LOW PRESSURE TRITIATION OF PROTEINS

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By
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LOW PRESSURE TRITIATION OF PROTEINS

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LIST OF ABBREVIATIONS

Tris•HCl  tris-(hydroxymethylamino)methane hydrochloride

O.D._{280}  optical density at 280 nm

SDS  sodium dodecyl sulfate

FAGLA  N-furylacryloylglycyl-L-leucine amide
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This thesis describes the tritiation of four proteins including type I rat skin collagen, human antithrombin III, human α₁-protease inhibitor and the zinc containing metalloendopeptidase thermolysin at low pressure in the presence of 1000-fold less tritium gas than is required by the method of Wilzbach (K.E. Wilzbach, J. Am. Chem. Soc. 79, 1013 (1957)). Very little degradation of the protein is observed with this method and recoveries of enzymatic activity have been as high as 95% after purification of the labeled product. Between 3 and 100 mg protein is lyophilized onto a semicylindrical stainless steel plate and evacuated in a reaction chamber to a base pressure of 1 x 10⁻⁶ torr. The protein is exposed to 5 mCi T₂ at 1 x 10⁻³ torr for from 6 to 100 h. After exposure, the excess T₂ is removed and the protein is purified by appropriate techniques in order to remove readily exchangeable tritium. In most cases this involves simple dialysis. The specific radioactivities obtained by this method are: thermolysin, 1-76 Ci/mole; collagen, 90 to 5600 Ci/mole; antithrombin III, 10 Ci/mole; and α₁-protease inhibitor, 1 Ci/mole. The tritiated products are indistinguishable from the native proteins by gel electrophoresis (collagen, α₁-protease inhibitor and antithrombin), enzyme activity (thermolysin) and trypsin inhibitory capability (antithrombin). In the case of thermolysin, the tritium label is stable to multiple dialysis and chromatography steps until the protein is denatured whereupon approximately 94% of the tritium is lost by exchange with solvent. It is apparent that low pressure tritiation leads to
exchange of T for H at N-H and O-H bonds in the interior of the enzyme molecule which are not exposed to solvent until the tertiary structure of the protein is destroyed. Exposure of proteins to low pressures of tritium gas offers a mild and convenient method for the introduction of tritium into these molecules. Proteins labeled by this technique should be useful in experiments which require tritium labeled proteins that are difficult or impossible to obtain by any other method.
CHAPTER I

INTRODUCTION AND HISTORICAL BACKGROUND

Suitability of Tritium as a Biological Tracer

Radioisotopes occupy a position of fundamental importance in medical and biochemical research and in many respects tritium is the ideal choice for biological tracer work. Its half life of 12.4 years is conveniently long so that correction for decay is seldom necessary during most biological experiments, yet it is sufficiently short so that very high specific activities are obtainable. Tritium, as opposed to carbon-14, can be obtained in a pure form. The weakness of the β-radiation makes tritium labeled compounds ideal for autoradiography. Because of the ubiquitous presence of hydrogen in biological molecules, any molecule of interest can potentially be labeled with tritium if a suitable labeling method can be devised. The search for a generally applicable method for the incorporation of tritium into proteins has led to the development of a gas exposure labeling technique that uses far less stringent conditions than are required by previously reported methods and it is this new technique that is described in this thesis.

Known Methods of Tritiation

Tritium labeled compounds can be prepared by many methods including direct chemical synthesis, chemical modifications, biosynthetic procedures and isotopic exchange techniques (Evans, 1974). Each of these techniques is useful and the choice of the one most appropriate
for a given application is governed by many factors. Small, simple tritium labeled molecules are usually most easily obtained via direct chemical synthesis. However, for the preparation of tritiated proteins, direct chemical synthesis is either impossible or prohibitively expensive so other methods must be found.

Chemical modification of proteins by the introduction of small, tritium labeled chemical groups such as $[\text{H}]\text{CH}_3\text{CO}^-$ is a method used with some success. For example, $[\text{H}]$ acetic anhydride of high specific activity has been used to label human growth hormone and porcine insulin (Colipp et al., 1965). Although such chemical derivatization can produce highly labeled products, caution must be exercised in the use of these derivatives in vivo and in vitro. Differential behavior between the macromolecule and its derivative has been observed with labeled derivatives of insulin (Chen et al., 1963, and Marlow and Sheppard, 1970). Similarly, $[\text{H}]$ acetyl-bradykinin binds to bradykinin antiserum more effectively than bradykinin (Rinderknecht et al., 1967). Other physiological properties such as antigenic determinants or the rate of trans-membrane transport of derivatized proteins could also be altered.

Biosynthetic procedures can produce tritiated proteins and other biological macromolecules of usefully high specific activity but these methods are usually highly specialized, expensive, and are seldom of general applicability. An example is the biosynthesis of actinomycin. Six tritiated amino acids were added to growing cultures of $\text{Streptomyces antibioticus}$ and $[\text{H}]$ actinomycin was isolated at 3-4 Ci/mole (Ciferri et al., 1964). Only five percent of the added tritium activity was incorporated into the antibiotic. For the production of tritium labeled
human proteins, biosynthetic procedures are not possible unless exogenous
tissue culture systems or other similar methods can be developed.

Clearly a more general technique for the tritiation of proteins is
desirable. Those methods grouped under the general heading of isotopic
exchange techniques provide just such general methods. Table 1 lists a
number of proteins labeled by these isotopic exchange techniques.
Wilzbach reported in 1957 that organic molecules exposed to curie
quantities of tritium gas \((T_2)\) for a number of days will undergo isotopic
exchange reactions to yield tritiated products (Wilzbach, 1957). Subse­
quent experiments with lysozyme and ribonuclease (Steinberg et al., 1957),
\(\gamma_2\)-globulin (Rajam and Jackson, 1959, and Pany, 1959), human albumin
(Pany, 1959) and insulin (Von Holt et al., 1960) demonstrated that
proteins could also be tritiated by the gas exposure method of Wilzbach.
Unfortunately a significant percentage of the tritium in Wilzbach labeled
compounds is found to be in labile positions and in highly tritiated
decomposition products which must be removed by vigorous purification
procedures. The few proteins labeled by this method which were carefully
purified had low final specific radioactivities.

