Effect of Ultraviolet Irradiation on Composition and Function of Carboxypeptidase A*

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ABSTRACT: Irradiation with ultraviolet light at 2537 A increases the esterase and decreases peptidase activities of carboxypeptidase A. These alterations in function can be related to three distinct changes in composition as indicated by amino acid and zinc analyses at different stages of irradiation. First, irradiation results in an irreversible loss of zinc and a proportionate decrease of both esterase and peptidase activities. Second, low doses of irradiation destroy tyrosyl residues. This is thought to account for an increase in esterase activity, similar to that previously observed on acylation or iodination (Vallee, B. L., 1964, *Federation Proc. 23*, 8). Third, with increasing doses of irradiation, tryptophan and histidine are destroyed also. The relationship between these changes in chemical composition and the activity profiles of the enzyme is discussed.

The inactivation of enzymes by ultraviolet radiation has been attributed to its chemical effects, *e.g.*, the photolysis of disulfide bonds of cystine and of aromatic residues (Luse and McLaren, 1963; Ferrini and Zito, 1963; McLaren and Shugar, 1964). Studies on carboxypeptidase have related the loss of peptidase activity on irradiation to changes in tryptophan or tyrosine content or both (Fujioka and Imahori, 1963). The effect on esterase activity was not examined. Acetylation or iodination of tyrosyl residues of carboxypeptidase markedly alters the dual specificity of the enzyme (Simpson *et al.*, 1963; Riordan and Vallee, 1963). It seemed important, therefore, to investigate whether a similar mechanism might underlie the changes in enzymatic activity induced by radiation.

The simultaneous examination of the enzymatic and chemical properties of carboxypeptidase suggest that the complexity of the system precludes a direct correlation of all activity changes due to ultraviolet irradiation with alterations in primary structure. Ultraviolet irradiation apparently produces its effects upon the activity of carboxypeptidase by at least three chemically distinct paths. A preliminary report of some aspects of this work has been presented (Piras and Vallee, 1965).

Experimental Section

Materials. Twice recrystallized bovine pancreatic carboxypeptidase A, prepared by the method of Anson (1937), was obtained from the Worthington Biochemical Corporation, 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 of $\epsilon_{278} = 6.42 \times 10^4$ M^{-1} cm^{-1}. All chemicals employed in these experiments were of reagent grade and were used without further purification. Special precautions were taken to prevent contamination by adventitious metal ions (Coleman and Vallee, 1960).

Methods. Irradiation was carried out with a mercury lamp provided with a filter (Mineralight, Model SL 2537, Ultraviolet Products, Inc., South Pasadena, Calif.). Virtually all (99%) of the integrated intensity came from the mercury resonance line at 2537 A.[1] The lamp was calibrated by chemical actinometry at 20°, employing the method of Hatchard and Parker (1956). Under the conditions used, a 0.006 M solution of ferrioxalate has an infinite absorption and the quantum yield is 1.25.

In order to eliminate absorption or scattering of radiation in the windows of sample cuvets, the solution was exposed to the mercury arc in the cell as shown in Figure 1. Five milliliters of solution was pipetted into the trough of the water-cooled chamber. The liquid layer was 3.5 mm deep. This was exposed directly to the window of the source which matched precisely the dimensions of the lucite cell (19 × 75 mm). The distance between the exit window and surface of the liquid layer was kept at 5 mm to assure reproducible conditions of irradiation. The trough was cooled with circulating, iced water to maintain a sample temperature of 4°. With this arrangement, the intensity of the lamp was found to be 1.6×10^6 ergs/cm²/min.

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[1] Radiation of wavelengths shorter than 2537 A (*e.g.* 1849 A) was excluded as a cause of the results, since identical alterations of activity (*vide infra*) were obtained with light filtered through 20% acetic acid.

