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Tritiation of Tryptophyl Residues in Proteins*

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ABSTRACT: *S*-Carboxymethyl-lysozyme, myoglobin, and wool keratin were treated with tritiated trifluoroacetic acid with the aim of selectively labeling their tryptophyl residues. The distribution of the tritium labels was determined by amino acid analysis of complete enzyme hydrolysates of the treated proteins. Most of the radioactivity introduced into the first two proteins was present as tryptophyl residues and a large part of the remainder as degradation products of these residues. This degradation could be minimized by curtailing the time of labeling or by adding 2-mercaptoethanol to inhibit aerial oxidation. The former modification gave best results with *S*-carboxymethyl-lysozyme; enzyme hydrolysis of the tritiated protein afforded a 97% yield of tryptophan which bore 95% of the total radioactivity. Tritiation of wool led to the incorporation of about 15 times as much radioactivity as expected if only tryptophyl residues were labeled. This extra radioactivity could be removed only by disruption of the wool's structure, *e.g.*, by reduction and carboxymethylation; the residual activity was present mainly in the tryptophyl residues.

Tryptophyl residues are frequently cited as major sites of photodegradation during irradiation of proteins (*e.g.*, Weil *et al.*, 1953; Luse and McLaren, 1963; Leaver and Ramsay, 1969) and there have been many photochemical studies of tryptophan and tryptophyl peptides (*e.g.*, Yoshida and Kato, 1954; Weil, 1965; Benassi *et al.*, 1967; Savige, 1971). However, the fate of tryptophyl residues during irradiation of proteins is less well known owing to the complexity of these systems. Studies of this nature (*e.g.*, Lapuk *et al.*, 1968; Gomyo and Fujimaki, 1970) would be simplified if it were possible to radioactively label the tryptophyl residues initially as this would facilitate subsequent isolation of photodegradation products.

The boron trifluoride complex of tritiated acetic acid has been used previously to label tryptophyl residues in β-lipoprotein (Gosztonyi *et al.*, 1965) but model experiments with tryptophan, tyrosine, phenylalanine, and serine indicate that the labeling is not specific for tryptophyl residues. A later procedure used by Bak *et al.* (1969) appears to show more promise. These authors showed by nuclear magnetic resonance that all five hydrogen atoms on the aromatic system of the single tryptophyl residue of glucagon were labeled when the protein was dissolved in deuterated trifluoroacetic acid. Seryl and threonyl residues were concomitantly trifluoroacetylated, but these groups were subsequently hydrolyzed during dialysis against water. Bak *et al.* (1969) also labeled glucagon by treatment with tritiated trifluoroacetic acid. Radioassay of solvent distilled from the mixture showed that, for each glucagon molecule, 66 hydrogen atoms had exchanged with tritium, 5 more than the number of hydrogen atoms present at O and N sites. Not all of these extra labels, which presumably reside on the tryptophyl residue, were stable to back-exchange; only two remained after prolonged dialysis.

The methods used by Bak *et al.* (1969) provide mainly indirect evidence for the labeling of the tryptophyl residue in glucagon and might not detect partial labeling at other sites. Therefore we have labeled myoglobin, *S*-carboxymethyl-lysozyme, and wool with tritiated trifluoroacetic acid and then

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examined hydrolysates of these proteins in order to establish the location of the labels present. The first two proteins were used in preference to glucagon because their ready availability makes them more suitable for photochemical study. Wool was used because its photoyellowing is a serious practical problem.

Materials and Methods

Proteins. Sperm whale myoglobin (Koch-Light) was freed from heme by the method of Teale (1959). Egg-white lysozyme (Sigma) was reduced and carboxymethylated according to O'Donnell and Thompson (1964). The keratin was a sample of solvent-scoured merino wool top (64's quality).

