THE FREE-RADICAL INTERCEPTOR TECHNIQUE AS A MEANS FOR THE PREPARATION OF TRITIATED PROTEINS

F. H. White, Jr., and P. Riesz

National Heart Institute and National Cancer Institute,
National Institutes of Health, Bethesda, Maryland

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A number of proteins have recently been tritiated by free-radical interception for the purpose of investigating the secondary (Henriksen et al., 1963) carbon free-radical distributions among their amino acids after gamma irradiation (Riesz et al., 1966; White et al., in press). This procedure consists of exposure of the lyophilized, gamma-irradiated protein to tritiated hydrogen sulfide (HST), whereupon the carbon free-radicals become labeled by abstraction of tritium from the gas. The reactions involved have been discussed elsewhere (Riesz et al., 1966). In the course of this investigation, tritiated ribonuclease (T-RNase) and tritiated lysozyme (T-lysozyme) exhibited a surprising degree of chromatographic homogeneity, with most of the radioactivity coinciding with the major protein components. Further, repeated crystallization did not appear to remove carbon-bound tritium from T-lysozyme. These results suggest that little or no structural alteration occurred during the tritiation procedure and therefore that this method may prove useful in the preparation of tritiated proteins which could be employed in the investigation of a wide variety of biological reactions.

EXPERIMENTAL

Irradiation and Exposure to Labeling Reagents. For all experiments, γ-radiolysis was carried out in a Co^{60} source to a dose of about 6 Megarads and the time of

subsequent exposure to HST was 4 hours. All other conditions of irradiation, tritiation, and exchange in water solution (to remove most of the exchangeable tritium on nitrogen and oxygen) have already been described (Riesz et al., 1966; White et al., in press).

Alternatively, H_2S^{35} was used as the labeling agent. This gas, obtained from New England Nuclear Corporation, contained 1 millicurie in 3.0 ml at N.T.P. at the time of synthesis. This amount of gas was employed for the exposure of 20 mg of protein, exactly as for labeling with HST.

Chromatography of Tritiated Proteins on XE-64. Essentially the method of Tallan and Stein (1953) was employed for the chromatography of lysozyme, with a column (0.9 × 30 cm) of XE-64 which had a flow rate of 5 ml per hour. The buffer was 0.2 M sodium phosphate, pH 7.18.

RNase was chromatographed by the procedure of Hirs et al. (1953) on a column of XE-64 of the above dimensions and flow rate, but with 0.2 M sodium phosphate of pH 6.17.

Crystallization of Lysozyme. A procedure similar to that of Fevold and Alderton (1949) was used. However, the starting concentration of protein was 30 mg/ml. For the second and fourth crystallizations, unlabeled lysozyme was added to ensure that the concentration was not less than 10 mg/ml, and the necessary corrections were made in specific activity to allow for differences in concentration. Crystals were separated from the supernatant liquid by centrifugation and redissolved in water for measurement of specific activity by scintillation counting (Bray, 1960). The concentration of the protein was determined from optical density at 280 mμ of an appropriately diluted aliquot [$E^{1\%}_{1\,cm} = 27.2$ (Ehrenpreis and Warner, 1956)].

Assays for Enzymatic Activity. Lysozyme was assayed by the procedure of Shugar (1952) and RNase as previously (White, 1961) with yeast RNA as the substrate.

RESULTS

Chromatography of T-Lysozyme. Fig. 1 demonstrates a chromatographic comparison of native lysozyme with T-lysozyme, and it can be seen that the major tritiated component elutes identically with the native protein. However, the small shoulder which precedes it does not appear for the native protein and remains to be investigated. The yield of tritiated protein was 88% and that of tritium 84%, while the yield of native lysozyme was 89%.

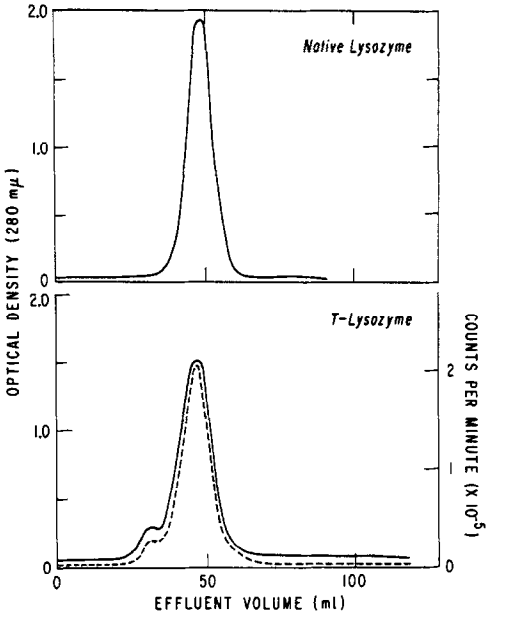

Figure 1. Chromatography of native lysozyme and T-lysozyme on XE-64. Optical density at 280 mμ, ———; tritium counts, -----.

Chromatography of Labeled RNases. Similar results were obtained with T-RNase, the tritiated components of this sample coinciding closely with peaks A and B of the native enzyme (Fig. 2). The yields of native and T-RNases (including peaks A and B) were 92% and 90%, respectively. That of tritium counts was 90%.