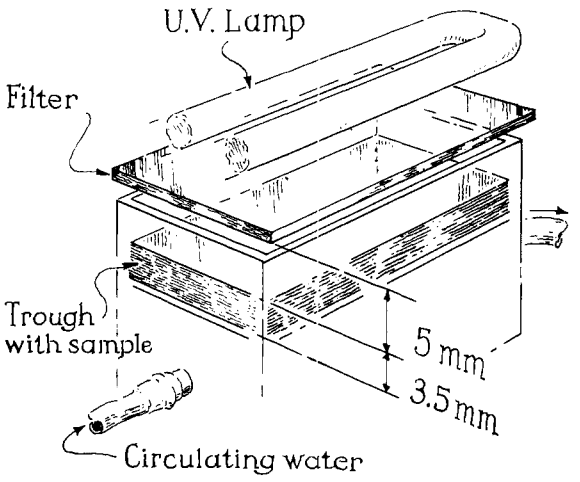

FIGURE 1: Diagram of the experimental arrangement employed for ultraviolet irradiation.

Carboxypeptidase A, 4×10^{-5} M, was irradiated in 1 M NaCl–0.01 M Tris–HCl buffer at pH 7.5. Aliquots were removed at intervals for enzymatic assay. When the total volume removed exceeded 5%, the experiment was terminated. The pH was determined with a Radiometer PHM-22 pH meter, equipped with a general purpose combined electrode. Protein concentration was determined by absorbance in a Zeiss PMQII spectrophotometer. A Cary Model 11 recording spectrophotometer was used to obtain continuous spectra.

ENZYMATIC ACTIVITIES. Peptidase activity was determined using carbobenzoxyglycyl-L-phenylalanine (Mann Research Laboratories) as the substrate (Snoke and Neurath, 1949). Activity is expressed as an apparent proteolytic coefficient C, defined as $\log a_0/a/\text{min}/\mu\text{mole enzyme}$, where a_0 and a are the concentration of substrate at time zero and t, respectively (Simpson *et al.*, 1963). The assays were performed at 0° in 1 M NaCl–0.02 M sodium Veronal buffer, pH 7.5, with an initial concentration of 0.02 M substrate. C was calculated from the linear portion of the first-order reaction plots, when hydrolysis did not exceed 15%.

Esterase activity was determined by pH titration (Snoke *et al.*, 1948) with 0.1 N NaOH of the hydrogen ions released on hydrolysis of the substrate, using a pH-Stat (Radiometer) and recorder (Ole Dich). Assays were performed at 25° with 3.0 ml of 0.01 M hippuryl-DL-β-phenyllactate in 0.2 M NaCl–0.005 M Tris–HCl buffer, pH 7.5. Activities are expressed as zero-order velocity constants, k, with units of $\mu\text{moles } H^+/\text{min}/\mu\text{mole of enzyme}$.

ANALYTICAL MEASUREMENTS. Zinc was determined by atomic absorption spectroscopy (Fuwa and Vallee, 1963); tryptophan by the colorimetric procedure of Spies and Chambers (1949), by *N*-bromosuccinimide oxidation (Patchornik *et al.*, 1958) as used by Bargetzi *et al.* (1963), and in some instances by spectrophotometry (Bencze and Schmid, 1957).

Total amino acid analysis followed the procedure recommended by Moore and Stein (1963). Enzyme (4.5 ml) was precipitated, immediately after irradiation, by addition of ethanol to a final concentration of 30%, followed by heating for 5 min in a boiling water bath and then cooling at 0°. Samples were then centrifuged twice and quantitatively transferred to heavy walled combustion tubes. Hydrolysis was carried out under vacuum in 4 ml of 6 N HCl at 105° for 24 hr. Appropriate aliquots were analyzed in a Spinco Model 120 B amino acid analyzer by the chromatographic technique of Spackman *et al.* (1958). Total recovery was above 90%. The number of residues of each amino acid per mole of enzyme was calculated assuming 19 alanyl residues per mole of carboxypeptidase (Bargetzi *et al.*, 1963).

