Structural Changes of Carboxypeptidase A on Ultraviolet Irradiation*

Romano Piras† and Bert L. Vallee

ABSTRACT: Ultraviolet irradiation (2537 A) alters the structure of carboxypeptidase A. Optical rotatory dispersion reveals a decrease in the magnitude of the Cotton effect at 233 mμ and a change of b_0 from −216 to −132. On ultracentrifugation, formation of rapidly sedimenting polydispersed material is apparent. Gel filtration on Sephadex G-75 demonstrates the existence of three species, two of which are denatured and have a high R_F value. One species emerges with the R_F value of native carboxypeptidase. The esterase activity of this material, however, is increased markedly, accounting for the increased esterase activity of the unseparated mixture.

Amino acid analysis of this fraction shows the destruction of one tyrosyl residue. Irradiation of lysozyme and α-chymotrypsin demonstrates that such structural changes are not restricted to carboxypeptidase. These results suggest caution in correlating alterations of enzymatic activities on irradiation with the destruction of specific amino acid residues, without concomitant examination of the physicochemical properties of the system.

Amino acid residues involved in the mechanism of enzyme action have generally been identified by correlating chemical modifications or destruction of a specific amino acid residue with changes in enzymatic activity (Balls and Jensen, 1952; Koshland *et al.*, 1962; Vallee, 1964). Irradiation with ultraviolet light has been utilized extensively for such purposes (Ferrini and Zito, 1963; McLaren and Shugar, 1964). However, *losses* in activity can be brought about *both* through site-specific modification and by nonspecific structural alterations (Fraenkel-Conrat, 1957; Bethune *et al.*, 1964). The latter must be ruled out before changes in activity can be attributed to the former. Thus, physicochemical characterization is an essential step in differentiating specific from nonspecific changes.

These comments are especially pertinent to studies on the results of ultraviolet irradiation, since an adverse effect on enzymatic systems has been attributed both to its action on specific amino acids and on the structural integrity of the enzyme (Augenstine, 1962; McLaren and Shugar, 1964). The changes in enzymatic activities of carboxypeptidase upon ultraviolet irradiation (Piras and Vallee, 1966) could not be attributed unequivocally to the modification or destruction of one or several specific residues. It seemed imperative, therefore, to study the physicochemical properties of the enzyme to define the basis of the enzymatic observation further.

The present data demonstrate that ultraviolet irradiation exerts its effects on the function of carboxypeptidase both by destruction of specific amino acid residues in the primary sequence and through profound alterations of structure as evident from the physicochemical characteristics of the irradiated protein. Enzymatic changes due to alterations in primary structure are distinctive, however, and can be differentiated from the concurrent structural changes. Similar effects of ultraviolet irradiation on the structure of other proteins, *i.e.*, lysozyme and chymotrypsin, have also been demonstrated. These results have appeared as a preliminary report (Piras and Vallee, 1965).

Experimental Section

Materials. Twice recrystallized bovine pancreatic carboxypeptidase A, prepared by the method of Anson (1937), was obtained from Worthington Biochemical Corp., Freehold, N. J. The enzyme suspension was centrifuged, and the crystals were washed three times with water before dissolving in the appropriate buffer. The zinc to protein ratio was 0.97–1.03 g-atoms/mole, based on a molar absorptivity $\epsilon_{278} = 6.42 \times 10^4\,M^{-1}\,cm^{-1}$ (Simpson *et al.*, 1963). Lysozyme and α-chymotrypsin (2× and 3× recrystallized, respectively, Worthington Biochemical Corp.), *N*-acetylimidazole (K and K Laboratories), and phenylmethanesulfonyl fluoride[1] (Cyclo Chemical Corp.) were used without further purification.

Methods. Ultraviolet irradiation and measurement of enzymatic activities, zinc, protein concentration, and amino acid composition were performed as previously

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[1] Abbreviation used int his work: PMSF, phenylmethanesulfonyl fluoride.