A number of modifications of the simple gas exposure technique
have been introduced. They all have as a common goal the activation of
the tritium gas in a way that will lead to higher reactivity of the gas
and a subsequent increase in the specific radioactivities obtainable.
Among the more successful of these modifications which have been applied
to proteins are the electrical discharge (Khairallah et al., 1962, and
Noyer et al., 1976), the free radical interceptor (White and Riesz,
1968, and White et al., 1969) and the microwave discharge methods
(Hembree et al., 1973, Ehrenkauf e r et al., 1973, and Wessels et al., 1978).
<table>
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<th>Tritiation Method</th>
<th>Sp. Act. ( \mu \text{Ci}/\text{mg} )</th>
<th>mole tritium/mole substrate</th>
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<td>ACTH</td>
<td>Wilzbach</td>
<td>1.8</td>
<td>0.00025</td>
<td>Nishizawa et al., 1965</td>
</tr>
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<td>Lysozyme ribonuclease</td>
<td>Wilzbach</td>
<td>--</td>
<td>0.0018</td>
<td>Steinberg et al., 1957</td>
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<tr>
<td>Insulin</td>
<td>Wilzbach</td>
<td>4.6</td>
<td>0.00083</td>
<td>Von Holt et al., 1960 Von Holt and Von Holt, 1958</td>
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<td>Thyrotropin releasing hormone (TRH) (a tripeptide)</td>
<td>Wilzbach</td>
<td>8000</td>
<td>0.1</td>
<td>Schally and Redding, 1970</td>
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<td>Vasopressin</td>
<td>Electrical discharge</td>
<td>400</td>
<td>0.01</td>
<td>Fong et al., 1960</td>
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<tr>
<td>Angiotensin II</td>
<td>Electric arc</td>
<td>300</td>
<td>--</td>
<td>Khairallah et al., 1962</td>
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<tr>
<td>Ribonuclease</td>
<td>Electrical discharge</td>
<td>--</td>
<td>0.09-0.3</td>
<td>M. Noyer et al., 1976</td>
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<tr>
<td>Adenocorticotropicin (ACTH)</td>
<td>Microwave discharge</td>
<td>3200</td>
<td>0.5</td>
<td>Hembree et al., 1973</td>
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<tr>
<td>( \beta_2 )-microglobulin</td>
<td>Microwave discharge</td>
<td>500-5000</td>
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<td>( \beta )-lipoprotein</td>
<td>Tritium exchange with CH(_3)CO(_2)T</td>
<td>0.72</td>
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<td>Gosztonyi et al., 1965</td>
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<td>Lysozyme ribonuclease</td>
<td>Free radical interruptor (( \gamma, )HST)</td>
<td>0.7</td>
<td>0.00033</td>
<td>White and Riecz, 1968</td>
</tr>
<tr>
<td>Lysozyme ribonuclease actin</td>
<td>Free radical interruptor (( \gamma, )electrical discharge, HST)</td>
<td>0.4-0.06</td>
<td>0.003</td>
<td>White et al., 1969</td>
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<tr>
<td>Collagen</td>
<td>Free radical interruptor (electrical discharge, HST)</td>
<td>0.7</td>
<td>0.0074</td>
<td>Labrosse et al., 1976</td>
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The electrical discharge and free radical interceptor methods are similar. In the case of the electrical discharge method, the protein to be labeled is deposited inside an evacuated tube and $T_2$ is admitted. An electrical discharge such as that produced by a Tesla coil leak detector is applied to the gas. The gaseous radicals and atoms thus formed then react with the substrate to yield tritiated products. In the free radical interceptor method, it is the protein itself that is exposed to the electrical discharge or some other means of activation such as exposure to $\gamma$-rays. Subsequent exposure to tritiated hydrogen sulfide yields tritiated products. Each of these methods is rather crude and hazardous to the molecular integrity of the protein thus treated. Many impurities were formed when lysozyme and ribonuclease were labeled by this method (White and Riesz, 1968).

The microwave discharge technique has been the most successful procedure for obtaining high specific radioactivities as demonstrated by the production of tritiated porcine adrenocorticotropic (ACTH) with a specific radioactivity of 14,500 Ci/mole (Hembree et al., 1973, and Ehrenkaufer et al., 1973). The purified $[3^H]^{-}$ACTH was shown to retain both its immunological and biological activities. However, a more recent study has shown that this technique can cause significant degradation of proteins during the labeling process (Wessels et al., 1978). Chromatography of a sample of $\beta_2$-microglobulin tritiated by the microwave discharge method showed multiple peaks indicating that considerable damage to the protein had occurred. It appears that the microwave discharge method is the best general exchange method presently available for general tritiation of proteins yet it does have the potential to cause extensive damage.
Seeking to develop a generally useful technique for the tritiation of biological macromolecules that would allow the production of products with high specific radioactivity and full biological activity, the low pressure tritium gas exposure labeling of some selected proteins has been studied. The bacterial metalloendopeptidase thermolysin, type I rat skin collagen, and two human serum glycoproteins, antithrombin III and \( \alpha_1 \)-protease inhibitor have been labeled by exposure to far less tritium gas at pressures much lower than that used with the original gas exposure technique of Wilzbach (Wilzbach, 1957, and Wenzel and Schulze, 1962). Exposure of thermolysin to approximately 5 mCi of \( T_2 \) at \( 1 \times 10^{-3} \) torr for from 6 to 100 h yields tritiated thermolysin with a specific radioactivity between 1 and 76 Ci/mole after extensive purification. Very little degradation of protein is observed and recovery of enzyme activity has been as high as 95%.
CHAPTER II

EXPERIMENTAL METHODS

Materials

Tritium was kindly supplied by Dr. R.W. Fink, Georgia Tech. Tritium gas was obtained from the solid titanium tritide deposited on the surface of a small copper disc which was originally used as a neutron generator target. A preparation of 10% Pd on charcoal was purchased from Alfa Ventron, Danvers, MA.

Thermolysin (E.C. 3.4.24.4) was purchased from Sigma Chemical Co., St. Louis, MO. The synthetic thermolysin substrate, N-furylacryloylglycyl-L-leucine amide (FAGLA) was purchased from BACHEM, Inc., Torrance, CA. Type I rat skin collagen preparations were kindly supplied by Dr. J. Travis, University of Georgia, Dr. P. Bornstein, University of Washington, and Dr. I.E. Liener, University of Minnesota. α1-Protease inhibitor (α1-antitrypsin) was also supplied by Dr. J. Travis. Drs. E.W. Davie and K. Kurachi, University of Washington, supplied the human antithrombin III. Desmosine was obtained from the Elastin Products Co., St. Louis, MO. All common chemicals and buffer salts used were at least reagent grade.

