

THE PREPARATION OF TRITIATED PROTEINS BY THE WILZBACH METHOD AND A SIMPLE METHOD FOR LIQUID SCINTILLATION COUNTING OF RADIOACTIVE PROTEINS*

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THE technique recently described by WILZBACH³ for introducing tritium into organic compounds holds great promise as a tool for application to biochemical studies. It should be possible in many instances to obtain a labeled precursor or substrate without the sometimes difficult problems of synthesis. However, initial enthusiasm must be tempered in view of the purification problems which to some extent replace the problems of synthesis. In all cases there are significant quantities of radioactivity in degradation products which must be removed before the labeled compound can be used.

In the case of proteins the only way of obtaining labeled material heretofore has been by the addition of labeled amino acids or other simple precursor molecules to biological systems with recovery then of the newly synthesized labeled protein by the usual isolation techniques. In view of the mild conditions used in the Wilzbach procedure it appeared to us possible that this method could be applied also to the labeling of proteins. Preliminary results with the tritiation of lysozyme and ribonuclease are described here together with a method for radioassay of the intact tritiated protein by liquid scintillation methods.

Crystalline lysozyme (237 mg) was exposed to 1.8 c of tritium gas for 72 hr (room temperature; pressure 0.39 atm).† After exposure, and before any further treatment, the specific enzymatic activity of the lysozyme was determined and found to be 90% that of the enzyme assayed prior to exposure. Clearly there is no wholesale destruction of the protein nor any extensive denaturation of the bulk of the protein during exposure to tritium gas under these conditions.

Labile tritium was next removed by dissolving the protein in water (5 mg/ml) and then lyophilizing. The protein was redissolved and lyophilized a total of three times. The specific radioactivity of the protein at this point, measured

* Preliminary reports of these studies have been published.^{1,2}

† Exposure to tritium gas was carried out by the New England Nuclear Corp., Boston, Mass.

using the liquid scintillation technique described below, was 3.6×10^5 counts/min mg.

An aliquot of the labeled protein was subjected to chromatography on IRC-50 according to the method of GONCALVES *et al.*⁴ As shown at top of Fig. 1, a single major protein peak was eluted corresponding in position of

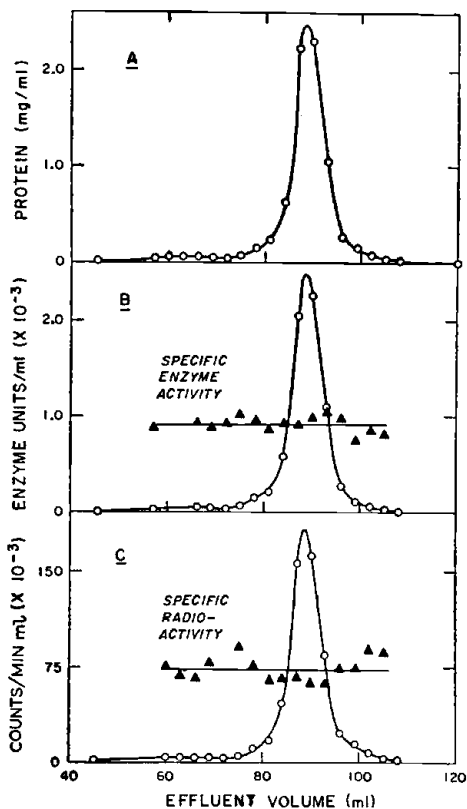


Fig. 1. Chromatography of tritiated lysozyme on IRC-50. A: Total protein by method of Lowry.⁵ B: Enzyme activity by method of TALLAN and STEIN.⁶ Solid triangles indicate specific enzyme activity in arbitrary units. C: Tritium radioactivity by method of VAUGHAN, *et al.*² Solid triangles indicate specific radioactivity in arbitrary units.

elution to that normally occupied by lysozyme. As has been reported by others previously, there was a very small component which was eluted before the main peak. The specific enzymatic activity of this peak corresponded well with the specific enzymatic activity of untreated lysozyme, as shown in panel B of Fig. 1. It will be noted that the minor forepeak also showed the specific enzymatic activity of lysozyme. Finally, as shown in panel C of Fig. 1, the specific radioactivity of both the major and the minor peaks was, within the limits of error of the method, quite constant throughout. The recoveries of

protein and of total enzymatic activity were 93% and 90% respectively. This, together with the uniformity with respect to enzymatic activity and radioactivity indicates that the bulk of the exposed enzyme protein is unaffected to any major extent by the labeling procedure. However, the recovery of radioactivity was very incomplete. Only about 50% of the radioactivity put on the column was recovered in the lysozyme peaks. Direct count of an aliquot of the resin in the column showed that there was considerable tritium left behind adsorbed to the resin.

To examine further the question of purity a fresh sample of labeled protein was subjected to repeated recrystallizations. It became clear that a large fraction of the total radioactivity was present in partially degraded protein since the specific radioactivity of the crystalline material fell with successive crystallization steps. After addition of carrier lysozyme one sample was recrystallized five times before constant specific radioactivity was reached. At this point the specific radioactivity had fallen to only about 16% of the original value.