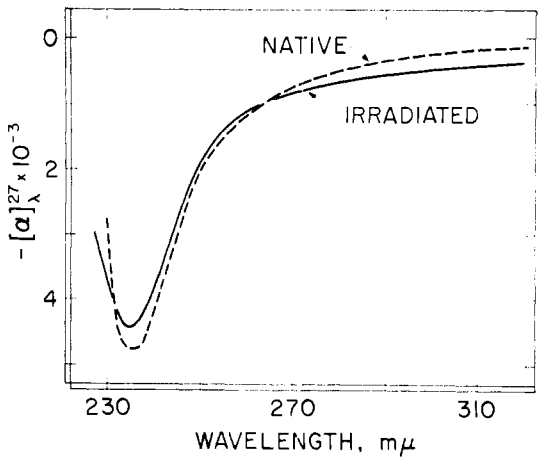

FIGURE 1: Optical rotatory dispersion of native (---) and irradiated (—) carboxypeptidase. Irradiation (2537 A) was carried out up to a dose of 3.2×10^7 ergs/cm². A Cary Model 60 recording spectropolarimeter with a 2-mm cell, and 1 M NaCl–0.01 M Tris–HCl, pH 7.5, was employed.

described (Piras and Vallee, 1966). Optical rotatory dispersion was measured with a Cary Model 60 recording spectropolarimeter between 220 and 320 mμ at 27° in a 2-mm cell. The slit width of the instrument was programmed to yield maximal and constant light intensities at all wavelengths. Specific rotations were based on protein concentration, and the instrument was calibrated, at all wavelengths, to give zero rotation for the buffer blank. The values are precise to ±50°.

Sedimentation analyses were performed in a Spinco Model E ultracentrifuge, at 50,700 rpm and 23°, at a protein concentration of 4.7 mg/ml.

Gel filtration was carried out with a 1.0 × 100 cm column of Sephadex G-75 (Pharmacia, Uppsala) equilibrated with 1 M NaCl–0.01 M Tris–HCl buffer, pH 7.5, at a flow rate of 0.3 ml/min. A 2-ml sample was applied, and 2-ml fractions were collected automatically and used for protein, zinc, and activity determinations. A 4.4 × 45 cm column was used for preparative purposes with similar results.

Results

Effect on Optical Rotatory Dispersion. The optical rotatory dispersion of native carboxypeptidase and that irradiated with 3.2×10^7 ergs/cm², retaining 55% of the original peptidase activity, differ significantly (Figure 1). On irradiation, the amplitude of the intrinsic Cotton effect at 233 mμ is diminished, and the dispersion at wavelengths greater than 262 mμ becomes more levorotatory. Such alterations are consistent with denaturation (Urnes and Doty, 1961; Ulmer and Vallee, 1965). However, optical rotatory dispersion measurements *per se* do not yield information to indicate whether all of the molecules or only a fraction of them has undergone changes. This question can best be answered by means of a transport method, such as sedimentation.

Effect on Sedimentation. Native carboxypeptidase sediments as a single symmetrical boundary (Figure 2A) with an $s_{20,w}$ of 3.3 S, consistent with values previously observed under similar conditions (Bethune, 1965). Irradiation of the enzyme, however, profoundly alters the sedimentation pattern (Figure 2B). After 6 and 14 min of centrifugation, material with a high S value appears and is visible as an inflection on the main peak. Moreover, the area under the boundary of the pattern obtained after 73 min is greatly reduced, although the total protein concentration is identical in both experiments. Only about one-third of the material present sediments at the same rate as the native enzyme.

