homogenous solution for assay in the scintillation counter.

The sample of protein or amino acid to be dissolved is weighed directly into the glass vial to be used for counting (7), and to it is added 1 ml of 1*M* Hyamine in the hydroxide form in methanol prepared according to Passman *et al.* (4). After the sample is completely dissolved, 10 ml of 600 mg percent diphenyloxazole (DPO) in toluene is added, and the contents of the vial are mixed.

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° to 60°C for 1 to 2 hours.

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 percent. Insulin causes a decrease in the observed counting rate for tritium of from 14 to 57 percent 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 per minute is not linear for these preparations. As is shown in Table 1, the magnitude of this quenching effect 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.

Table 1. Correction for quenching effect of trichloroacetic acid-precipitated protein.

Protein weight (mg)	Observed count/min minus background	Specific activity observed (count/min mg)	Increment in count/min owing to addition of standard*	Specific activity corrected for quenching (count/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 count/min of tritium-labeled Δ4-cholestenone.

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

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 count/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 count/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. This effect of temperature is also observed when Hyamine is used with DPO in dioxane rather than in toluene solution.

A simple method for preparing samples of tritium or C^{14}-labeled amino acids and proteins for radioassay in the liquid scintillation counter has been described here. It will be evident that there are many possibilities for modifying the composition of the counting solution. For example, microgram quantities of ribonuclease dissolved in 0.5 ml of formamide can be counted satisfactorily after the addition of a solution of DPO in dioxane. However, the method that has been outlined would appear to be satisfactory for many purposes, and thus far none of the variations explored have been found to be of particular advantage. The ability to assay tritium directly in amino acids and proteins, coupled with the ease of labeling these compounds or their precursors with tritium by the Wilzbach technique (2) should provide a new tool for application to studies of protein synthesis and metabolism.

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12 June 1957

Preparation of Tritiated Proteins by the Wilzbach Method

Wilzbach has recently described a simple procedure for introducing tritium into organic compounds (1). The compound to be labeled is exposed to 5 to 10 c of carrier-free tritium gas for several days at room temperature. Toluene, cholesterol, and digitoxin were labeled and found, on purification, to have specific radioactivities of 22.2, 64.3, and 90 mc/g, respectively. The labeling procedure caused some degradation, but this was considerably less extensive than that observed with the triton-recoil method (2). In view of the mild conditions used, it appeared possible that the method could be applied to the labeling of proteins. Preliminary results on the tritiation of lysozyme and ribonuclease by the Wilzbach method are described here.

Crystalline lysozyme (*3*) in the amount of 237 mg was exposed at room temperature to 1.8 c of tritium gas at a pressure of 0.39 atm for 72 hours (*4*). The specific enzymatic activity of the tritium-labeled protein was 90 percent of that of the nonlabeled material. Labile tritium was removed by dissolving the protein in water (5 mg/ml), then lyophilizing. This was repeated twice. A sample of the protein (22.5 mg) was then taken up in 5 ml of *p*H 6.5, 0.2*M* phosphate buffer and chromatographed on a 0.9 by 20-cm column of IRC-50, according to the method of Goncalves *et al.* (*5*).

The effluent fractions were examined for (i) optical density at 280 mμ; (ii) total protein, by the method of Lowry (*6*), with a reference curve prepared with nonlabeled lysozyme; (iii) enzymatic activity, by the method of Tallan and Stein (*7*); (iv) tritium radioactivity, by the method described in the accompanying report by Vaughan, Steinberg, and Logan (*8*) to permit direct assay in a liquid scintillation counter (*9*).

As is shown in Fig. 1, a single major protein peak was eluted, which emerged at the point where untreated lysozyme usually appears. The curves for enzymatic activity and radioactivity coincided exactly with the curve for total protein, as is indicated by the relatively uniform values for specific enzymatic activity and specific radioactivity in successive tubes.