Pronase AF (Kaken Chem. Co., Tokyo) was purified by fractional precipitation from aqueous acetone followed by chromatography on diethylaminoethylcellulose. The leucine aminopeptidase was a commercial sample from P-L Biochemicals (Milwaukee, Wis.). Prolidase was extracted from porcine kidneys and purified to step 2 by the method of Davis and Smith (1957); various preparations had proteolytic coefficients (C_1) in the range 12–21 (substrate, glycyl-L-proline).

Radioactive Reagents. Tritiated water (5 Ci/ml) from the Radiochemical Centre (Amersham, England) was diluted to 5 mCi/ml with deionized water for use. Tritiated trifluoroacetic acid was prepared by careful addition, with cooling, of an equimolar amount of the tritiated water to trifluoroacetic anhydride that had been purified by distillation from phosphorus pentoxide immediately before use.

Radioassay. Assays were carried out with a Packard Tri-Carb liquid scintillation spectrometer (series 314 EX). The scintillation mixture contained 1,4-bis[2-(5-oxazolyl)]benzene (0.05 g), 2,5-diphenyloxazole (4.0 g), and naphthalene (120 g) dissolved in dioxane (1000 ml). Standard solutions (A and B) of tritiated water were prepared by diluting the stock solution 4000 and 40,000 times, respectively. The specific activity of the stock solution was determined by radioassay of B in the presence of scintillation mixture (1:12). Solution A was used as an internal standard when subsequently counting protein solutions and column effluents. Protein samples (2 mg) were dissolved in 0.4 M NaOH (0.5 ml) and diluted to 5 ml with water and then aliquots (1.0 ml) were added to scintillation mixture (12 ml) for counting. It was necessary to heat the keratin samples with alkali at 100° for 1 hr to effect dissolution. Samples of column effluent, after evaporation under reduced pressure, were dissolved in 0.2 M ammonia (0.5 ml) and then counted in the presence of scintillation mixture (6.0 ml).

Tritiation of Proteins. All treatments were carried out in a dry box containing both sodium hydroxide and phosphorus pentoxide. The dry protein (100 mg) was treated with tritiated trifluoroacetic acid (2–5 ml) at 20–25° for the appropriate time (0.25, 4, or 24 hr); in some cases 2-mercaptoethanol (20–50 μl) was also present. In the case of wool keratin the trifluoroacetic acid was removed by repeated washing with water for 24 hr. The other reaction mixtures were diluted with four volumes of water and lyophilized. The treated myoglobin was shaken with water for several hours until dissolved and then dialyzed against water. However it was necessary to dissolve the treated *S*-carboxymethyl-lysozyme in 4 M urea before dialysis against water. The resultant protein solutions were stored at 0° prior to enzyme hydrolysis.

Enzyme Hydrolysis. Wool keratin was reduced and carboxymethylated (O'Donnell and Thompson, 1964) before enzyme digestion; myoglobin and *S*-carboxymethyl-lysozyme required no pretreatment. The proteins (50–200 mg) were digested at pH 7–8 and 40° with Pronase (2% on the weight of protein) for 24 hr and then under the same conditions with a mixture of leucine aminopeptidase (2%) and prolidase (2%) according to Milligan *et al.* (1971).

Derivation of Tryptophyl Peptides from S-Carboxymethyl-lysozyme. A sample of *S*-carboxymethyl-lysozyme that had been treated with tritiated trifluoroacetic acid for 15 min was used. (1) A suspension of the tritiated protein (50 mg) in 0.1 M HCl (40 ml) was treated with cyanogen bromide (23.5 mg) for 24 hr at 20°. The product, after lyophilization, was dissolved in 1 M NH_4OH (5 ml) and subjected to gel filtration on Sephadex G-50 using 0.05 M ammonium carbonate as eluent. The first two main components to be eluted contained tryptophan, whereas the third did not. (2) An aqueous solution of the tritiated protein (50 mg) at pH 8 was incubated at 38° with trypsin (2 mg) for 2 hr and then lyophilized. The residue was subjected to paper electrophoresis at pH 1.9 for 75 min using a voltage gradient of 45 V/cm. The positions of the tryptophyl peptides were determined on narrow strips using Ehrlich's reagent and the appropriate bands were then excised and the peptides eluted with 0.1 M NH_4OH. Sequence assignments were made on the basis of amino acid content after acid hydrolysis.