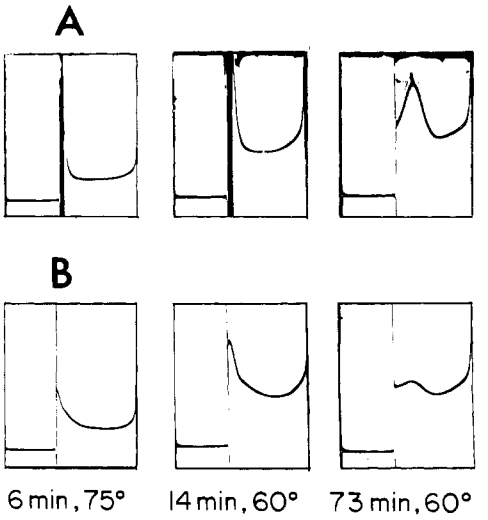

FIGURE 2: Effect of irradiation (2537 A) on sedimentation of carboxypeptidase, 4.7 mg/ml, in 1 M NaCl–0.01 M Tris–HCl, pH 7.5. Sedimentation patterns of native (A) and irradiated (1.3×10^8 ergs/cm²) enzyme (B) were photographed at the times and angles indicated. Sedimentation is from left to right at 50,700 rpm.

Effect on Gel Filtration. In view of the results of sedimentation analysis, gel filtration seemed a suitable method for the separation and identification of the species produced by irradiation. Native carboxypeptidase emerges from Sephadex G-75 as a single, symmetrical peak (Figure 3A), but after irradiation additional fractions of apparently higher molecular weight appear. On exposure to increasing doses of irradiation, these fractions progressively accumulate, as is apparent from the relative areas under the peaks (Figures 3B and C). Moreover, the elution profile indicates the heavier material is heterogeneous; at least two fractions are seen, one emerging at the void volume of the column. Peptides could not be detected

in effluent fractions expected to contain material of low molecular weight.[2]

Enzymatic activities were measured in the peak fraction of all three species. In Figure 3, Fractions I and II correspond to the two peaks of high R_F values and Fraction III corresponds to that of the native enzyme. From the onset of irradiation, both esterase and peptidase activities of Fraction I decrease, while esterase activity of Fraction III increases to 180% of the control value, and peptidase decreases to 44% (Table I). The activities in Fraction II are intermediate between those of Fractions I and III. Though Fraction III emerges from the column in the same position as the native enzyme, its zinc content is only 0.62 g-atom/mole. Correcting for the loss or zinc, the esterase in Fraction III is three times that of the native enzyme.

Larger quantities of the heterogeneous mixture resulting from irradiation were separated on a 4.4 × 45 cm column of Sephadex G-75, and Fraction III was analyzed for zinc, enzymatic activities, and amino acid composition. The increase in esterase activity of carboxypeptidase on irradiation is clearly due to the material in this fraction (Table II). Amino acid analysis of Fraction III demonstrates the loss of only one of the nineteen tyrosyl residues, but tryptophan and histidine are unaltered. As would be expected, the absorption spectrum of Fraction III is virtually unchanged in the ultraviolet region, since one tyrosyl residue contributes only a small fraction to the total absorbance.

TABLE I: Effect of Ultraviolet Irradiation on Esterase (k) Peptidase (C) Activities of Carboxypeptidase Measured in Fractions I, II, and III.[a]

Dose (10^7 ergs/cm^2)	Fraction I		Fraction II		Fraction III	
	$k \times 10^{-3}$	C	$k \times 10^{-3}$	C	$k \times 10^{-3}$	C
0	...	...	...	...	7.1	36
0.8	7.0	8	8.0	13.5	8.9	34
2.4	2.8	3.5	5.9	9	10.5	24
4.8	1.5	1.5	5.2	7.5	12.5	20
7.2	1.5	...	4.0	...	...	..
9.6	0.9	0.8	4.0	...	12.7	14

[a] Samples of 2 ml of irradiated carboxypeptidase, 4×10^{-5} M, were separated as in Figure 3, and tubes corresponding to elution volumes of 32, 38, and 46–48 ml were used for enzymatic assays of fraction I, II, and III, respectively. Activities are calculated on the basis of protein concentration. For definitions of fractions, see text.

