter dramatically increases the specific activity of the samples. However, once deposited on a surface, the specific activity was found to be dependent on the sample thickness (mg/m^2) reaching a maximum when a sample thickness equal to or less than a monolayer was achieved ($\sim$ 0.45 mg on the filters investigated) and decreased at a rate proportional to the reciprocal of amount labeled when a monolayer was exceeded $[\alpha/(\beta+x)]$. A survey of the effect of the nature of the filter surface on labeling was carried out using 20 different filters with L-proline as the model compound. Enhancement of the labeling was observed in nearly all cases investigated. Labeling on the hydrophilic cellulosic filters resulted in the highest observed specific activities. It is not known whether this trend is general for all classes of compounds or if differences may be observed for less polar and nonpolar compounds. Lastly, for most (15 of 20) filters investigated we observed a linear relationship between the specific activity and the percent tritium incorporation (or tritium "yield") in the parent proline. Exceptions were mainly found among the hydrophobic filters.

EXPERIMENTAL

Materials and Methods

The amino acids L-valine and L-proline were purchased from Schwarz/Mann, L-alanine from the California Foundation for Biochemical Research, and L-phenylalanine from Nutritional Biochemicals. The peptide L-valyl-L-alanyl-L-alanyl-L-phenylalanine was purchased from Fox Chemicals (Now Vega-Biochemicals). The amino acids and peptides were used without prior purification.

Surface areas were determined by the Micrometrics Instrument Co. (Norcross, Ga.) on a Model 2100 D Orr Surface Area-Pore Volume Analyzer by the standard multipoint B.E.T. technique using krypton adsorption.

Scanning electron microscopy was carried out at Brookhaven National Laboratory on a Materials Analysis scanning electron microscope Model 800, a magnification of 5000 was used.

Microporous membrane filters were obtained from the following companies: Millipore Corp. (Bedford, MA), Sartorius (South San Francisco, CA), Amicon (Lexington, MA), Gelman (Ann Arbor, MI), and Schleicher and Schuell (Keene, NH).

Liquid scintillation counting was carried out on a Beckman LS-II spectrometer in Aquasol or Biofluor (New England Nuclear) scintillator solutions. Samples were efficiency corrected by the use of an internal standard (^{3}H-toluene).

Sample preparation

Powders of the amino acids and the peptide were labeled as received from the supplier. L-proline used in the filter labeling studies was applied to the filters in 200 µl water solution (or other appropriate solvent as noted in Table III) and evaporated to dryness in a vacuum dessicator.

Labeling

Labeling of the L-valine, L-alanine, L-phenylalanine and the tetrapeptide L-val-L-ala-L-ala-L-phe, was carried out in the system described in ref. (1) with modifications found in ref. (7a). Reaction conditions were: 4 mg sample, 4 torr $^3H_2$ gas ($\sim$ 1 Ci), liquid nitrogen cooling, 20 watts microwave power, a cycling pump rate of 175 cycles/min, and a reaction time of 5 minutes.

Proline samples were labeled as a powder and on microporous membrane filters in the large single sample liquid nitrogen cooled system designed for filter labeling described in reference (10). Reaction conditions were: 4 torr $^3H_2$ gas ($\sim$ 2 Ci), 30 watts microwave power, a pumping rate of 180 cycles/min and a reaction time of five minutes.

Purification

Tritiated L-alanine and L-valine samples were purified by successive chromatographies on a 10 ml pipette column of AG 50W-X8 cation exchange resin (200-400 mesh), 0.1 M pyridine-acetic acid buffer pH 3.1, followed by chromatography on 0.9 x 60 cm AG 50W-X8 with the same buffer at 37°C, and a flow rate of 20 mL/hr. L-phenylalanine was chromatographed initially on AG 50W-X8 10 mL pipette column using a gradient from 0.1 M pyridine-acetate, pH 3.1 to 0.5 M at a pH of 5.0. The L-phe was subsequently chromatographed on 3 successive 0.9 x 100 cm AG 50W-X8 columns which were developed first isocratically with 0.1 M pyridine-acetate (pH 3.1) followed by a gradient 0.1 M → 0.5 M at 37°, 20 mL/hr. These extra chromatographies were found to be necessary due to the presence of a radioactive impurity with ion exchange properties similar to that of L-phe. (This may be β-cyclohexylalanine. See ref. #8a). The tetrapeptide L-val-L-ala-L-ala-L-phe was purified initially on a 10 mL pipette AG 50W-X2 column with a 0.1 M (pH 3.1) up to 1.0 M (pH 5.0) pyridine-acetate, followed by chromatography on 0.9 x 100 cm AG 50W-X2 (200-400 mesh) with a gradient using the same buffers at 37° and a flow rate of 20 mL/hr. Proline samples were eluted from the filters in 0.1 N HCl (2 mL). Carrier proline (1.0 mg) was added to the 100 μg proline sample for easier purification. The proline was then purified by successive ion exchange chromatographies as described in reference (10). Final purification was by HPLC ion exchange chromatography on H-70 ion exchange resin (Hamilton Corp. see ref. (10).

In all cases specific activity profiles were determined serially across the desired amino acid (or peptide) peak to ensure radiochemical purity. Purified L-alanine and L-valine samples were also subjected to glc analyses to demonstrate their purity (see ref. 7a, 7b for glc derivatives and analysis conditions).

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APPENDIX

Specific Activity as a Function of Sample Thickness

1. Estimate of proline cross sectional area.
   a) Assumed to be similar to that of benzene (43 $\mathring{A}^2$)*
   b) Estimated from crystal structure by superimposing Van der Waals radii radii (33 $\mathring{A}^2$). When packed into a reasonable geometric lattice a cross-sectional area of 42.8 $\mathring{A}^2$ was obtained.

   Using 43 $\mathring{A}^2$/proline a value of 0.42 mg/m^2 constitutes a monolayer of L-proline or 0.445 mg/filter (calc. from Table II assuming 1.07 m^2/filter at a loading of between 0.1 to 1.0 mg pro/filter).

2. Maximum tritium activity for a monolayer was calculated from the specific activity of the 0.1 mg sample labeled since this is below the monolayer amount. At 250 mCi/mmol 0.1 mg would contain 217.5 μCi; a monolayer of 0.445 mg would therefore contain 968 μCi.

3. To determine the sample thickness (mg/m^2) of varying amounts of proline (since the surface area varies slightly with amount of sample on the filter) a plot was made to known points (1.00 mg, 0.94 mg/m^2; 10.00 mg, 11.7 mg/m^2 - see Table II) and intermediate values were determined from the graph.

4. Functional form of the specific activity.

$$\text{Specific activity (mCi/mg)} = \frac{\alpha}{(\beta + x)}$$

*McClellan, A.S. and Harnsberger, H.F., J. Colloid and Interface Sci., 23: 577 (1967).

where:

α = Total tritium activity for a monolayer (mCi)

β = Amount of sample in a monolayer (mg)

$(\beta + x)$ = Total sample weight (mg)

For the L-Proline/microporous filter system described: $\alpha = 0.968$ mCi

$\beta = 0.445$ mg

Sample Values Used to Generate Curve in Figure 1.

Wt. of Sample (mg)	Thickness (mg/m^2)	Calc. sp. act. (mCi/mmol)
0.445	0.42	250 maximum
1	0.94	111
2	2.20	56
3	3.30	37
5	5.70	22
7	8.10	16
10	11.70	11

Actual experimental values plotted (circles in Figure 1) are shown in Table II.