When H_2S^{35} was employed as the labeling reagent, the incorporated S^{35} counts amounted to a specific activity of 0.089 μCi/mg and could not be removed by the

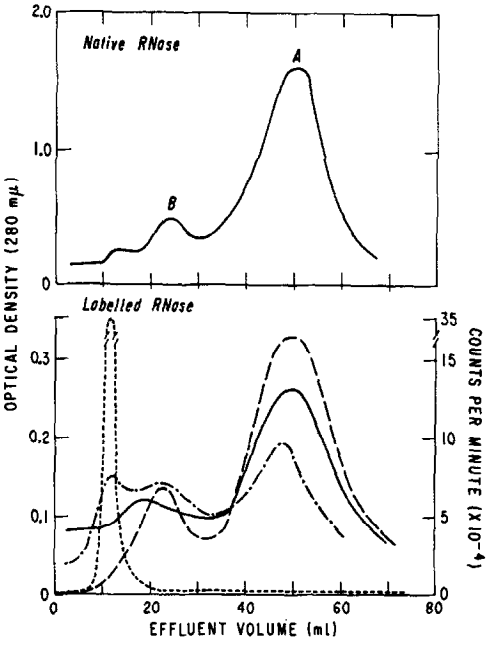

Figure 2. Chromatography of native and labeled RNases on XE-64. Optical density curves at 280 mμ for native and T-RNases, ———; optical density curve for S^{35}-RNase, -·-·-; counts for T-RNase, - - - - -; counts for S^{35}-RNase, ·····.

usual exchange procedure in water or by chromatography on Sephadex G-25. It therefore appeared that the S^{35} had become covalently bound to the protein. Chromatography of this material on XE-64 (Fig. 2) resulted in a single S^{35} peak, while the optical density curve approximated those of native and T-RNases. This experiment demonstrates that sulfur and tritium are incorporated to form separate molecular species which are easily separable by chromatography.

Crystallization Studies. As a further check on the homogeneity of T-lysozyme, it was repeatedly crystallized to determine the effects on specific activity. It is improbable that any breakdown products resulting from irradiation or subsequent treatment of the protein would escape tritiation on exposure of the irradiated sample

to HST. Further, it is unlikely that tritiated breakdown products would be incorporated into the crystal structure, and therefore crystallization of the tritiated protein should result in a lowering of its apparent specific activity if such products are present. Fig. 3 indicates a lowering of specific activity of T-lysozyme, but the activity closely approaches the value of 0.77 μCi/mg shown by the broken line. This value is the specific activity of an acid hydrolysate of T-lysozyme after drying and two lyophilizations from aqueous solution. Further lyophilizations do not further lower the specific activity, and this stability indicates removal of all exchangeable tritium, which was present on nitrogen and oxygen (White et al., in press). It therefore appears that the decrease in specific activity on repeated crystallization resulted only from removal of exchangeable tritium, while the tritium attached to carbon was on a molecular species still capable of crystallization. Therefore by this criterion no radioactive breakdown products were detectable.

Enzymatic Activities. The major chromatographic components of T-lysozyme and T-RNase exhibited enzymatic activities that were identical within experimental error to those of the corresponding components of the native enzymes. Therefore no

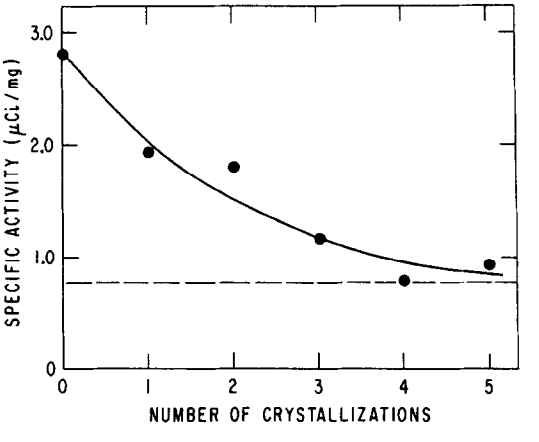

Figure 3. Effects of repeated crystallization upon the specific activity of T-lysozyme.

significant changes occurred in activity as a result of irradiation, exposure to HST, or exchange.

DISCUSSION

Tritiated proteins could be of use as substrates or precursors in a large number of biological investigations. The method of free-radical interception would have an advantage over the commonly used external labeling methods (e.g. iodination) in that the isotope is broadly distributed, with nearly every amino acid being affected (Riesz et al., 1966; White, in preparation), permitting the fates of all parts of the protein chain to be studied without introduction of labeling groups that are foreign to the protein and which might therefore have subtle effects upon the protein conformation.

The desirability of such tritiated materials has long been recognized. Steinberg et al. (1958) have, in fact, explored the possibility of labeling proteins by the method of Wilzbach (1957), which involves the use of tritium gas as the labeling agent. When lysozyme, so labeled, was chromatographed on IRC-50 (XE-64), a yield of approximately 50% of the total counts was observed, and a considerable loss of tritium was found on repeated crystallization. A similar degree of chromatographic heterogeneity was indicated for tritiated RNase. The relatively high yields of apparently homogeneous tritiated products obtained with the free-radical interceptor method suggests that HST may prove to be the reagent of choice, having the advantage that approximately one hundredth as much gaseous radioactivity is involved, with correspondingly less radiation hazard and breakdown of the protein by radiolysis. Much work remains, however, to explore the possibility of heterogeneity due to minor structural damage. This investigation is in progress.

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