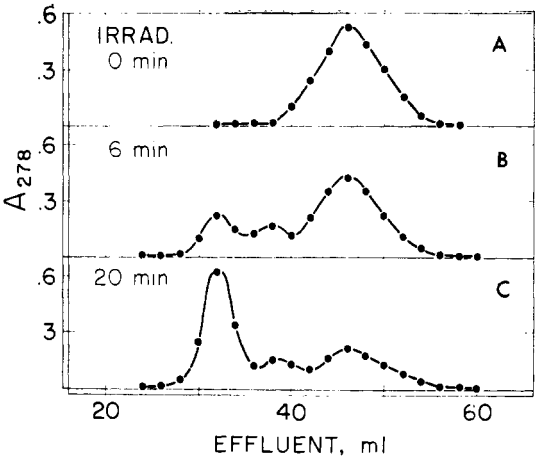

FIGURE 3: Gel filtration of native (A) and irradiated (2537 A) carboxypeptidase (B, C). Two milliliter samples of carboxypeptidase, 4×10^{-5} M, irradiated for the times shown, were each passed over a Sephadex G-75 column (1 × 100 cm) at a flow rate of 0.3 ml/min. Absorbance at 278 mμ was determined in successive 2-ml fractions.

TABLE II: Properties of Fraction III Separated on Sephadex G-75 from Irradiated Carboxypeptidase.[a]

	Dose of Irradiation	
	0	10^8 ergs/cm^2
Zn Content	0.97 g-atom/mole	0.64 g-atom/mole
Esterase,[b] k	7.2×10^3	22×10^3
Peptidase,[b] C	38	30
Amino acid analysis[c]		
Tryptophan	6.9 residues/mole	7.0 residues/mole
Histidine	7.6 residues/mole	7.5 residues/mole
Tyrosine	18.8 residues/mole	18.0 residues/mole

[a] Carboxypeptidase (16 ml), 4×10^{-5} M, was applied to a 4.4 × 45-cm column and separated with 1 M NaCl–0.01 M Tris–HCl buffer, pH 7.5. Tubes corresponding to fraction III (see text) were pooled. [b] Based on the amount of active enzyme present, as determined by zinc content. [c] No significant change in the composition of other amino acids was detected.

[2] The detergent Brij-35 has been employed to prevent precipitation when carboxypeptidase is irradiated at room temperature (Fujioka and Imahori, 1963). Even though this problem does not arise when irradiation is carried out at 4°, some experiments were performed with Brij-35 at this temperature to examine whether or not it might prevent all or part of the structural changes. The presence of the detergent had no detectable effect on any of the physical changes; hence, it was omitted in all experiments reported here.

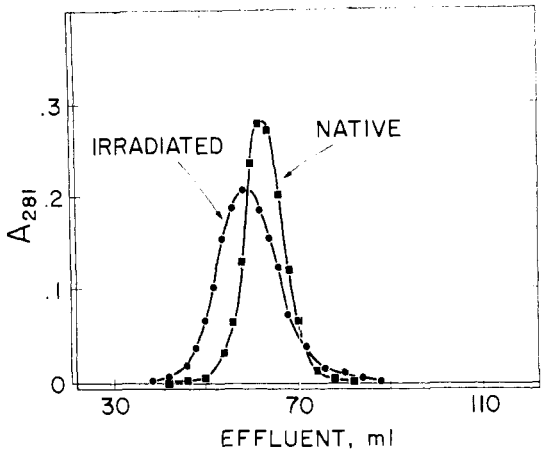

FIGURE 4: Gel filtration of native (—■—) and irradiated (2537 A) (—●—) lysozyme. Two-milliliter samples of native and irradiated (1.6×10^7 ergs/cm^2) lysozyme, 4×10^{-5} M, in 0.04 M phosphate buffer, pH 7.1, were each separated under conditions similar to those in Figure 3.