Results

Effect on Zinc Content of Carboxypeptidase. Preliminary studies confirmed that ultraviolet irradiation decreases the peptidase activity of carboxypeptidase. Since zinc is required for activity (Vallee and Neurath, 1955; Vallee *et al.*, 1960b), we examined whether or not loss of zinc might account for part or all of the observed changes. The zinc content of the enzyme was measured before and after increasing doses of irradiation. Prior to analysis zinc, dissociated from the enzyme, was removed by extensive dialysis against metal-free buffer or by gel filtration through Sephadex G-25 (1 × 30 cm column). The zinc content decreased progressively from 0.98 to 0.44 g-atom/mole of enzyme (Table I), and this could not be prevented by the presence of excess Zn^{2+} during irradiation. All enzymatic activities to follow are based, therefore, on active enzyme as determined by its zinc content.

TABLE I: Effect of Irradiation (2537 A) on the Zinc Content of Carboxypeptidase.[a]

Dose ($E \times 10^{-7}$, ergs/cm²)	Gram-Atoms of Zinc/Mole Enzyme
0	0.98
0.8	0.85
2.4	0.74
4.8	0.61
9.6	0.50
14.4	0.44

[a] Samples of 1 ml of irradiated enzyme, 4×10^{-5} M, were passed over a Sephadex G-25 column (1 × 30 cm) at a flow rate of 1 ml/min, and the protein peak was collected. The zinc content and the absorbance at 278 mμ were measured to determine the ratio of metal to protein.

Effect on Enzymatic Activities. Ultraviolet irradiation

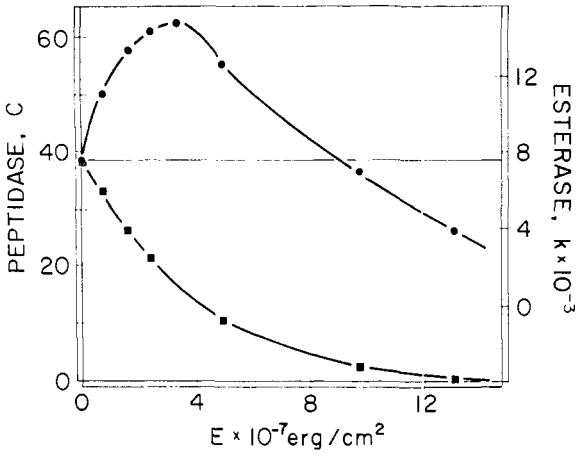

FIGURE 2: Effect of ultraviolet irradiation (2537 A) on esterase (●) and peptidase (■) activities of 4 × 10^{-5} M carboxypeptidase in 1 M NaCl–0.01 M Tris–HCl, pH 7.5, at 4°. Assays were carried out as under Methods. The horizontal line represents the control values.

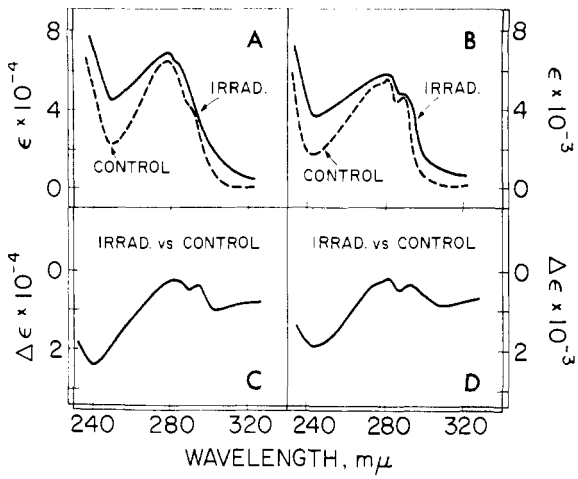

FIGURE 3: Absorption spectra of native and ultraviolet-irradiated carboxypeptidase, A; tryptophan and irradiated tryptophan, B. Difference spectra of irradiated *vs.* native carboxypeptidase, C; and irradiated tryptophan *vs.* tryptophan, D. Irradiation with 3 × 10^7 ergs/cm² was performed as described under Methods. A Cary Model 11 recording spectrophotometer with 1-cm cells was employed. All spectra were obtained in 1 M NaCl–0.01 M Tris–HCl, pH 7.5, room temperature.

of carboxypeptidase at 4°, pH 7.5, markedly alters both the catalytic rates and the specificity (Figure 2). As the dose of irradiation is increased, peptidase activity is rapidly abolished (apparent first-order kinetics). In contrast, at doses of irradiation up to 3.7 × 10^7 ergs/cm², esterase activity increases to 200% of the control value. Doses greater than 4 × 10^7 ergs/cm² decrease the activity.