Tritiation Apparatus

The tritiation apparatus constructed for these experiments is diagramed in Figure 1. The reaction chamber is a stainless steel vessel 29 cm in length by 7.2 cm diameter which is sealed by a high vacuum flange using an aluminum gasket. A high vacuum is achieved by the use
Figure 1. Low pressure tritiation apparatus.
of a small glass mercury diffusion pump together with a Sargent-Welch Directorr Model 8805 oil roughing pump. A trap containing 4Å molecular sieves is included in the fore pump line to absorb any tritium pumped from the reaction chamber before it can contaminate the roughing pump (Zimmerman et al., 1968). The base pressure achieved by this system is $1 \times 10^{-6}$ torr. The pressure is measured by a Pirani thermocouple gauge for pressures above $10^{-3}$ torr and a Penning ionization gauge for pressures down to $10^{-7}$ torr. Two liquid nitrogen cold traps are included in the system. The reaction chamber is isolated from the pumps during reactions by closing the high vacuum stopcock between the two traps.

In order to maintain a high vacuum, it is necessary to keep the two traps filled with liquid nitrogen at all times. An automatic filling system was constructed for this purpose. When the liquid nitrogen level drops below the temperature probe of the Torr Vacuum Products (Van Nuys, CA) model T-11-A Cryo-miser, two solenoid valves are activated causing the vent to close and gaseous nitrogen pressurizes the liquid nitrogen reservoir. The cold trap dewars are refilled and the level controller turns off the solenoid valves when the temperature probe is again immersed in nitrogen. The system can run unattended for 10 to 12 h with 4 L liquid nitrogen.

The tritium gas handling system consists of two quartz vessels connected to the stainless steel valves (see Figure 1) via black wax sealed glass standard taper joints and glass-to-metal epoxy seals. Each quartz vessel is equipped with a simple oven capable of heating to 500°C. T$_2$ is released from the neutron generator target by heating the vessel to 450°C and is purified by absorption onto Pd-charcoal where it is
stored until needed. Heating the Pd to 350° to 400°C readily releases T₂. At the end of a reaction, the remaining T₂ is reabsorbed onto the Pd and any gas not absorbed is pumped away.

**Labeling Procedure**

A sample is prepared for labeling by lyophilization as a very thin film on the surface of a semicylindrical steel plate 9 cm across and 15 cm long. The final density of the film is approximately 60-600 µg/cm², depending upon the amount of sample used. Sample sizes are typically between 3 and 100 mg. The lyophilized sample is sealed inside the reaction chamber and the system is evacuated to 1 x 10⁻⁶ torr. Because of the slow pumping speed of the present apparatus, 24 to 48 hours are required to achieve a good vacuum.

Once a good vacuum is obtained, 5 mCi T₂ is admitted to a pressure of 1 x 10⁻³ torr by heating the Pd to approximately 400°C. The sample is allowed to remain in the T₂ atmosphere for an appropriate length of time ranging from 6 to 100 h. At the end of the experiment, the T₂ is reabsorbed onto the Pd and the reaction chamber is pumped to base pressure for at least 12 h. The sample is then removed and characterization is begun.

**General Methods Used for Protein Purification**

Protein concentrations were determined by measurement of the absorbance at 280 nm. Dialysis was performed using cellulose dialysis tubing at 4°C in buffers appropriate for the protein. Protein hydrolyses for amino acid analysis and for determination of residual tritium content were performed in sealed evacuated ampules containing 6 N HCl that were
heated for 20 h at 110°C. The samples were dried and redissolved in water repeatedly after hydrolysis to remove all traces of volatile tritium. Tritium was determined by liquid scintillation spectrometry using a Beckman Instruments model LS-100C liquid scintillation spectrometer. The counting cocktail used was a toluene-Triton X-100 emulsion system recommended by Dr. A. Moghissi (Lieberman and Moghissi, 1970). The liquid scintillation counter was kindly made available for use by the Georgia Tech School of Biology. Polyacrylamide gel electrophoresis was performed according to the method of Weber and Osborn in 0.6 x 7 cm tube gels (Weber and Osborn, 1969). Amino acid analyses were kindly performed on a Beckman Instruments model 119 CL analyzer by Dr. J. Travis, University of Georgia.

Chromatography of the denatured proteins was performed using a Sephadex G-10 column (0.6 x 80 cm) equilibrated with 8 M urea buffer (0.10 M Tris-HCl, pH 7.0). 1.0 ml fractions were collected and each was analyzed for protein absorbance and tritium content.

Specific Protein Methods

Thermolysin

Thermolysin concentrations were calculated from O.D.280 using $E_{280}(1%) = 17.6$ (Pangburn et al., 1973). Commercial enzyme preparations were purified before and after tritiation by affinity chromatography on a specific resin prepared by Dr. N. Nishino, Georgia Tech. The ligand-support was $\text{HONHCOCH}_2 \text{C}_6 \text{H}_5 \text{CO-Ala-Gly-NH(CH}_2)_3 \text{-O-Agarose}$. The enzyme is bound tightly to this column at pH 7.2 (0.10 M Tris-HCl, 0.01 M CaCl$_2$) and active thermolysin is released readily when the pH is raised to pH 9.0 and the Ca$^{2+}$ concentration is increased to 0.10 M. A typical
elution profile is shown in Figure 2. The purified enzyme is dialyzed against 1 mM calcium acetate at pH 6.0 to remove the buffer salts before lyophilization onto the target plate. The low concentration of calcium is required to stabilize the enzyme against autolysis (Dahlquist et al., 1976) so it is necessary to label thermolysin in the presence of calcium acetate. A single experiment in which calcium chloride was used resulted in the total loss of enzyme activity for unknown reasons.

Thermolysin activity was assayed using the synthetic substrate FAGLA (Feder, 1968). The kinetic parameter \( \frac{k_{\text{cat}}}{K_m} \) was obtained by determining the initial enzyme activity at a series of FAGLA concentrations ranging from 0.45 to 3.0 mM. These data were analyzed by Lineweaver-Burk plots (Segal, 1975).

**Collagen**

Collagen contains very few aromatic amino acid residues and its absorbance at 280 nm is very low (Ramachandran and Reddi, 1976). Collagen concentrations were estimated based upon dry weight. Collagen solutions were prepared by cutting weighed amounts of the protein into small pieces and stirring gently in 0.1 M acetic acid at 4°C for several hours. The type I rat skin collagen used was acid soluble. Collagen samples were prepared for labeling by lyophilizing onto the plate from 0.1 M acetic acid so that only protein was present on the plate.