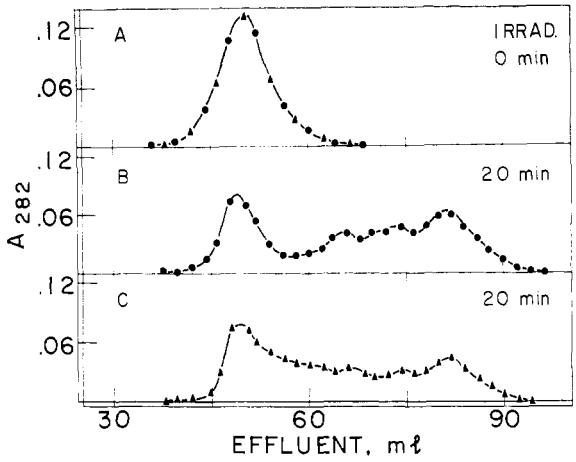

FIGURE 5: Gel filtration of native (—●—) and PMSF inactivated (—▲—) chymotrypsin, A; irradiated (3.2×10^7 ergs/cm^2) chymotrypsin (—●—), B; and PMSF-inactivated and irradiated chymotrypsin (—▲—), C. Two-milliliter samples of enzyme, 2.3×10^{-5} M, were each separated under conditions similar to those in Figure 3.

Hydrogen peroxide modification (Piras and Vallee, 1966) changes activities in a manner similar to that observed on irradiation. Therefore, carboxypeptidase treated with 4 mM hydrogen peroxide was also subjected to gel filtration, and the pattern obtained is qualitatively similar to that observed on irradiation (Figure 3B or C).

Effect of Ultraviolet Irradiation on Lysozyme and Chymotrypsin. Since irradiation affects the three-dimensional structure of carboxypeptidase, it is possible that other enzymes are similarly damaged. It has been reported that lysozyme exposed to 1.6×10^7 ergs/cm^2 (2537 A) loses 75% of its original activity concomitant with the destruction of histidine (Ferrini and Zito, 1963). When irradiated under conditions similar to those used by these authors the enzyme emerges as a single fraction from Sephadex G-75 (Figure 4). However, the peak is displaced toward the region of higher molecular weight material, and it is much broader than that of the native enzyme, suggesting heterogeneity.

Chymotrypsin irradiated with 3.2×10^7 ergs/cm^2 in 0.04 M phosphate buffer, pH 7.1, undergoes much more pronounced changes (Figure 5). Only 36% of the total protein emerges in the position of native chymotrypsin. The remainder is scattered in several small and poorly resolved peaks, which are of lower rather than higher R_F than that of the native enzyme (Figure 5B). To rule out the possibility that this material might be the result of self-digestion, α-chymotrypsin was first inactivated completely with PMSF (Fahrney and Gold, 1963) and then irradiated under the same conditions. The elution pattern obtained from Sephadex is essentially identical with that obtained in the absence of inhibitor (Figure 5C).

Discussion

It has been shown previously that ultraviolet irradiation drastically alters the enzymatic properties of carboxypeptidase. Two distinct changes in the primary structure could be detected, and their significance has been discussed (Piras and Vallee, 1966). First, an irreversible release of zinc and destruction of cysteine is observed. Second, the destruction of tyrosyl residues accounts for increases in esterase and losses of peptidase activities, similar to those previously seen on chemical modification. These latter changes are superimposed upon those due to loss of zinc. The additional destruction of tryptophan and histidine and the nature of the esterase activity curve, biphasic as a function of the intensity of irradiation, strongly suggested that yet a third, independent process might operate, ultimately resulting in a loss of the increased esterase activity. Structural changes of carboxypeptidase were postulated to be responsible for these findings, perhaps representing a third mechanism for changes in activity. The present study was undertaken to investigate and evaluate the possible existence of such alterations. Optical rotatory dispersion, sedimentation analysis, and gel filtration served as the experimental tools to examine the physicochemical properties of the irradiated enzyme.