Buffer composition (Tris–HCl, Tris–citrate, phosphate, Veronal), NaCl concentration (0.2–2 M), pH of irradiation (5–8.2), and presence or absence of oxygen do not affect the over-all characteristics of the enzymatic changes. As judged by the enzymatic measurements, the protein concentration (0.1–3.4 mg/ml) affects the rate, but not the pattern of activities.

On the basis of these results, a standard procedure was adopted: carboxypeptidase, 4 × 10^{-5} M, in 1 M NaCl–0.01 M Tris–HCl buffer, pH 7.5, at 4° was irradiated at an intensity of 1.6 × 10^6 ergs/cm²/min. Under these conditions, the quantum yield of inactivation for the peptidase activity of carboxypeptidase is 4.8 × 10^{-3}. The value was calculated from the $D_{0.37}$ dose of the first-order plot (Settlow and Pollard, 1962), using a molar absorptivity $\epsilon_{254} = 2.57 \times 10^4\ M^{-1}\ cm^{-1}$.

Effect on Spectrum. Alterations in the absorption spectrum between 230 and 330 mμ indicated modification of aromatic amino acid residues (Figure 3A). Compared with the native enzyme the absorbance of irradiated carboxypeptidase is increased over the entire spectral range examined, similar to previous observations on this and other enzymes (Fujioka and Imahori, 1963; McLaren and Shugar, 1964). The increased absorbance of irradiated tryptophan, at a concentration corresponding to that of 4 × 10^{-5} M carboxypeptidase, resembles that of irradiated carboxypeptidase (Figure 3B). The difference spectra (Figure 3C and D) are virtually indistinguishable, implying that tryptophan is destroyed on irradiation of carboxypeptidase.

Effect on Amino Acid Composition. The number of moles of tryptophan modified was measured both with *N*-bromosuccinimide and by the colorimetric procedure of Spies and Chambers (1949). With a dose of 4.8 × 10^7 ergs/cm², approximately one of the seven residues[2] of tryptophan per mole of native enzyme is lost (Table II). At this point the ratio of esterase to peptidase activity is maximal. Tyrosine and histidine are also destroyed progressively at different stages of the irradiation as shown by amino acid analyses (Table II). Half-cystine was not determined quantitatively by amino acid analyses of performic acid oxidized or alkylated enzyme. However, destruction of cysteine was evident qualitatively by repeated analyses of carboxypeptidase air oxidized at pH 6.5 for 4 hr (Moore and Stein, 1963). The area under the cystine peak was greatly decreased, indicating that irradiation partially destroys the two half-cystines known to be present (Bargetzi *et al.*, 1963). With the possible exception of insignificant amounts of lysine

[2] Bargetzi *et al.* (1963) found eight tryptophans per mole of carboxypeptidase A. Similar results have also been obtained in our laboratory (Simpson and Vallee, unpublished). However, with the enzyme used in the present study, seven residues per mole were found consistently with all three methods employed, *i.e.*, *N*-bromosuccinimide, colorimetry, and spectrophotometry (Patchornik *et al.*, 1958; Spies and Chambers, 1949; Bencze and Schmid, 1957). While this difference remains unexplained, it is not germane to the objectives of the present experiments (Table II).