**Antithrombin III**

The concentration of this protein was calculated from 0.0280 absorbance at 280 nm using \( E_{280}(1\%) = 5.7 \) (Kurachi et al., 1976a). The purity of the protein was monitored by SDS gel electrophoresis. Antithrombin inhibitory activity was determined by the ability of preparations to inhibit trypsin (Kurachi et al., 1976a, and Kurachi et al., 1976b).
Figure 2. Affinity chromatography of commercial thermolysin.
α₁-Protease Inhibitor

The concentration of this protein was calculated from O.D.₂₈₀ using $E_{2₈₀}(1\%) = 5.2$ (Baugh and Travis, 1976). SDS gels were used to monitor protein purity and the ability of the preparation to inhibit elastase was used to measure its biological activity (Baugh and Travis, 1976). The inhibition studies were kindly performed by Dr. D. Johnson, University of Georgia.
CHAPTER III

EXPERIMENTAL RESULTS

Performance of the Apparatus

It is necessary, before presenting the results, to discuss some characteristics of the tritiation apparatus which bear upon those results. This apparatus was the first constructed for the purpose of tritiating proteins at low pressure and there are many problems associated with its operation. Reaction conditions are very difficult to control and almost impossible to reproduce. Seemingly identical experiments have given widely varying results.

The first major difficulty associated with the system is that the pumping speed is very slow and as long as 72 hours may be required to reach base pressure. The reason for this is that the mercury diffusion pump is small and has a jet less than 6 mm in diameter. An additional problem that was present during the early experiments was the lack of control of the liquid nitrogen level in the cold traps. Wide variations in the pressure during exposure experiments were the result. This problem was solved by the installation of the $\text{N}_2(1)$ control system.

A second problem is that there is a leak in the vacuum chamber. This very slow leak was not discovered until the $\text{N}_2(1)$ control system was installed. Once the main stopcock is closed, isolating the reaction chamber, a very slow leak is observed over the time period of the reaction. The pressure increases linearly at a rate of $1 \times 10^{-3}$ torr/h over
200 h. The source of the leak is unclear. The result is that the leak limits the time of the reaction somewhat but not severely.

The third and most important problem involves the $T_2$ itself. The present system provides no way to actually measure the amount of $T_2$ present during any given experiment. The closed Pd vessel is heated to 450°C for 30 min to 1 h before the leak valve is opened and the pressure allowed to rise to the desired value. The only measure of the amount of $T_2$ present is the total pressure in the chamber. Heating the Pd to this high temperature will surely cause the outgassing of other non-condensible gases which will contribute to the total pressure. There is no means to determine the partial pressure of the $T_2$ present in the system so all values given for the amount of $T_2$ during a reaction must be considered as upper limits.

A final factor that causes variations in the results obtained is the difficulty of reproducing the distribution of the protein on the surface of the target plate. The lyophilized protein on the surface of the plate is very fragile and is disturbed very easily. It is difficult to deposit the material on the plate in a uniform, reproducible manner. Collagen is the easiest protein to apply to the plate because of its fibrous nature. Thermolysin is more difficult. Since the surface area of the exposed protein is probably a very important factor in the labeling process, the uneven distribution of the material will cause variations in the degree of tritiation.

The majority of these problems will be solved by the new apparatus designed as a result of these studies. The new system is described in the Appendix. The preparation of the surface for labeling will still be
a major factor in the process and more reproducible methods for sample preparation will need to be found.

**Tritiation of Thermolysin**

Thermolysin was chosen as a model for these studies because of its stability, availability and low cost. A convenient assay procedure is also available (Feder, 1968). Any degradation of the protein as a result of the labeling process can easily be observed as a loss of catalytic activity. Seventeen labeling experiments with thermolysin resulted in specific activities ranging from 1 to 76 Ci/mole after extensive purification. Recovery of catalytic activity ranged from 40 to 95% depending upon the conditions of labeling. Activity was totally lost in only one experiment when the enzyme was labeled in the presence of calcium chloride instead of calcium acetate. The reason for the destruction of the enzyme in this case is not known. The destruction was possibly caused by formation of $^3$H Cl on the plate. Since thermolysin requires calcium for stability (Dahlquist et al., 1970), about 3 mg of calcium acetate was always present during labeling.

**Purification of $[^3H]$ Thermolysin**

Extensive purification of the tritiated protein is required. The primary reason for this is that a large majority of the tritium incorporated into the protein is exchangeable with water. Greater than 95% of the tritium incorporated into thermolysin was found to exchange immediately with water when dialyzed. After dialysis, chromatography procedures were employed to further purify the protein.

The first chromatography of the enzyme was always on the hydroxamate affinity column. Figure 3 is the chromatogram obtained in a typical
Figure 3. Affinity chromatography of $^3$H thermolysin.
experiment. It can be seen that a relatively small amount of labeled protein passes through the column without binding. This inactive protein does not appear to be significantly more radioactive than the active enzyme which is eluted when the pH is changed to 9.0. Highly radioactive decomposition products have not been observed in these experiments but are quite common with Wilzbach labeled proteins (Evans, 1974).

The second chromatography was performed after dialysis to pH 6.0 buffer (0.01 M Na OAc, 2 nM Ca(OAc)$_2$, 1.0 M NaCl) and concentration by ultrafiltration on a PM 30 membrane. The column was a 1 x 24 cm Sephadex G-75 column equilibrated with the same buffer (Titani et al., 1972). The specific radioactivity is observed to be constant across the protein peak (see Figure 4).

The G-75 chromatography was followed by dialysis back to pH 7.20 buffer and re-chromatography on the affinity column. The result of this chromatography is shown in Figure 5. The inactive protein peak is not observed indicating that the protein is completely active. The results of this experiment are summarized in Table 2. The specific radioactivity obtained after these steps remains stable as long as the enzyme retains its native conformation. However, as will be discussed later, denaturation of the enzyme leads to the loss of greater than 90% of this apparently stable tritium.

Specific Enzyme Activity and $k_{cat}/K_m$ of $[^3H]$ Thermolysin

A number of experiments were performed to determine if the tritium labeled enzyme behaved kinetically the same as native thermolysin. The specific enzyme activity for the hydrolysis of FAGLA was routinely employed at each step of the purification procedure as a measure of the
Figure 4. G-75 chromatography of $[^3H]$ thermolysin.
Figure 5. Re-chromatography of [3H] thermolysin on the affinity column.
Table 2. Tritiation of Thermolysin

Gas Exposure Conditions: \( 1 \times 10^{-3} \text{ Torr T}_2 \) (5 mCi), 62 hr

<table>
<thead>
<tr>
<th>[(^3\text{H})] Thermolysin</th>
<th>DPM/( \mu \text{g} )</th>
<th>Ci/mole</th>
<th>Specific Enzyme Activity (( \mu \text{mole/sec mg} ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude product dissolved in buffer</td>
<td>( 1.6 \times 10^5 )</td>
<td>2500</td>
<td>0.38</td>
</tr>
<tr>
<td>after dialysis and first affinity chromatography</td>
<td>( 1.2 \times 10^4 )</td>
<td>190</td>
<td>0.47</td>
</tr>
<tr>
<td>after dialysis, concentration and Sephadex G-75</td>
<td>( 4.1 \times 10^3 )</td>
<td>64</td>
<td>0.45</td>
</tr>
<tr>
<td>after dialysis and second affinity chromatography</td>
<td>( 4.6 \times 10^3 )</td>
<td>72</td>
<td>0.45</td>
</tr>
<tr>
<td>after 6N hydrolysis and 2x lyophilization</td>
<td>52</td>
<td>0.82</td>
<td>--</td>
</tr>
</tbody>
</table>
purity of the labeled enzyme preparation. The specific enzyme activity is calculated from equation (1):