On irradiation the enzyme undergoes a significant change in conformation, as indicated by a diminution in the amplitude of the intrinsic Cotton effect at 233 mμ and a decrease of b_0 from -216 to -132. Such changes are thought to be typical of denaturation (Urnes and Doty, 1961; Ulmer and Vallee, 1965). Previous optical rotatory dispersion studies of irradiated carboxypeptidase (Fujioka and Imahori, 1963) detected a decrease of b_0 from -226 to -198 when the peptidase activity was reduced to 30% of the initial value. Due to the then existent instrumental limitations, the previous investigations were confined to the longer wave-

lengths range, thus probably accounting for the small magnitude of the experimental changes in that study.

The sedimentation pattern of irradiated carboxypeptidase differs completely from that of the native enzyme. Rapidly sedimenting material due to protein aggregation, observed here as an inflection of the main peak, is a known consequence of ultraviolet irradiation in other systems (McLaren and Shugar, 1964). In the present study, moreover, this aggregated material is polydisperse, since it does not sediment as a single symmetrical peak. It separates continuously from the main boundary, the sedimentation constant of which is characteristic of native carboxypeptidase.

While confirming these results, gel filtration also provided an analytical procedure for the separation and identification of the products of irradiation. From the onset of irradiation, gel filtration on Sephadex G-75 reveals at least two fractions in addition to native carboxypeptidase. Fraction I, which appears first, probably represents an aggregate of the enzyme, and is, therefore, excluded from the gel matrix. This interpretation is consistent with the sedimentation pattern, and the observation is similar to the aggregation of ribonuclease subsequent to γ-irradiation (Shapira, 1963). Thus, ultraviolet irradiation profoundly alters the structure of carboxypeptidase.

The biphasic nature of the esterase activity as a function of the dosage of irradiation suggested at least two separate mechanisms to account for activation and inactivation, respectively, the latter becoming predominant at higher intensities of irradiation (Piras and Vallee, 1966).

The separation of the different species by means of gel filtration allows the measurement of the enzymatic activities in each fraction as a function of irradiation (Table I). The enzymatic changes in Fractions I and II are nonspecific since both esterase and peptidase activities decrease in parallel from the beginning. The increased esterase activity of Fraction III, on the other hand, accounts for the total increased esterase activity of the unseparated mixture. Fraction III emerges in the position of the native enzyme, suggesting that it has undergone specific modifications without there being detectable structural changes.

Like the unseparated mixture of the products of irradiation, however, Fraction III loses zinc (Table II); hence, it contains both apocarboxypeptidase and zinc carboxypeptidase. Further, approximately one tyrosyl residue is destroyed, but there is no change either in tryptophan or histidine. Since in Fraction III only tyrosine is destroyed, the enzymatic changes which are virtually indistinguishable from those observed for the unseparated mixture (Piras and Vallee, 1966) should be attributed to the modification of this residue.

The losses of tryptophan, histidine, and possibly additional tyrosyl residues in the total irradiation material (Piras and Vallee, 1966) may well be manifestations of the process which ultimately leads to the formation of Fractions I and II and denaturation.

The consequences of hydrogen peroxide treatment of carboxypeptidase (Piras and Vallee, 1966) resemble those of ultraviolet irradiation in many ways. The analogies in regard to zinc content and enzymatic and spectral changes previously described are now found to extend to the pattern obtained on gel filtration. The reagent is not thought to attack histidine (Hachimori *et al.*, 1964). Thus, tryptophan and tyrosine are presumably the only residues modified both by hydrogen peroxide and ultraviolet irradiation. The data suggest that tryptophanyl residues may participate in maintaining the three-dimensional structure of the enzyme; once they are destroyed, carboxypeptidase can no longer maintain its native configuration, leading to denatured products which eventually aggregate.