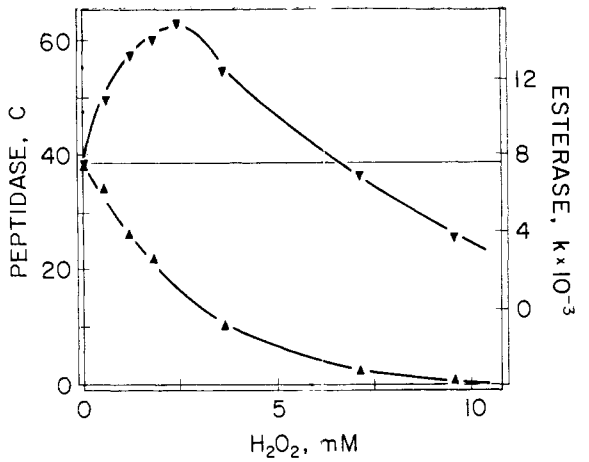

FIGURE 4: Effect of variation of the hydrogen peroxide concentration on esterase (▼) and peptidase (▲) activities of carboxypeptidase. Carboxypeptidase, 2×10^{-5} M, was treated at 0° in 10% dioxane, 0.5 M Na_2CO_3, final pH 8.4, for 3 hr with the hydrogen peroxide concentrations indicated. Activities were determined as under Methods. The horizontal line represents the control values.

and isoleucine, all other amino acids remained unchanged.

Comparison of Effects of Ultraviolet Irradiation and of Chemical Modifications. The changes in activity upon irradiation could not be correlated unequivocally to the destruction of a specific amino acid residue. Hence, chemical modification of tryptophan and irradiation of acetylcarboxypeptidase were carried out to ascertain the extent to which the enzymatic changes induced by ultraviolet irradiation might be attributable to the modification of either of these residues.

(a) HYDROGEN PEROXIDE MODIFICATION. Hydrogen peroxide in a solution of carbonate–dioxane modifies tryptophanyl residues in proteins (Hachimori *et al.*, 1964). On exposure of carboxypeptidase to increasing concentration of hydrogen peroxide, the number of tryptophanyl residues modified increases, as judged by the change in absorbance at 282 mμ (Hachimori *et al.*, 1964). At a hydrogen peroxide concentration of 15 mM or greater, approximately six tryptophans of carboxypeptidase are altered. Further, zinc is lost and enzymatic activities are changed drastically. Peptidase activity decreases as the hydrogen peroxide concentration is increased, but esterase activity first increases to a maximum of 200%, and then decreases (Figure 4). When the difference between the two activities is maximal, approximately two tryptophans are modified. The similarity of the effects of ultraviolet irradiation and of hydrogen peroxide oxidation on zinc content, enzymatic activities, and spectrum is evident. It seemed appropriate, therefore, to examine whether chemical modification of tyrosyl residues could bring about additional changes in activity.

(b) ACETYLATION. Acylation of two tyrosyl residues at the active center of native carboxypeptidase induces a 6–7-fold increase of esterase and decreases peptidase activity when measured under standard conditions (Simpson *et al.*, 1963; Riordan and Vallee, 1963). In contrast to its effect on the native enzyme, from the beginning irradiation decreases the esterase activity of acetylcarboxypeptidase. The loss, which proceeds by apparent first-order kinetics, does not seem due to simple deacetylation since reacetylation of the irradiated acetyl-enzyme does not further affect esterase activity (Table III, lines 2, 3, and 4).

The sequence in which irradiation and acetylation are performed might provide additional insight into the presumable nature of the underlying process. Hence, native carboxypeptidase was first irradiated (Table III, line 5) and then acetylated (line 6). The residual peptidase activity of the irradiated enzyme (line 5) indicates the degree to which acetylation can be expected to further enhance esterase activity (line 6). Deacetylation of the irradiated acetyl-enzyme (line 7) with hydroxylamine (Simpson *et al.*, 1963) restores peptidase and esterase activities to the level expected at the dose of radiation employed (lines 2, 4, 5, and 7). Thus, the active

TABLE II: Amino Acid Composition[a] of Native and Ultraviolet-Irradiated Carboxypeptidase.

	Dose of Irradiation (ergs/cm²)			
Amino Acid	0	2.4×10^7	4.8×10^7	1.3×10^8
Lysine	14.7	14.5	14.3	14.1
Histidine	7.9	7.4	7.0	6.4
Arginine	10.0	10.2	9.8	9.9
Aspartic acid	26.4	26.1	26.2	25.9
Threonine	22.1	21.7	22.0	21.2
Serine	27.3	27.1	27.4	26.9
Glutamic acid	24.3	24.1	24.0	24.0
Proline	9.7	9.5	9.9	9.9
Glycine	22.4	22.1	22.6	22.6
Alanine	(19)	(19)	(19)	(19)
Valine	15.0	14.5	14.7	14.9
Methionine	2.7	2.7	2.6	2.4
Isoleucine	17.6	17.4	17.0	16.8
Leucine	22.9	22.6	22.8	21.6
Tyrosine	18.7	18.0	17.4	15.5
Phenylalanine	14.6	14.4	14.3	14.3
Tryptophan[b]	6.8	6.2	5.6	4.7
Tryptophan[c]	7.0	6.0	6.0	5.3