Equation (1)

$$\text{mole/s\cdot mg} = \frac{(V_{\text{total}}(\Delta O.D.\cdot 345/s))}{(317 \text{ M}^{-1} \text{ cm}^{-1})(\varepsilon)(V_{\text{enzyme}})[E]}$$

$V_{\text{total}}$ is the total volume of the assay in mL, $\Delta O.D.\cdot 345/s$ is the change per second in optical density at 345 nm, $317 \text{ M}^{-1} \text{ cm}^{-1}$ is the change in the molar extinction coefficient for the hydrolysis of FAGLA (Walsh et al., 1974), $\varepsilon$ is the path length of the cell, $V_{\text{enzyme}}$ is the volume in mL of the enzyme aliquot added and $[E]$ is the concentration in mg protein/mL of the enzyme aliquot added as calculated from O.D. $280$. The specific enzyme activity thus determined is a function of the substrate concentration since the useful range of FAGLA concentrations is below the $K_m$ value of this substrate. 2.00 mM FAGLA solutions were routinely used during this study and the highest specific activity observed was 0.72 $\mu$moles/s\cdot mg.

The kinetic parameter $k_{\text{cat}}/K_m$ (Segal, 1975) was determined before labeling and after purification of the tritiated thermolysin in order to determine if these values were the same for each enzyme. The native enzyme gave a value of 17,500 L\cdot mole$^{-1}\cdot$s$^{-1}$ and $[^3\text{H}]$ thermolysin gave a value of 19,800 L\cdot mole$^{-1}\cdot$s$^{-1}$. These numbers are easily within the experimental error of such determinations and are considered to be the same. Therefore it is apparent that the labeling process has not altered the catalytic apparatus of thermolysin.
Denaturation of $[^3\text{H}]$ Thermolysin

Denaturation of $[^3\text{H}]$ thermolysin causes the release of greater than 90% of the tritium which is otherwise stable under conditions that favor the native conformation of the enzyme. Figure 6 is a chromatogram of 560 µg $[^3\text{H}]$ thermolysin with an initial specific radioactivity of 53 Ci/mole which was chromatographed on G-10 Sephadex in the presence of 8 M urea at pH 7.0. The peak of $[^3\text{H}]\text{H}_2\text{O}$ representing 90.5% of the applied tritium is well separated from the protein peak. 9.5% of the tritium remains associated with the protein and corresponds to a residual specific radioactivity of 4.8 Ci/mole.

Amino Acid Analysis of $[^3\text{H}]$ Thermolysin

3.2 nmole $[^3\text{H}]$ thermolysin with an initial specific radioactivity of 62 Ci/mole was hydrolyzed for amino acid analysis. The residual specific radioactivity was 0.85 Ci/mole. This sample was applied to the analyzer and fractions were collected for liquid scintillation counting. Unfortunately the radioactivity was too low to give statistically significant counts above background. It was not possible to determine the distribution of tritium among the various residues. The analysis of the sample was used to determine that the amino acid composition of the $[^3\text{H}]$ thermolysin was not altered by the tritiation process. Table 3 gives the analysis obtained for $[^3\text{H}]$ thermolysin along with the published amino acid composition of thermolysin.

A second analysis was performed using 13 nmole of the same $[^3\text{H}]$ thermolysin sample. This large amount of hydrolyzate overloaded the analyzer causing most of the minhydrin peaks to go off scale. However, it was possible to obtain some useful counting data from this run. The
Figure 6. Denaturation chromatography of [³H] thermolysin.
tritium distribution per amino acid residue is given in Table 3. The largest tritium containing peak in the spectrum which eluted prior to Lys was unidentified. Since this sample was hydrolyzed for only 20 h, this peak is probably a dipeptide containing Lys which resulted from incomplete hydrolysis of the protein. There were also 4 other unidentified peaks containing small amounts of tritium. The most prominent tritium peaks in the amino acid analysis were assigned to Asp, Thr + Ser, Gly, Tyr, Lys and Arg.
Table 3. Amino Acid Analysis of $[^3H]$ Thermolysin

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Literature $^a$</th>
<th>Found $^b$</th>
<th>Total dpm $^c$</th>
<th>% dpm $^d$</th>
<th>dpm/nmol AA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asx</td>
<td>44</td>
<td>45.8</td>
<td>4645</td>
<td>17.9</td>
<td>8.1</td>
</tr>
<tr>
<td>Thr</td>
<td>25</td>
<td>26.4</td>
<td>2482</td>
<td>9.5</td>
<td>3.7</td>
</tr>
<tr>
<td>Ser</td>
<td>27</td>
<td>32.4</td>
<td>435</td>
<td>1.7</td>
<td>1.1</td>
</tr>
<tr>
<td>Glx</td>
<td>22</td>
<td>18.8</td>
<td>5006</td>
<td>19.2</td>
<td>10.7</td>
</tr>
<tr>
<td>Pro</td>
<td>8</td>
<td>6.8</td>
<td>48</td>
<td>0.2</td>
<td>0.13</td>
</tr>
<tr>
<td>Gly</td>
<td>36</td>
<td>39.0</td>
<td>87</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>Ala</td>
<td>28</td>
<td>28.2</td>
<td>2.0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cys</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Val</td>
<td>22</td>
<td>18.0</td>
<td>15.2</td>
<td>0.5</td>
<td>0.3</td>
</tr>
<tr>
<td>Met</td>
<td>18</td>
<td>16.0</td>
<td>126</td>
<td>0.5</td>
<td>0.3</td>
</tr>
<tr>
<td>Ile</td>
<td>16</td>
<td>16.0</td>
<td>827</td>
<td>3.2</td>
<td>2.3</td>
</tr>
<tr>
<td>Leu</td>
<td>10</td>
<td>9.4</td>
<td>130</td>
<td>0.5</td>
<td>1.0</td>
</tr>
<tr>
<td>Tyr</td>
<td>8</td>
<td>7.6</td>
<td>201</td>
<td>0.77</td>
<td>1.9</td>
</tr>
<tr>
<td>Phe</td>
<td>11</td>
<td>11.0</td>
<td>2630</td>
<td>10.1</td>
<td>18.4</td>
</tr>
<tr>
<td>His</td>
<td>10</td>
<td>9.2</td>
<td>425</td>
<td>1.6</td>
<td>3.3</td>
</tr>
</tbody>
</table>

unidentified peaks ——> 9120 35

$^a$Titani et al., 1972.