Tryptophan contents of the various peptides were determined by the colorimetric procedure described by Opienska-Blauth *et al.* (1963) or by direct spectrophotometry at 287 nm.

Analysis of Enzyme Hydrolysates. The complete enzyme hydrolysates were chromatographed at 50° on a column (45 × 2 cm diameter) containing Technicon Chromobeads (type C) using pyridine–acetic acid buffers (pH 3.26, 4.26, and 5.28, successively). Fractions (250; 5 ml) were collected, buffer changes being made after 600 and 900 ml had been collected. Alternate fractions were radioassayed and in some runs all fractions were monitored for amino acids with ninhydrin.

The hydrolysates were also analyzed using a Beckman-Spinco amino acid analyzer (Model 120B). A lithium citrate buffer system, which permits resolution of asparagine and glutamine from the other amino acids (Holt *et al.*, 1971), was used in some cases. The basic amino acids were analyzed on a longer column than usual to effect better resolution of tryptophan.

Results and Discussion

In order to establish the location of the tritium labels introduced by treatment with tritiated trifluoroacetic acid, it was necessary to hydrolyze the treated proteins to amino acids under conditions favoring minimal destruction and tritium exchange. Obviously acid hydrolysis is unsuitable because it causes extensive decomposition of tryptophan, the expected site of labeling. Therefore we have used the three enzymes, Pronase, leucine aminopeptidase, and prolidase, to effect hydrolysis (Milligan *et al.*, 1971), a procedure related to that of Hill and Schmidt (1962).

Amino acid analyses of both acid and enzyme hydrolysates of the *S*-carboxymethyl-lysozyme and myoglobin used in this work are shown in Tables I and II, respectively. Generally, there is good agreement between the two sets of analyses although low yields of glycine, arginine, asparagine, and glutamine were obtained by enzyme hydrolysis of *S*-carboxymethyl-lysozyme. However, the hydrophobic amino acids, including tryptophan, were obtained in good yield from both proteins. These amino acids were also obtained in good yield from *S*-carboxymethyl-kerateine (Holt *et al.*, 1971).

S-Carboxymethyl-lysozyme was initially labeled using the

TABLE I: Amino Acid Analyses[a] of *S*-Carboxymethyl-lysozyme.

Amino Acid	Untreated			Tritiated[c]
	Theory[b]	Acid Hydrolysis	Enzyme Hydrolysis	Enzyme Hydrolysis
Lys	6	5.8	5.2	5.1
His	1	1.0	1.0	1.1
Arg	11	10.5	8.8	8.1
Trp	6	0	5.9	3.0
S-CMC	8	6.5	6.3	6.6
Asp	8	21.3	9.1	10.7
Asn	13		3.9	*d*
Glu	2	5.3	2.8	3.0
Gln	3		1.5	*d*
Thr	7	6.7	6.1	*d*
Ser	10	9.8	8.9	*d*
Pro	2	2.1	1.1	2.1
Gly	12	12.6	8.2	7.3
Ala	12	12.3	11.3	10.4
Val	6	5.3	6.0	5.2
Met	2	1.0	0.7	0.4
Ile	6	5.1	5.9	5.7
Leu	8	8.0	8.0	8.0
Tyr	3	2.5	3.0	3.0
Phe	3	3.2	3.0	3.0

[a] Residues/mole; results are normalized to leucine = 8.0 residues/mole. [b] Canfield (1963). [c] Tritiated trifluoroacetic acid, 4 hr, 20–25°. [d] Asparagine, glutamine, serine, and threonine were not resolved in this analysis.