[a] Values correspond to 24-hr hydrolysis and are expressed in residues per mole of carboxypeptidase, assuming 19 alanines per mole (Bargetzi *et al.*, 1963). Destruction of cysteine was apparent from the magnitude of the area under the cystine peak subsequent to analysis of carboxypeptidase air oxidized at pH 6.5 for 4 hr. [b] Method of Spies and Chambers (1949). [c] *N*-Bromosuccinimide method (Patchornik *et al.*, 1958) in 10 M urea (Bargetzi *et al.*, 1963).

TABLE III: Enzymatic Activities of Carboxypeptidase on Successive Modifications.

Enzyme[a]	Modification[b]	Peptidase, C	Esterase ($k \times 10^{-3}$)
1. [(CPD)Zn]	...	35.6	7.8
2. [(Ac_ICPD)Zn]	...	1.7	42.0
3. [(Ac_ICPD)Zn]	Ultraviolet	1.2	30.5
4. [(Ac_ICPD)Zn]	Ultraviolet, A_I	1.4	30.2
5. [(CPD)Zn]	Ultraviolet	21.2	12.5
6. [(CPD)Zn]	Ultraviolet, Ac_I	1.6	26.7
7. [(Ac_ICPD)Zn]	Ultraviolet, NH_2OH	23.0	6.7

[a] [(CPD)Zn] symbolizes carboxypeptidase, and [(Ac_I-CPD)Zn] acetylcarboxypeptidase prepared by reaction with acetylimidazole. [b] Irradiation with 2×10^7 ergs/cm^2 was carried out under standard conditions. When modifications were performed successively, the sequence was in the order in which they are listed in the second column. Acetylation was performed with a 60 M excess of *N*-acetylimidazole, and deacetylation with 1 M NH_2OH, pH 7.5, 10 min, 25° (Simpson *et al.*, 1963).

center, once modified either by irradiation or acetylation, cannot be altered to increase activity further.

Discussion

Correlation of losses of activity with destruction of aromatic and sulfur-containing amino acids of enzymes has been thought to make ultraviolet irradiation a suitable agent for site-specific and selective modifications of proteins. A number of enzymes have been examined from this point of view. Previous studies on carboxypeptidase have directly related the observed changes in tryptophan or tyrosine content on irradiation to the loss of peptidase activity (Fujioka and Imahori, 1963). Since these investigations preceded the rapidly evolving understanding of the chemical details of the active site of carboxypeptidase, an extension of such studies in the light of present knowledge seemed indicated.

Since zinc is required for the enzymatic activity of carboxypeptidase (Vallee and Neurath, 1955), attention was given to the effect of irradiation on the metal content of the enzyme. Zinc of carboxypeptidase is bound to cysteine (Vallee *et al.*, 1960a), a residue readily modified by ultraviolet radiation. Its destruction, qualitatively apparent on amino acid analysis (Table II), is, no doubt, partially responsible for the irreversible loss of metal (Table I), though disruption of secondary and tertiary structure cannot be dismissed as contributory factors.

Loss of the zinc atom thus represents one distinctive consequence of irradiation. The ensuing loss of activity of this and similar metalloenzymes should be monitored by metal analyses to differentiate this mechanism of inactivation from others that may operate. It is, therefore, difficult to compare the present data to those of studies in which zinc was not measured (Fujioka and Imahori, 1963). This also applies to the changes in esterase activity, which here served as an additional criterion for the functional effects. The loss of peptidase activity far exceeds that which can be accounted for solely based on the loss of zinc (Figure 2). While peptidase activity decreases, esterase activity increases to more than twice that of the control (Figure 2). These effects must be independent of those due to loss of zinc, since the latter would result in the decrease of *both* activities.