From the results on recrystallization and the results on column chromatography it must be concluded that the labeling procedure led to the formation of a small amount of highly radioactive but partially degraded material. The nature of this material has not been further investigated. The fact that on repeated recrystallization the specific radioactivity fell even below that found for column purified lysozyme suggests that some of the molecules are subtly modified. They appear to behave normally on IRC-50 but are lost, perhaps because of decreased stability, in the course of repeated recrystallization procedures.

Crystalline ribonuclease has also been subjected to the Wilzbach procedure.* In this case 570 mg was exposed at 25°C to 6.73 c of tritium gas for 48 hr. The specific enzymatic activity immediately after the exposure was 82% of that of the untreated material indicating that most of the protein remained unaffected. Labile tritium was removed in this case by treatment with 0.1 N NH₄OH and subsequent lyophilization. The protein was taken up in 0.2 M pH 6.17 phosphate buffer and chromatographed on IRC-50, according to the method of HIRS *et al.*⁷ A peak of radioactivity containing no enzymatic activity emerged almost with the front. This degraded fraction was incompletely resolved from the enzymatically active peak which followed. Since resolution was so incomplete the degree of homogeneity of the labeled ribonuclease cannot be rigidly evaluated. However, the peaks of radioactivity, enzymatic activity and total protein (optical density at 280 μ) coincided with each other. Furthermore, at least in the descending limb of the peak, the specific enzymatic activity and the specific radioactivity were reasonably constant in successive tubes.

* We are grateful to Dr. K. E. WILZBACH for his kindness in carrying out this tritiation procedure for us.

It may be worth while mentioning a possible explanation for the origin of the highly radioactive degradation products in these studies despite the rather good recoveries of the total protein and of enzyme activity. The protein is exposed to tritium gas as a powder but the dimensions of each granule may be significant when related to the extremely short range of the beta-particles from tritium. It may be that the layer of protein at the periphery of each granule, being subjected to an extremely high flux of beta-particles, exchanges a high proportion of its hydrogen for tritium but at the same time and for the same reason suffers a greater degree of degradation than the protein molecules internally located in the granule. Presumably, if this hypothesis has any validity, there is a layer of protein occupying an 'optimal' position somewhere just beneath the surface which receives enough beta flux to become labeled but not enough to cause extensive degradation. Following this line of reasoning it would seem logical to modify the technique of exposure by preparing a very thin layer of the protein and exposing it for the minimum time necessary to obtain a useful degree of labeling. In this way it might be possible to avoid some of the problems involved in separating the degradation products from the native material. We have encountered a similar phenomenon in the labeling of amino acid analogues, that is, the occurrence of high specific radioactivity degradation products, and so the same explanation may apply in other cases and a similar solution may be valid.

The results of the present study demonstrate the applicability of the Wilzbach procedure to the preparation of tritiated proteins. Purification must be rigorous but it is possible to obtain good yields of chromatographically pure, enzymatically active protein. In addition a simple and sensitive method of radioassay is necessary for the effective use of the tritium-labeled compounds in biological studies. PASSMAN *et al.*⁶ have reported the use of a methanolic solution of a quaternary amine, Hyamine,* to form a complex with $C^{14}O_2$ which is soluble in the organic solvents required for solution of the phosphors employed in liquid scintillation counting. It has been found that Hyamine can also form complexes with amino acids and proteins. The phosphors dissolved in toluene can then be added to the methanolic solution of protein-amine complex providing a clear, homogeneous solution for assay in the liquid scintillation counter.

The sample of protein or amino acid to be dissolved is weighed directly into the glass vial to be used for counting and to it is added 1 ml of 1M Hyamine in the hydroxide form in methanol prepared according to PASSMAN *et al.* as modified by Eisenberg.† After the sample is completely dissolved 10 ml of 600 mg % diphenyl-oxazole (DPO) in toluene is added and the contents of the vial are mixed.

* *p*-Di-isobutylcresoxymethoxyethyl dimethylbenzyl ammonium chloride monohydrate = Hyamine - 10X, Rohm and Haas Co., Philadelphia, Pa.

† EISENBERG, FRANK; this volume.

Alanine, leucine, phenylalanine, tyrosine and tryptophan are each soluble to a concentration of at least 20 mg/ml of Hyamine solution. Aspartic acid is somewhat less soluble than the afore-mentioned amino acids, and as little as 5 mg of arginine cannot be completely dissolved in 1 ml. It has been found that up to 10 mg of the following crystalline proteins dissolve readily at 37°C: insulin, ribonuclease, lysozyme, ovalbumin, and bovine serum albumin. Mixed tissue proteins (precipitated and washed with trichloroacetic acid (TCA), followed by ethanol-ether (1/1) and ether) dissolve with more difficulty, but 10 mg can be taken up in 1 ml of the amine solution by capping the vials and heating to 55°-60°C for 1-2 hr.