$^b$From a 24 h hydrolysis of 3.2 nmole $[^3H]$ thermolysin.

$^c$From a 24 h hydrolysis of 13 nmole $[^3H]$ thermolysin. This hydrolysis was complete because there was a pin hole in the ampule present during hydrolysis.

$^d$3.0 x 10$^4$ dpm were applied to the analyser and 2.6 x 10$^4$ dpm (86%) were recovered. % dpm is calculated from the total tritium recovered.

$^e$Met was destroyed by oxidation during this hydrolysis.
Tritiation of Collagen

Purification and Characterization of $[^3\text{H}]$ Collagen

Type I rat skin collagen was labeled in the same way as thermolysin except that collagen was lyophilized onto the plate from acetic acid solution leaving only protein on the plate. Collagen is a fibrous protein and the lyophilized surface on the plate was like a very thin, gossamer fabric stretched loosely over the plate. Specific radioactivities ranging from 100 to 5600 Ci/mole were obtained. These are values calculated after extensive dialysis to remove readily exchangeable tritium. No chromatography procedures were employed with this protein for purification. Repeated dissolution and lyophilization of the dialyzed $[^3\text{H}]$ collagen led to some decrease in the apparent specific radioactivity. SDS gel electrophoresis showed the expected pattern of bands and $[^3\text{H}]$ collagen was indistinguishable from native collagen.

Effect of Increased Exposure Time to $T_2$

Because collagen gives a lyophilized surface that is uniform and more easily reproduced than the other proteins labeled in these studies, it was used in a series of experiments designed to determine the effect of increasing the time of exposure to $T_2$. A constant amount of collagen was used while attempting to keep all other parameters constant. The results are presented in Table 4. The increase in crude activity is not strictly linear but a definite increase in tritium incorporation is observed with increasing time of exposure. The experimental limits of the apparatus, as discussed earlier, severely hinder this type of experiment because it is almost impossible to duplicate exposure conditions from one run to the next.
Table 4. Exposure of 5 mg Collagen to 5 mCi T\textsuperscript{2} at $1 \times 10^{-3}$ Torr as a Function of Time

<table>
<thead>
<tr>
<th>Time of exposure (h)</th>
<th>Crude Specific Radioactivity $\mu$Ci/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>1.9</td>
</tr>
<tr>
<td>6</td>
<td>2.4</td>
</tr>
<tr>
<td>12</td>
<td>17</td>
</tr>
</tbody>
</table>
Denaturation of [³H] Collagen

[³H] collagen was also chromatographed in 8 M urea in order to determine the effect of denaturation on the stability of the label. As with thermolysin, a high percentage of the tritium was found to be exchangeable when [³H] collagen was denatured. Figure 7 shows the same chromatogram obtained when [³H] collagen was chromatographed on the 8 M urea column. Only 3% of the initial label remained with the protein upon denaturation and the remaining tritium was found to be exchangeable.

Amino Acid Analysis of [³H] Collagen

[³H] collagen was also subjected to amino acid analysis in an attempt to determine the distribution of tritium among the amino acid residues. Unfortunately, as with [³H] thermolysin, the residual specific radioactivity was too low to allow an adequate determination. It was possible only to determine that Hyp, Gly, Val and Lys definitely contained tritium. The remaining tritium counts were too close to the background levels to be distinguished. In order to obtain these data, much more sample would have to be analyzed and then the resolution of individual peaks would be lost because of overloading. A residual specific radioactivity of at least 10 Ci/mole would be required before a good amino acid analysis could be obtained without overloading the analyzer. The residual specific radioactivities of hydrolyzed [³H] collagen samples were in the range of 1 Ci/mole.

Tritiation of Human Antithrombin III and α₁-Protease Inhibitor

These two similar serum glycoproteins were tritiated three times each in preliminary experiments designed to determine if these proteins could also be labeled by low pressure T₂ exposure.
Figure 7. Denaturation chromatography of $[^3H]$ collagen.
yielded tritiated products with specific radioactivities after dialysis of 8.7, 10 and 11 Ci/mole. Lyophilization reduced these values to around 1.0 Ci/mole. Similarly, α₁-protease inhibitor yielded post-dialysis specific radioactivities of 2.3, 0.75 and 1.0 Ci/mole. No additional purification steps were performed with either of these proteins. 6 N HCl hydrolysis yielded a residual specific radioactivity of 0.7 Ci/mole for antithrombin and 0.1 Ci/mole for α₁-protease inhibitor.

Because these values are low and because the possibility exists that acid catalyzed exchange with [³H]H₂O could occur during hydrolysis, a blank experiment was performed. A sample of unlabeled thermolysin was hydrolyzed in the presence of 1 x 10⁶ dpm [³H]H₂O and treated in the same manner as the tritiated proteins to determine the residual tritium content. No significant tritium was found remaining with the amino acids after this treatment.

Both [³H] antithrombin and [³H] α₁-protease inhibitor were found to retain biological activity as demonstrated by the antithrombin inhibition of trypsin (Kurachi et al., 1976b) and the α₁-protease inhibitor inhibition of elastase (Baugh and Travis, 1976). The residual specific radioactivities were too low for amino acid analysis.

Denaturation of [³H] Antithrombin and [³H] α₁-Protease Inhibitor

Each of these proteins was denatured and chromatographed on the 8 M urea column. In contrast to [³H] collagen and [³H] thermolysin, a larger percentage of the tritium label was found to remain with these serum glycoproteins after denaturation. [³H] antithrombin retained 25 and 70% of the label in two separate chromatographies. [³H] α₁-protease inhibitor retained 61% of its label after chromatography. Figures 8 and
Figure 8. Denaturation chromatography of $[^3]$H antithrombin III.
9 are the chromatograms obtained for $[^{3}\text{H}]$ antithrombin and $[^{3}\text{H}] \alpha_{\text{T}}$-protease inhibitor, respectively.