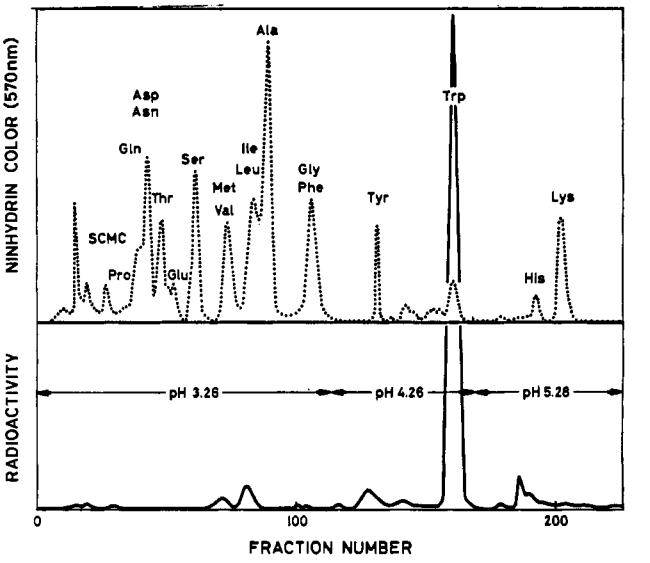

FIGURE 1: Ion-exchange chromatogram of an enzymic hydrolysate of *S*-carboxymethyl-lysozyme that had been treated for 4 hr with tritiated trifluoroacetic acid. The fractions were monitored for radioactivity (unbroken line) and optical density at 570 nm after treatment with ninhydrin (dotted line).

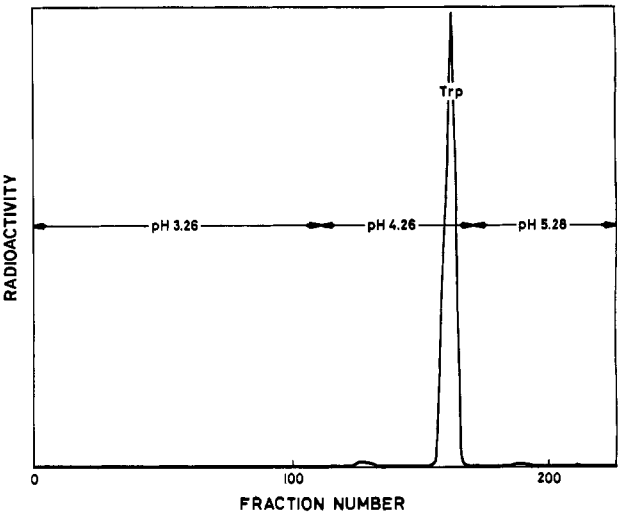

FIGURE 2: Ion-exchange chromatogram of an enzyme hydrolysate of *S*-carboxymethyl-lysozyme that had been treated for 15 min with tritiated trifluoroacetic acid.

conditions recommended by Bak *et al.* (1969), *viz.*, by treatment with tritiated trifluoroacetic acid at room temperature for 4 hr. The distribution of radioactivity in the enzyme hydrolysates of the treated protein was determined after separation of the amino acids by ion-exchange chromatography. The tryptophan peak contained most of the radioactivity (see Figure 1) but numerous smaller peaks of radioactivity were also present. Surprisingly, none of these (with the possible exception of tyrosine) coincided with the positions of other amino acids normally present in protein hydrolysates. These extra peaks could be due to peptides of tryptophan arising from incomplete hydrolysis of the tritiated protein but this seems unlikely in view of the excellent yields of tryptophan obtained by enzyme hydrolysis of the untreated protein. A more plausible explanation is that the extra peaks of radioactivity arise as the result of degradation of tryptophyl residues during tritiation. Certainly much lower yields of tryptophan are obtained from tritiated than from untreated *S*-carboxymethyl-lysozyme, whereas the yields of other amino acids are unchanged within experimental error (see Table I).[1]

Other workers have observed the decomposition of tryptophan-containing proteins in trifluoroacetic acid. Uphaus *et al.* (1959) found that solutions of ovalbumin, lysozyme, and glycyltryptophan in trifluoroacetic acid developed a green fluorescence which they attributed to cyclization products of the tryptophyl residues. The facile oxidation of tryptophan and its derivatives in trifluoroacetic acid has also been reported (Marshall, 1967).