Possible differences in the amino acid composition of the native and irradiated enzymes (Table II) might clarify this problem. The amino acid composition of the native enzyme, the base line for the losses of residues on irradiation, corresponded closely to those of Bargetzi *et al.* (1963) (Table II) but differ by more than four to five residues, in some instances, from those found in a previous study, precluding a direct comparison (Fujioka and Imahori, 1963).

The destruction of tyrosine observed here and previously (Fujioka and Imahori, 1963) and some of the enzymatic consequences of irradiation are reminiscent to those subsequent to acetylation or iodination, known to involve tyrosyl residues of carboxypeptidase (Simpson *et al.*, 1963; Riordan and Vallee, 1963; Vallee, 1964) (Table II), and suggest similar mechanisms for the functional changes.

The results of successive irradiation and acetylation of the enzyme, and variation in the order of these procedures, also imply common denominators for their action. Each of these modifications changes esterase and peptidase activities only to the degree that the other has failed to do so; *i.e.*, the changes are additive, not multiplicative (Table III). These results are similar to those obtained by successive modifications of carboxypeptidase with chemical reagents, specific for tyrosyl residues (Vallee, 1964). The data show that tyrosyl residues are affected both by acetylation and irradiation, and their modification apparently constitutes a second, distinct radiation effect.[3]

However, on acetylation esterase activity increases monotonically, commensurate with the decrease of peptidase activity. On irradiation, in contrast esterase activity increases initially, though to a lesser degree, and then falls, resulting in a biphasic profile. Peptidase, however, decreases continually but more rapidly.

[3] The most effective substrates and inhibitors of carboxypeptidase contain an aromatic residue which is readily destroyed by irradiation, rendering such compounds unsuitable for protection experiments. Glycyl-L-leucine, however, is bound firmly to the active site (Coleman and Vallee, 1962), is hydrolyzed slowly, and is not readily altered by ultraviolet irradiation. It reproducibly afforded protection by reducing the magnitude of the activity changes by about 20%.

Jointly these phenomena suggest the existence of other concurrent reactions, characteristic of irradiation (*vide infra*) (Figure 2). The modification or destruction of yet other residues, *e.g.*, tryptophan (Table II) (Fujioka and Imahori, 1963), was considered as a possible basis for activity changes, a possibility considered previously, based on other considerations (Vallee *et al.*, 1963).

The similarity between the difference spectra of the irradiated *vs.* the native enzyme and of irradiated tryptophan *vs.* tryptophan are consistent with the demonstrated destruction of this amino acid (Figure 3 and Table II). Hydrogen peroxide in dioxane has been thought to modify tryptophan preferentially (Hachimori *et al.*, 1964). The effects of hydrogen peroxide on enzymatic activities (Figure 4), the zinc content, and spectrum of carboxypeptidase are virtually indistinguishable from those of irradiation. However, the concentration of hydrogen peroxide required to modify the tryptophans of carboxypeptidase is significantly higher than that necessary for a number of other enzymes. Thus, 3 mM hydrogen peroxide modifies at least 75% of the tryptophanyl residues of lysozyme, trypsinogen, trypsin, chymotrypsinogen, and chymotrypsin (Hachimori *et al.*, 1964), while it alters less than 30% of those of carboxypeptidase. Similarly, Bargetzi *et al.* (1963) found that 10 M urea is necessary to titrate all the tryptophanyl residues of carboxypeptidase with *N*-bromosuccinimide. The relative unavailability of tryptophan in carboxypeptidase, requiring high concentrations of hydrogen peroxide, may, in fact, decrease the specificity of the reagent, so that the functional and spectral changes might not be due entirely to tryptophan. In this context, it should be mentioned that hydrogen peroxide may also modify tyrosine, albeit to a lesser degree than tryptophan (Hachimori *et al.*, 1964). In fact ribonuclease, which does not contain tryptophan, undergoes some spectral changes in the 280-mμ region on treatment with hydrogen peroxide–dioxane (Piras, unpublished observations). Thus, for ribonuclease, at least, the spectral alterations cannot be attributed to tryptophan and may, in fact, be due to tyrosine. The lack of specificity of these methods suggests caution in attributing a specific, functional significance to the destruction of tryptophanyl residues either by ultraviolet irradiation or hydrogen peroxide. Further studies of this problem are in progress.