**Desmosine**

Desmosine is a complex cross linking amino acid found in elastin. 11 mg of this amino acid was labeled in a single experiment in order to determine if any tritium would be incorporated under the conditions used in the protein experiments. The crude specific radioactivity obtained was 1.4 Ci/mole. The crude labeled product was applied to a column of Dowex 50 (H$^+$) X-8 cation exchange resin in order to remove labile tritium. This treatment resulted in a lowering of the specific radioactivity to 0.003 Ci/mole. No further characterization of this product was attempted.
Figure 9. Denaturation chromatography of $[^{3}H] \alpha_1$-protease inhibitor.
CHAPTER IV

DISCUSSION

These studies began with experiments intended to determine if a molecular ion beam of vibrationally excited $T_2^+$ ions could be used to generally label proteins with tritium in a way that would lead to formation of stable carbon-tritium bonds with little or no damage to the protein. It was known prior to this work that proteins and other molecules could be tritiated by exposure to Curie quantities of $T_2$ (Wilzbach, 1957). It was believed that the ionizing radiation present with large concentrations of $T_2$, which amounts to $1.8 \times 10^{19}$ eV/Ci day, was necessary for the incorporation of T into the target substrate (Evans, 1974). In order to produce a molecular ion beam much lower pressures and less $T_2$ were required than in the Wilzbach method. The apparatus constructed for these experiments proved to be inadequate to produce a useful molecular ion beam. However, it was surprising to discover that the products of reactions performed with the apparatus were significantly radioactive even in the absence of activation of the $T_2$ by an electron beam. This observation prompted the continued study of the low pressure tritiation of proteins. As a result, it has been shown that Curie quantities of $T_2$ are neither necessary nor desirable for the gas exposure tritiation of proteins. It has been demonstrated conclusively that significant amounts of tritium are incorporated into proteins when they are exposed to low concentrations of $T_2$ at low pressure.
The important difference between low pressure $T_2$ exposure and the Wilzbach method is that the flux of high energy $\beta$ particles is negligible with the millicurie amounts of $T_2$ used in the low pressure method. The specific radioactivities obtained are similar in both methods yet the highly tritiated decomposition products produced by Wilzbach labeling have not been observed with the low pressure method. This is presumably because the flux of high energy particles which can cause fragmentation of the protein molecules is very low at lower concentrations of $T_2$. Some other mechanism of exchange is present in both methods which leads to exchange without damage to the substrate. The nature of this exchange mechanism is not known.

Each labeling method suffers from one major disadvantage. A majority of the tritium found in the crude product is readily exchangeable with water, presumably because tritium exchange in the gas phase occurs preferentially at N-H and O-H positions. Rigorous purification of the tritiated product is necessary to achieve a stable, constant specific radioactivity. The important difference is that with Wilzbach-labeled proteins it is also necessary to remove highly tritiated decomposition products which are chemically very similar to undamaged tritium labeled molecules. A significant portion of the radioactivity present can be due to such decomposition products. Such products have not been found in proteins labeled by low pressure $T_2$ exposure.

Thermolysin was the model system chosen for the initial experiments for several reasons. Since it is an enzyme for which a convenient and simple assay is known, damage as a result of the labeling process could be readily detected as a loss in catalytic efficiency. Also, a simple and highly specific affinity chromatography support was available
for the purification of the enzyme after tritiation. It was by the use of this affinity resin that it was demonstrated that highly tritiated decomposition products were not produced during the tritiation of thermolysin. It can be seen in Figure 3 that a small peak of inactive protein is eluted at the void volume and that this material is only slightly more radioactive than the active protein eluted later. A portion of the tritium associated with this inactive peak is $[^3\text{H}]\text{H}_2\text{O}$ which would also be eluted at the void volume of the column. It was therefore concluded that the inactive protein was not significantly more radioactive than the active enzyme.

Further support for this conclusion was provided by kinetic measurements. Recoveries of catalytic activity were high, ranging as high as 95%. $[^3\text{H}]$ thermolysin was shown to have the same specific enzyme activity as the native enzyme. The tritiated enzyme was also shown to have the same value for the kinetic parameter $k_{\text{cat}}/K_m$, further supporting the contention that tritiation did not alter the catalytic apparatus of the enzyme.

Having demonstrated the technique with thermolysin, attention was turned to collagen, antithrombin III, $\alpha_1$-protease inhibitor and desmosine in order to determine if the technique was general. It was found that each of these molecules was indeed labeled by exposure to T2 and that the degree of tritiation was dependent upon the nature of the substrate. Collagen yielded much higher specific radioactivities, as high as 5600 Ci/mole. Antithrombin and $\alpha_1$-protease inhibitor were found to incorporate less tritium. Desmosine, the only non-protein labeled, is a small molecule and was found to incorporate very little tritium.
Collagen is a very large (F.W. approx. 360,000), fibrous protein with a triple helical structure. Thermolysin, on the other hand, is a small (F.W. 34,800) globular protein. Antithrombin III (F.W. 56,600) and \( \alpha_1 \)-protease inhibitor (52,000) are both medium sized globular glycoproteins. The differences in structure surely contribute to the observed differences in the degree of tritiation. It is these differences in molecular architecture that account for the observed release of tritium from the proteins when they are denatured.

\(^{3}H\) thermolysin can be chromatographed, lyophilized and dialyzed repeatedly and the specific radioactivity remains reasonably constant as long as the native conformation of the enzyme is maintained. This is also true for \(^{3}H\) collagen, \(^{3}H\) antithrombin and \(^{3}H\) \( \alpha_1 \)-protease inhibitor. However, when any of these proteins is denatured by 8 M urea, formerly non-exchangeable tritium becomes labile. With \(^{3}H\) thermolysin and \(^{3}H\) collagen, the release amounts to greater than 90\% of the tritium associated with the protein. \(^{3}H\) antithrombin and \(^{3}H\) \( \alpha_1 \)-protease inhibitor also lose some tritium upon denaturation but the percentage lost is lower. Gas exposure labeling of proteins apparently leads to the preferential exchange of T for H at N-H and O-H positions. This exchange is not limited solely to sites on the surface of the protein molecule because internal, solvent-inaccessible positions are also found to be labeled.

These internal N-T and O-T positions are protected from exchange with water when the protein is dissolved in buffer and exchange-out of the tritium at these positions is not possible until the sites are exposed to the buffer after denaturation. Corroborative support for
this contention is found in $[^3\text{H}]\text{H}_2\text{O}$ exchange studies with many proteins. Such experiments have demonstrated that many proteins have hydrophobic inner cores in which peptide amide protons are inaccessible to external solvent and therefore fail to exchange with $[^3\text{H}]\text{H}_2\text{O}$. In hydrogen-tritium exchange studies with thermolysin, it was found that 100 peptide hydrogens, 32% of the entire polypeptide backbone, were inaccessible to and failed to exchange with $[^3\text{H}]\text{H}_2\text{O}$ (Voordouw and Roche, 1975).

Hydrogen-tritium exchange studies with collagen have also shown the presence of protons protected from exchange with water. Exposure of ichthyocol collagen to $[^3\text{H}]\text{H}_2\text{O}$ at low pH and low temperature for days failed to give a product in which all of the potentially exchangeable amide bonds were labeled. Higher temperatures, near the melting transition temperature where collagen begins to unwind, were required to achieve total exchange (Yee et al., 1974). A similar study with insoluble collagen showed that total exchange of the theoretical number of amide protons would occur only if the exchange were allowed to take place in the presence of 0.75 M hydroxylamine and 5.5 M lithium bromide, which are hydrogen-bond disrupting agents (Kingham and Brisbin, 1968).