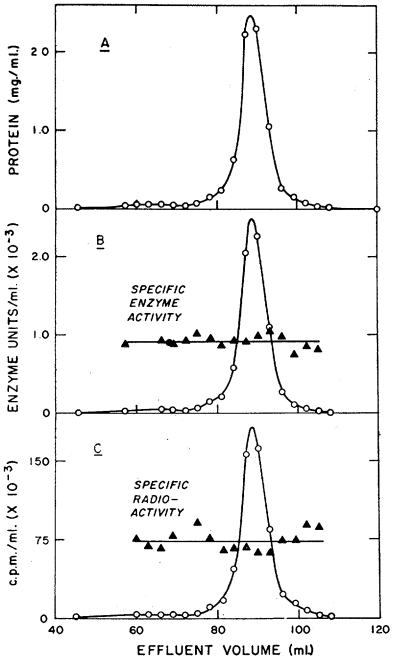

Fig. 1. Chromatography of tritiated lysozyme on IRC-50. (*A*) Total protein by method of Lowry (*6*). (*B*) Enzyme activity by method of Tallan and Stein (*7*). Solid triangles indicate specific enzyme activity in arbitrary units. (*C*) Tritium radioactivity by method of Vaughan *et al.* (*8*). Solid triangles indicate specific radioactivity in arbitrary units.

The specific enzymatic activity in the peak was 23 percent higher than that for the unfractionated labeled material. The specific radioactivity, on the other hand, was distinctly lower—only 1.87×10^5 count/min mg compared with 3.63×10^5 count/min mg before chromatography—and none of the other effluent fractions collected contained significant amounts of tritium. Only about one-half of the total nonlabile tritium was recovered in the peak, whereas the recoveries for total protein and enzymatic activity were 93 percent and 90 percent, respectively. This suggests the presence in the starting material of an enzymatically inactive component of high specific radioactivity, presumably formed by degradation during exposure to tritium, which remained tightly bound to the resin. A direct count on an aliquot of the resin confirmed the presence of tritium-containing material not eluted under the conditions used for chromatography.

In addition to the major peak, there was a very small but definite minor peak eluted just before it. The protein of this minor component was found to have the same specific enzymatic activity and specific radioactivity as that in the major peak.

Crystalline ribonuclease (*10*) in the amount of 570 mg was exposed at 25°C to 6.73 c of tritium gas for 48 hours (*11*). The specific enzymatic activity of the tritium-labeled protein, determined by the method of Anfinsen *et al.*, was 82 percent of that for the unlabeled material (*12*). Labile tritium was removed by dissolving the protein in 0.1*N* NH_4OH and then lyophilizing. The protein was taken up in 0.2*M* *p*H 6.17 phosphate buffer and chromatographed on IRC-50, according to the method of Hirs, Moore, and Stein (*13*). A degraded fraction of very high specific radioactivity but without enzyme activity emerged with the front. This was incompletely resolved from the enzymatically active peak which followed. Because of the incomplete resolution, the degree of homogeneity of the enzymatically active peak could not be evaluated. However, the peaks of radioactivity, enzyme activity, and total protein (optical density at 280 mμ) coincided, and, in the descending limb at least, the enzyme specific activity and the specific radioactivity were reasonably constant in successive tubes.

These studies demonstrate the applicability of the Wilzbach method to the preparation of tritiated proteins. Although there is some degradation during the labeling procedure, good yields of chromatographically pure, enzymatically active protein are obtained, at least in case of lysozyme and ribonuclease. Since only a very small fraction of the enzyme molecules is actually labeled, it is impossible to be certain that the tritium-containing protein molecules are themselves enzymatically active, nor can subtle changes in structure be completely ruled out. Studies to characterize these labeled proteins further are in progress.

Many applications of this method suggest themselves. The fate of intact, internally labeled proteins in biological systems can be studied without the complications introduced when iodination or other "external" labels are used. The method should be particularly valuable for the preparation, in labeled form, of proteins present in the organism in very small quantity, such as the protein hormones, and for the preparation of labeled human proteins, since in these cases it is difficult or impossible to obtain satisfactory labeling by biosynthetic methods. Complex peptides of known structure can be prepared in labeled form by specific proteolytic degradation of tritium-labeled pure proteins or by tritiation of specific fragments derived from breakdown of unlabeled proteins. These labeled peptides, difficult to prepare by synthetic methods, should be valuable in studies of protein biosynthesis.

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Cerebral Synaptic Inhibition by Serotonin and Iproniazid

In 1955 we (*1*) described the highly potent cerebral synaptic inhibitory action exercised by serotonin. On this basis, we suggested for this natural constituent of mammalian brain a highly important role as a neurohumoral synaptic inhibitor, overshadowing adrenaline and noradrenaline by virtue of its much greater potency.