In the hope of minimizing tryptophan degradation, the time of treatment with tritiated trifluoroacetic acid was reduced from 4 hr to 15 min. This resulted in a much better recovery of tryptophan (97%) and a marked improvement in the specificity of labeling, 95% of the activity being present within the tryptophyl residues (see Figure 2).

An alternative method for minimizing tryptophan degradation during tritiation was also examined. Marshall (1967) has shown that the degradation of tryptophan and its derivatives in strong acids can be minimized by adding 2-mercaptoethanol to inhibit aerial oxidation. The tritiation of *S*-carboxymethyl-lysozyme (4-hr treatment) was repeated, therefore, in the

[1] Hydrophobic amino acids, such as tryptophan, tyrosine, phenylalanine, and leucine, can be determined with a much higher degree of reproducibility than can hydrophilic amino acids, which are released less readily by enzyme hydrolysis of proteins (Holt *et al.*, 1971).

TABLE II: Amino Acid Analyses[a] of Myoglobin.

Amino Acid	Untreated			Tritiated[c]	Tritiated[d]
	Theory[b]	Acid Hydrolysis	Enzyme Hydrolysis	Enzyme Hydrolysis	
Lys	19	19.4	19.2	17.2	18.6
His	12	11.8	12.0	10.8	10.8
Arg	4	3.6	4.1	3.1	3.6
Trp	2	0	1.85	1.1	1.65
Asp	6	8.5	4.9	4.1	4.4
Asn	2		*e*	*e*	*e*
Glu	14	20.2	12.8	10.5	12.9
Gln	5		*e*	*e*	*e*
Thr	5	5.1	*e*	*e*	*e*
Ser	6	6.0	*e*	*e*	*e*
Pro	4	4.4	4.1	4.5	3.8
Gly	11	11.7	10.7	10.7	10.3
Ala	17	17.9	16.1	16.6	16.6
Val	8	7.4	7.6	8.4	8.0
Met	2	1.4	2.1	1.6	1.7
Ile	9	7.5	8.9	9.4	8.8
Leu	18	18.0	18.0	18.0	18.0
Tyr	3	2.7	3.2	3.5	3.3
Phe	6	6.1	6.0	6.1	6.0

[a] Residues/mole; results are normalized to leucine = 18 residues/mole. [b] Edmundson (1965). [c] Tritiated trifluoroacetic acid, 4 hr, 20–25°. [d] Tritiated trifluoroacetic acid containing 2-mercaptoethanol (1%), 4 hr, 20–25°. [e] Asparagine, glutamine, serine, and threonine were not resolved in these analyses.

TABLE III: Specificity of Labeling of Tryptophyl Residues in Proteins by Treatment with Tritiated Trifluoroacetic Acid under Different Conditions.

Protein	Treatment Time (hr)	Radioactivity Present as Trp[b]	Hydrogen Atoms Labeled/Trp Molecule	Yield of Trp[c]
S-Carboxymethyl-lysozyme	0.25	95	1.22	97
	4	70	2.15	52
	4[a]	85	3.11	69
Myoglobin	0.25	71	1.35	
	4	65	2.62	60
	4[a]	81	3.10	90
Wool	0.25	70	0.95	97
	4	80	2.10	75
	4[a]	80	1.69	92
	24	58	2.30	46

[a] The treatment was carried out in the presence of 2-mercaptoethanol (1%, v/v). [b] As a percentage of the total nonvolatile activity eluted from the ion-exchange column. [c] As a percentage of the amount present in an enzyme digest of the original protein.

presence of a little 2-mercaptoethanol. This additive increased both the percentage of radioactivity present as tryptophan and the yield of tryptophan released by enzyme hydrolysis (see Table III). However, the percentage of radioactivity present as tryptophan, and the yield of tryptophan released by hydrolysis, were still lower than the corresponding values for *S*-carboxymethyl-lysozyme that had been tritiated for only 15 min. The latter would seem to be the procedure of choice.