It is clear that low pressure $T_2$ exposure causes hydrogen-tritium exchange in the hydrophobic interiors of proteins. Hydrogen-bonded positions in the inner, helical core of collagen become tritiated and remain so until these bonds are broken. The mechanism of this process is obscure. It could be a result of the dissolution or absorption of $T_2$ in the hydrophobic regions of the protein and subsequent exchange of H for T. The effect is apparently dependent upon the nature of the molecule itself. Extensive internal exchange occurs with collagen and
thermolysin, and a large percentage of the final specific activity after purification of these proteins is due to this internal tritium. On the other hand, purified antithrombin III and $\alpha_1$-protease inhibitor are labeled internally to a much lesser degree. It may be significant that these two proteins are similar in size and that each is a glycoprotein.

Two possible explanations exist. It is possible that these proteins have tightly packed structures which effectively exclude T$^\circ$2. This seems unlikely since thermolysin was shown to have a tightly compacted structure in solution that excluded $[^3\text{H}]\text{H}_2\text{O}$ yet T$^\circ$2 was able to penetrate to give exchange reactions. The alternate possibility is that these serum glycoproteins are labeled internally to the same degree as thermolysin and collagen yet the solution conformations are sufficiently flexible to allow most of this internal tritium to exchange out during dialysis. This seems to be a more likely possibility.

The residual tritium content experiments showed that all of the proteins labeled by low pressure T$^\circ$2 exposure had a residual specific radioactivity close to 1 Ci/mole. This value compared closely with the residual specific radioactivities calculated from the denaturation chromatographies. It seems certain that any tritium remaining after hydrolysis in 6 N HCl must be carbon bound T. Unfortunately, these residual specific radioactivities were too low to allow a determination of the distribution of the carbon bound T among the amino acid residues. With $[^3\text{H}]$ collagen it was possible only to establish that some T was associated with Hyp, Gly, Val and Lys.

Low pressure gas exposure tritiation of proteins is not likely to become a generally useful technique because of the problems associated
with obtaining a product with a stable tritium label. The tendency of
the tritium label to "dance around" is sufficient to cause concern in
most experiments which would use radioactivity labeled proteins.
However, specialized uses could be found for the products of low pressure
tritiation. *In vitro* experiments, the conditions of which would not
denature the labeled protein, could be productive. For example, as long
as $[^3\text{H}]$ thermolysin maintains its native conformation, the specific
radioactivity could be considered to be constant and therefore stable.
The ideal situation would be to purify the tritiated protein in such a
way that all of the exchangeable label could be removed. The technique
could prove useful in physical studies of the nature of exchangeable
hydrogens when used in conjunction with solution exchange experiments.

The major contribution of this work has been the establishment of
a protocol for the purification and characterization of proteins
tritiated by gas exposure and the design of a new apparatus which can be
used for highly controlled, reproducible gas exposure experiments. The
new apparatus, which is described in the Appendix, will make possible
experiments with a $^{2+}\text{T}_2$ molecular ion beam. The knowledge gained in these
experiments will permit the evaluation of the labeling experiments to be
performed with the ion beam. Proteins labeled by this technique should
be useful in experiments which require tritium labeled proteins that are
difficult or impossible to obtain by any other method.
APPENDIX

During the course of the study of the low pressure gas exposure tritiation of proteins, a design was formulated for a new, versatile vacuum system to be used in future experiments. The primary goal sought was the construction of a pumping system that would permit the evacuation of the reaction chamber to pressures as low as $10^{-8}$ torr. The drawings included with this appendix show the design which was produced.

Figures 10, 11 and 12 show three different views of the reaction chamber. The sample will be mounted inside the vessel on a stainless steel plate similar to that used in the old system. A cold finger trap will be mounted on the bottom flange in order to trap any condensible gases present in the system. One end port will serve as an access port through which the sample will be moved. The other end port will be an electrical feedthrough where an electron gun will be mounted. One of the 2.75 CFF flanges on top will be used for a dual liquid feedthrough which can be used to cool the sample stage. The other will be used for a Penning gas ionization gauge. The long half nipple on top will serve as a pump port and will have a small right angle valve. The arrangement of the chamber in the system is shown in Figure 13.

A quadrupole mass spectrometer will sample gas from the chamber via one of the 1.33 Mini CFF ports. The mass spectrometer will serve as a gas analyzer and will permit the precise determination of the amount of tritium present during any reaction. This will solve the major problem present in the original apparatus.
The $T_2$ handling system can be seen in Figures 14 and 15. The gas will be purchased in sealed ampules which will be opened inside the evacuated system and absorbed onto uranium powder in one of the narrow tubes. It will be stored on the uranium and released for use by heating the storage tube with a small oven. Gas will be carefully controlled while being admitted to the reaction chamber by operation of the leak valve. Any desired $T_2$ pressure can be obtained in this way.

The pumping system will consist of a 4-inch oil diffusion pump with a cryotrap and a slide body valve. A roughing pump will also be required. Varian Associates will provide a pre-assembled, leak tested pump station with the appropriate valves and traps which will mate the 9-inch ASA flange seen in Figure 13. The system should be capable of reaching pressures down to $10^{-7}$ to $10^{-8}$ torr.

The primary purpose for the new system will be to test an electron beam activation of $T_2$ that will hopefully yield higher and more stable specific radioactivities in the labeled proteins. The new system is more versatile and much more easily controlled so that experimental conditions will be more well defined and easily reproduced. The time required to run an experiment will also be greatly reduced because the pumping speed of the system will be far superior to that of the original system.
Figure 10. Proposed tritiation apparatus, reaction chamber, right side view.
Figure 11. Proposed tritiation apparatus, reaction chamber, left side view.
Figure 12. Proposed tritiation apparatus, reaction chamber, top view.
Figure 13. Proposed tritiation apparatus, overall system.
Figure 14. Gas handling system.
LITERATURE REFERENCES


VITA

Mark O. Lively, III, son of Mr. and Mrs. Mark O. Lively, Jr., was born in Albany, Georgia, on July 14, 1952.

He graduated from Lakeside High School, Atlanta, Georgia, in June, 1970. He attended Georgia Institute of Technology as a Cooperative Division student and worked for the United States Environmental Protection Agency. He graduated with his Bachelor of Science degree in Chemistry in March, 1975, and immediately began his graduate study in the School of Chemistry at Georgia Tech.