Lysozyme and chymotrypsin also undergo structural changes on irradiation (Figures 4 and 5); thus, disruption of the three-dimensional structure is not restricted to carboxypeptidase. The similarity of the Sephadex patterns of irradiated chymotrypsin and of irradiated PMSF–chymotrypsin indicate that the small molecular weight materials produced in this instance are a direct consequence of irradiation, rather than the result of self-digestion subsequent to irradiation. α-Chymotrypsin may be considered as three peptide chains linked by —S—S—bonds (Desnuelle and Rovery, 1961). Since ultraviolet irradiation destroys disulfide bonds of proteins (McLaren and Shugar, 1964) the highly heterogeneous low molecular weight material arising from irradiation of chymotrypsin may be attributed to the release of one or more of the three peptide chains. Lysozyme, on the other hand, consists of a single peptide chain, stabilized by disulfide bonds; hence, scission of some of these would probably open its structure and result in a molecule more readily excluded on gel filtration than the native protein.

Inactivation of trypsin by ultraviolet irradiation is thought to be accompanied by the disruption of hydrogen bonds. It has been proposed that in this instance a sequence of specific structural modifications in a cluster of weak bonds, involved in maintaining the native conformation, might be the basis of the observed changes in activity (Augenstine *et al.*, 1961; Augenstine, 1962). In addition to factors here cited, such a mechanism may contribute to the changes in conformation reported in the present examples. Since carboxypeptidase does not contain disulfide bonds, its structure is likely stabilized both by hydrogen and hydrophobic bonds. The destruction of tryptophan and histidine may be pertinent to a mechanism similar to that postulated by Augenstine.

The present results suggest caution in correlating alterations of enzymatic activities on irradiation with the destruction of specific amino acid residues, in the absence of physicochemical data. The delineation of different processes, all leading to inactivation, may prove to be a difficult task, unless the system is particularly susceptible to experimental resolution, as in carboxypeptidase. The increase in the esterase activity of carboxypeptidase—previously shown by other methods to be due to the modification of tyrosyl residues—served as a convenient criterion for the identification of those molecules in which only tyrosine was

destroyed, thereby accounting for specific functional changes. These could be differentiated from nonspecific changes, due to denaturation.

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Iodination of the Normal and Buried Tyrosyl Residues of Lysozyme. I. Chromatographic Analysis*

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ABSTRACT: Iodination of lysozyme with ^{131}I-labeled KI_3 solution (up to 14 moles/mole of lysozyme) at pH 8.5, in H_2O or 8 M urea at either 0° or 24°, followed by enzymatic hydrolysis and chromatography revealed that: (1) the rate of iodination of the first two tyrosyl residues was faster in 8 M urea and at 24°. The recovery of organic iodine was greater under these conditions with a maximum of 7 g-atoms of I/molecule of lysozyme. Oxidative side reactions were also maximal under these conditions. (2) Only two of the three tyrosyl residues were iodinated in H_2O, whereas in 8 M urea all three were iodinated. Monoiodotyrosine was quantitatively important only at low levels (<2 moles of I_2/mole of lysozyme) of iodination. Diiodotyrosine was the major product of all three tyrosyl residues but monoiodohistidine (and some diiodohistidine) appeared at molar ratios of $I_2 > 2$, *i.e.*, after one tyrosyl residue was fully iodinated. Thyroxine was not found.

It has been known for some time that the tyrosyl groups of lysozyme ionize with abnormally high pK values. Initially, all three[1] phenolic dissociations were thought to be identical, with an apparent pK value of 10.8 (Fromageot and Schneck, 1950; Tanford and Wagner, 1954; Donovan *et al.*, 1961). However, the titration curves were broader than the expected and the possibility that this was due to appreciably different pK values was entertained (Tanford and Wagner, 1954). At present it seems most probable that lysozyme contains three tyrosyl residues, two of which ionize instantaneously with an apparent pK of 10.4–10.5, while the third ionizes very slowly and with a midpoint in the titration curve at 12.8 (Inada, 1961; Edelhoch and Steiner, 1962). Ionization of this buried tyrosyl residue is time dependent (Inada, 1961). The enzyme is incompletely denatured in 8 M urea, whereas 4 M guanidine consistently causes major configurational changes, making possible titration of several masked

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[1] Fromageot and Schneck (1950) titrated only two tyrosyl residues and these had a normal titration curve.