Radioassay of the tryptophan isolated from *S*-carboxymethyl-lysozyme showed that the number of hydrogen atoms labeled increased with the time of tritiation (see Table III). This could be due to the failure of some tryptophyl residues, because of limited accessibility, to exchange during the relatively short reaction time. Alternatively, all of the tryptophyl residues may have been tritiated, but only to a limited extent due to the short time allowed for exchange. In order to differentiate between these alternatives *S*-carboxymethyl-lysozyme, tritiated for 15 min, was split into a number of tryptophyl peptides and the ratio of radioactivity to tryptophan content was determined for each peptide. The tryptophyl peptides were obtained by cleaving the protein at methionyl residues with cyanogen bromide and also by tryptic digestion. Table IV shows that the number of hydrogen atoms labeled per tryptophyl residue was almost the same for each of the derived tryptophyl peptides and for the original tritiated protein. Thus all of the tryptophyl residues in *S*-carboxymethyl-lysozyme appear to be labeled uniformly within 15 min, the overall level of tritiation increasing with increasing time of treatment.

Although all five hydrogen atoms on the aromatic system of tryptophan exchange in tritiated trifluoroacetic acid (Bak *et al.*, 1967), it seems that only two or three of the labeled sites are stable to back-exchange in aqueous solutions. Tritiation of tryptophan for 4 hr, followed by addition of water and lyophilization, led to the stable labeling of only 2.4 hydrogen atoms per molecule, close to the figure (2.2) found for the tryptophyl residues of *S*-carboxymethyl-lysozyme. This suggests that all of the tryptophyl residues in the protein have been fully tritiated in 4 hr. The results of Bak *et al.* (1969) on the tritiation of glucagon support this view. However, partial tritiation of tryptophyl residues in *S*-carboxymethyl-lysozyme is probably satisfactory for most purposes, provided that all of the tryptophyl residues are equally labeled. Treatment with tritiated trifluoroacetic acid for 15 min at room temperature is therefore the best procedure, because it leads to very selective labeling of tryptophyl residues with minimal degradation.

Myoglobin was also treated with tritiated trifluoroacetic acid under the same three sets of conditions used for *S*-carboxymethyl-lysozyme. Tritiation for 4 hr (no mercaptoethanol present) again led to nonspecific labeling, the distribution of radioactivity after ion-exchange chromatography of an enzyme hydrolysate being very similar to that in the case of *S*-carboxymethyl-lysozyme. The percentage of the total radioactivity present as tryptophan, the number of hydrogen atoms labeled per molecule of tryptophan, and the yield of tryptophan after hydrolysis are shown in Table III. Surprisingly, tritiation for a shorter time (15 min) did not substantially improve the specificity of labeling (see Table III). In this case, tritiation in the presence of mercaptoethanol for 4 hr gave a much better result; enzyme hydrolysis gave a 90% yield of tryptophan which bore 81% of the total radioactivity. A chromato-

TABLE IV: Extents of Tritiation of Tryptophyl Residues in Peptides Derived from Tritiated *S*-Carboxymethyl-lysozyme.[a]

Reagent Used for Cleavage	Peptide	Theor Trp Content (Residues/Mole)	Hydrogen Atoms Labeled/Trp Molecule
CNBr	Lys_{13}-Met_{105}	3	0.76
CNBr	Asn_{106}-Leu_{129}	3	0.86
Trypsin	Gly_{117}-Arg_{125}	1	0.79
Trypsin	Trp_{62}-Arg_{68}	2	0.90
	Ile_{98}-Arg_{112}	2	
Trypsin	Gly_{22}-Lys_{33}	1	0.80

[a] Prepared by treatment with tritiated trifluoroacetic acid for 15 min. This preparation had 0.88 hydrogen atom labeled per tryptophyl residue.