After solution is complete and the vials have cooled to room temperature, 10 ml of DPO in toluene is added, as with the other samples. The vials are then cooled to the temperature of the counting chamber (-10°C). Samples of protein or amino-acid containing salt (such as are obtained from column chromatography) can be taken up in the Hyamine solution satisfactorily, even though the salt does not dissolve.

With the exception of insulin, the afore-mentioned crystalline proteins do not cause quenching in amounts up to 10 mg per sample, and the counting efficiency with this method is approximately 5%. Insulin causes a decrease in the observed counting rate for tritium of from 14 to 57% as the amount of insulin is increased from 5 to 20 mg. Quenching is marked with the TCA-precipitated tissue proteins. Solutions of these denatured proteins frequently have a yellow-brown color. The intensity of the color varies with protein preparation, concentration of protein, and time of heating. The relationship between amount of protein and observed counts/min is not linear for these preparations. As is shown in Table 1, the magnitude of this quenching effect

TABLE 1
Correction for Quenching Effect of Trichloroacetic Acid-precipitated Protein

Protein weight (mg)	Observed counts/min minus background	Specific activity observed (counts/min mg)	Increment in counts/min owing to addition of standard*	Specific activity corrected for quenching (counts/min mg)†
2	3933	1966	12081	2685
4	6658	1664	11260	2440
6	8295	1382	9578	2383
8	9523	1190	7613	2581
10	10685	1068	6722	2620

* Standard = 16,500 counts/min of tritium-labeled Δ -4-cholestenone.

† Observed counts/min background $\times \frac{16,500}{\text{increment in counts/min owing to addition of standard}} \div \text{sample weight}$.

can be determined by adding a standard amount of tritium-containing compound to all of the sample vials and recounting them. By means of the ratio of the increment in counting rate owing to the addition of standard for each vial to the counting rate for the standard alone, the counting rates of the unknown samples can be corrected for quenching. The corrected counting rates are linearly related to the amount of tritiated protein in the range from 2 to 10 mg. This method also corrects for differences in quenching that result from variable losses of methanol during heating and for variation in the counting vials. Because the intensity of color of the solution in the vials tends to increase on standing, it is important to make the quenching correction shortly after the sample is counted.

Although an internal standard should always be used in order to correct for quenching due to the methanolic Hyamine, the magnitude of this correction can often be decreased considerably by the proper choice of the 'window', thus increasing the accuracy of the radioassay. In order to minimize errors in the correction factor the increment in count rate observed due to the addition of standard ought to be well in excess of the count rate due to the sample itself.

In the course of these studies, it has been noted that vials containing only methanol, Hyamine, toluene, and DPO give spuriously high counting rates (up to 500 counts/min) when warm. As the temperature of the vial falls to that of the counting chamber, the counting rate returns to the normal background level (about 35 counts/min). This phenomenon is observed only at the high voltages used for counting tritium. It does not occur when Hyamine is omitted from the solution—that is, with toluene and DPO alone—although it is evident when other quaternary amines are substituted for Hyamine.

It is most likely a thermal activated chemiluminescence. That it is not due merely to a temperature differential between the sample and the phototube was shown by counting vials containing hot water. These gave no increase in count rate over background.

Two other sources of spurious counts must be mentioned. We have confirmed the observation reported by DAVIDSON and FEIGELSON⁹ that ultraviolet irradiation of empty counting vials just prior to their insertion into the counter can give extremely high counting rates, up to 10,000 counts/min. Presumably any light source with an appreciable ultraviolet component could induce such a phenomenon. However, since the counting vials are stored in a closed refrigerated box overnight they are protected from irradiation and this has not been a problem. Another potential source of artifact has been called to our attention by Dr. RICHARD J. HERBERG* and by Dr. CHARLES HEIDELBERGER.* If Hyamine is not thoroughly purified, solutions of nonradioactive protein in methanolic Hyamine (3 ml of Hyamine and 100 mg protein) cause a rather

*Personal Communication.

high counting rate. This rate is greatly increased immediately after dilution with toluene even in the absence of DPO and returns only very slowly to background level. However, when Hyamine has been properly purified, so that no trace of colored impurities remain, and when Hyamine and protein are used in the amount suggested here (1 ml of Hyamine to 5–10 mg of protein) this chemiluminescence is minimal even at the time of preparation of the solution and the count rate returns to background levels within a few hours. It should be stressed, then, that Hyamine as sold commercially is impure and requires 1–4 recrystallizations in order to remove impurities.

Many applications of these procedures suggest themselves. For example, the fate of intact, internally labeled proteins in biological systems could be studied without the complications introduced when iodination or other 'external' labeling methods are used. Particularly exciting are the prospects of applying this method in the preparation of labeled proteins and polypeptides normally present in the organism in very small amounts, since it is difficult or impossible to obtain these materials by synthesis and biosynthetic methods yield material of only low specific radioactivity. Some of the problems relating to the fate and function of the polypeptide hormones may now be approached using this new tool.

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