The loss of a histidyl residue on irradiation further complicates the interpretation (Table II). The present data do not clarify the possible involvement of histidine in the catalytic mechanism of carboxypeptidase, previously considered (Vallee *et al.*, 1963; Riordan and Vallee, 1963).

The destruction of tryptophan and histidine may well be related to the biphasic changes in esterase activity found upon prolonged irradiation, but they may exert their effects indirectly, *e.g.*, through inducing alterations of secondary and tertiary structure. Thus, further investigations on the structure of the irradiated enzyme seemed indicated before the enzymatic changes could be interpreted fully. The results of this investigation are recorded in the following paper (Piras and Vallee, 1966).

References

Anson, M. L. (1937), *J. Gen. Physiol. 20*, 663, 777.

Bargetzi, J. P., Sampath Kumar, K. S. V., Cox, D. J., Walsh, K. A., and Neurath, H. (1963), *Biochemistry 2*, 1468.

Bencze, W. L., and Schmid, V. (1957), *Anal. Chem. 29*, 1193.

Coleman, J. E., and Vallee, B. L. (1960), *J. Biol. Chem. 235*, 390.

Coleman, J. E., and Vallee, B. L. (1962), *J. Biol. Chem. 237*, 3430.

Ferrini, V., and Zito, R. (1963), *J. Biol. Chem. 238*, PC3824.

Fujioka, H., and Imahori, K. (1963), *J. Biochem. (Tokyo) 53*, 341.

Fuwa, K., and Vallee, B. L. (1963), *Anal. Chem. 35*, 942.

Hachimori, Y., Horinishi, H., Kurihara, K., and Shibata, K. (1964), *Biochim. Biophys. Acta 93*, 346.

Hatchard, C. G., and Parker, C. A. (1956), *Proc. Roy. Soc. (London) S A235*, 518.

Luse, R. A., and McLaren, A. D. (1963), *Photochem. Photobiol. 2*, 343.

McLaren, A. D., and Shugar, D. (1964), Photochemistry of Proteins and Nucleic Acids, New York, N. Y., MacMillan Co.–Pergamon.

Moore, S., and Stein, W. H. (1963), *Methods Enzymol. 6*, 819.

Patchornik, A., Lawson, W. B., and Witkop, B. (1958), *J. Am. Chem. Soc. 80*, 4747.

Piras, R., and Vallee, B. L. (1965), *Federation Proc. 24*, 440.

Piras, R., and Vallee, B. L. (1966), *Biochemistry 5*, 855 (this issue; following paper).

Riordan, J. F., and Vallee, B. L. (1963), *Biochemistry 2*, 1460.

Settlow, R. B., and Pollard, E. C. (1962), Molecular Biophysics, Reading, Mass., Addison-Wesley, p 283.

Simpson, R. T., Riordan, J. F., and Vallee, B. L. (1963), *Biochemistry 2*, 616.

Snoke, J. E., and Neurath, H. (1949), *J. Biol. Chem. 181*, 789.

Snoke, J. E., Schwert, G. W., and Neurath, H. (1948), *J. Biol. Chem. 175*, 7.

Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.

Spies, J. R., and Chambers, D. C. (1949), *Anal. Chem. 21*, 1249.

Vallee, B. L. (1964), *Federation Proc. 23*, 8.

Vallee,B. L., Coombs, T. L., and Hoch, F. L. (1960a), *J. Biol. Chem. 235*, PC45.

Vallee, B. L., and Neurath, H. (1955), *J. Biol. Chem. 217*, 253.

Vallee, B. L., Riordan, J. F., and Coleman, J. E. (1963), *Proc. Natl. Acad. Sci. U. S. 49*, 109.

Vallee, B. L., Rupley, J. A., Coombs, T. L., and Neurath, H. (1960b), *J. Biol. Chem. 235*, 64.