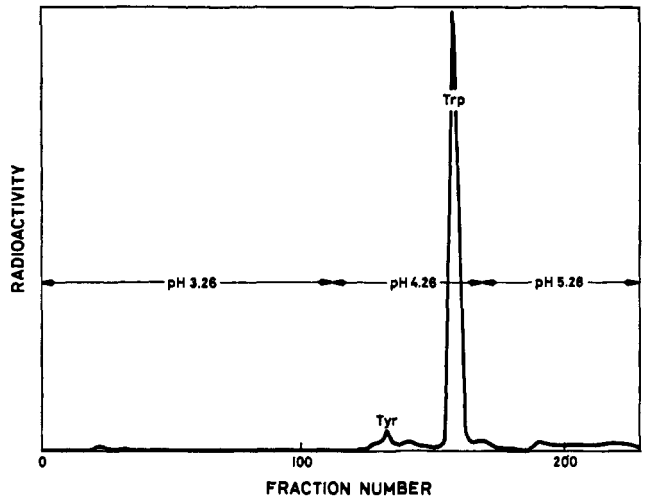

FIGURE 3: Ion-exchange chromatogram of an enzyme hydrolysate of myoglobin that had been treated for 4 hr with tritiated trifluoroacetic acid containing 2-mercaptoethanol (1%).

gram of the enzyme hydrolysate is shown in Figure 3. In this case a small amount of tyrosine has also been labeled; the other small radioactive peaks do not correspond to any of the usual amino acids. The amino acid analyses of enzyme hydrolysates of myoglobin that had been tritiated in the absence and presence of mercaptoethanol are shown in Table II. Although there are only small changes in the amino acid contents, we believe the changes in tryptophan content after tritiation to be significant (see footnote 1).

Unlike the above two proteins, wool is insoluble in tritiated trifluoroacetic acid. Nevertheless a large amount of radioactivity was incorporated during the treatment (4 hr), some 15 times that expected if only tryptophyl residues were tritiated. This extra radioactivity was not removed by treating the wool with boiling water, with dimethyl sulfoxide, with hot 0.1 M sodium carbonate, or with 8 M urea solution but was partially extracted by prolonged treatment with anhydrous formic acid. Only by disruption of the wool structure, *e.g.*, by reduction and carboxymethylation, or by dissolution in boiling 0.4 M sodium hydroxide could the level of nonvolatile radioactivity be reduced to the expected value. Presumably the extra tritium is bound at exchangeable sites which only become accessible to water after extensive disruption of the wool protein.

Ion-exchange chromatography of an enzyme hydrolysate of tritiated wool that had been reduced and carboxymethylated showed the presence of small amounts of radioactive by-products in addition to the major peak of radioactivity corresponding to tryptophan. The distribution of radioactivity was only a little less complex than that shown in Figure 1. Presumably some of these radioactive by-products arose by decomposition of tryptophan during tritiation, this amino acid being obtained in only 75% yield (see Table III). Prolonged tritiation (24 hr) led to further degradation of tryptophyl residues. In addition, tyrosyl residues were partially tritiated, 12% of the total nonvolatile radioactivity in the enzyme hydrolysate being present as tyrosine compared with 58% as tryptophan. However this is not a practical procedure for labeling tyrosyl residues in wool, only 0.04 tritium label/tyrosine being incorporated.

The degradation of tryptophyl residues during tritiation was markedly decreased by reducing the time of treatment to 15 min but, surprisingly, there was no improvement in the specificity of labeling (see Table III). Best results were obtained by tritiating wool for 4 hr in the presence of 2-mercaptoethanol. The presence of this antioxidant virtually prevented degradation of tryptophyl residues although it failed to improve the percentage of total radioactivity present as tryptophan (80%). Nevertheless, the selectivity of labeling is quite high when the low tryptophan content of wool (0.7%) is taken into account. Part of the remaining activity (*ca.* 5%) was present as tyrosine and the rest as a number of small unidentified peaks on the chromatogram. It must be borne in mind that this distribution of radioactivity only prevails in the derived *S*-carboxymethyl-kerateine. The original wool contains a considerable amount of extra tritium and therefore is probably unsuitable for studying the fate of tryptophyl residues during irradiation.

Although this paper describes modified tritiation procedures which lead to more selective labeling of tryptophyl residues than the procedure of Bak *et al.* (1969), there is still scope for further improvement in the selectivity of labeling. Conditions which give excellent results with *S*-carboxymethyl-lysozyme do not necessarily do so with myoglobin or wool, and it seems that each protein must be treated as an individual case in developing optimal conditions for tritiating its tryptophyl residues.

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Electrostatic Methods for Measuring the Binding of Ionic Ligands to Proteins*

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ABSTRACT: The ΔpH method of Scatchard and Black has been used successfully to measure the binding of small ions to proteins. However, the method has been shown to fail in measuring quantitatively the binding of large asymmetric organic ions to bovine serum albumin. The value of w, the electrostatic interaction parameter, for long-chain ligands, differs from its value for smaller or more symmetrical inorganic ions, and is subject to large uncertainties. We now present two methods for determining the interactions of ionic ligands with proteins based on the electrostatic effect of the bound ligand on the hydrogen ion equilibria of the protein. Unlike the ΔpH method, neither depends upon a known value for w nor upon a known relationship between the equivalents of ligand bound and the measured prototropic response. Dependence upon an uncertain theoretical relationship, or determination of an empirical stoichiometric relationship between the measured response and the equivalents of ligand bound, is avoided by obtaining data at two or more protein concentrations. One of the methods uses ΔpH as the measured response to the association of ligand with protein. The response measured in the other method is the equivalents of acid or base which must be added to maintain a constant pH as binding occurs. Values of molal ratios bound and free-ligand concentrations result. The results obtained with both methods agree with those obtained with the familiar standard thermodynamic methods.

Hydrogen ion equilibria of the ionizable groups of proteins are influenced by the net charge of the protein. A theoretical relationship (Tanford, 1961) describing this is

$$\mathrm{pH} = \mathrm{p}K_a - \log (h/n - h) - 0.87wZ \qquad (1)$$

where h is the number of protonated acid groups out of a total of n with dissociation constant K_a; Z is the net charge of the protein; and w is an electrostatic interaction factor which according to the Debye–Huckel theory is given by

$$w = (e^2/2DkT)\left[\frac{1}{b} - \kappa/(1 + \kappa a)\right]$$

in dilute solutions, where e is the protonic charge, D is the solvent dielectric constant, k is Boltzmann's constant, T is the absolute temperature, a and b are the distance of closest approach and the radius of the central ion, respectively, and κ has its usual meaning in the Debye theory. The validity of eq 1 and its implications have been repeatedly established from its successful use in analyzing the titration curves of many proteins (Steinhardt and Reynolds, 1969; Steinhardt and Beychok, 1964).

A modification of the equation has also been used to measure the binding of inorganic ions to proteins (Scatchard and Black, 1949). Upon binding charged ligand the net charge of the protein changes by ΔZ, where ΔZ is the charge of the ligand. No change occurs in $\mathrm{p}K_a$ and, if the protein is not too dilute, only a negligible change occurs in $h/n - h$ except at strongly acidic or basic pH. Thus a pH change will be observed which is related to the binding, *i.e.*

$$\Delta Z = \bar{\nu} = \Delta\mathrm{pH}/0.87w \qquad (2)$$

where $\bar{\nu}$ is the mole ratio of bound ligand.

The reliability of the ΔpH method for measuring the binding of organic ions to proteins has recently been examined (Cassel and Steinhardt, 1966). It appeared that eq 2 was not valid for the binding of large asymmetrical organic ions since results obtained with the ΔpH method did not agree with results obtained by more definitive techniques such as equilibrium dialysis and measurements of potentials across permselective membranes. Moreover, it was obvious that for ions of high affinity at the protein concentration employed (0.1% bovine

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