



Photochemical evidence for the participation of histidine in the active center of carboxypeptidase-A γ
by Kenneth Allen Freude

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of
DOCTOR OF PHILOSOPHY in Chemistry

Montana State University

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Abstract:

Dye-sensitized photooxidation using either methylene blue or Rose Bengal as sensitizers rapidly diminishes both the peptidase and esterase activities of carboxypeptidase-A γ . It is significant that both activities are simultaneously diminished by this chemical modification procedure, since heretofore a dual inactivation has been accomplished only by removal of the metal from the enzyme. All other chemical modifications employed on carboxypeptidase-A previously reported, including methylene blue sensitized photooxidation of carboxypeptidase-A δ have resulted in an increase in esterase activity and a decrease in peptidase activity. There is a considerable difference in the mechanism of action of Rose Bengal and methylene blue. Rose Bengal functions as a very effective sensitizer for photooxidation of both carboxypeptidase-A γ and apocarboxypeptidase-A γ , while methylene blue sensitizes the photooxidation of only the metal free form of the enzyme. Both sensitizers show a marked pH dependence and give identical sigmoidal curves which are typical of the pH dependency of the dye-sensitized photooxidation of the imidazole moiety. Crystal violet, a sensitizer which is reported to be specific for cysteine residues, was found to be completely ineffective on apocarboxypeptidase-A γ . Amino acid analysis of photooxidized enzyme revealed the loss of only histidine residues. Photosensitive amino acids such as cysteine, tyrosine, tryptophan, and methionine remained essentially unaltered. Significant protection against photooxidation was demonstrated using the substrates hippuryl-L-phenylalanine, glycylytyrosine, and the competitive inhibitor g-phenylpropionate. Kinetic studies indicate that Rose Bengal also functions as a competitive inhibitor of carboxypeptidase-A γ and prevents the exchange of metal ions at the active center of the enzyme. The photolability of several metallo derivatives of carboxypeptidase-A γ was investigated. Conformational changes subsequent to photooxidation and the photolability of the enzyme in various buffer systems were also investigated.



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PHOTOCHEMICAL EVIDENCE FOR THE PARTICIPATION OF HISTIDINE
IN THE ACTIVE CENTER OF CARBOXYPEPTIDASE-A_v

by

KENNETH ALLEN FREUDE

A thesis submitted to the Graduate Faculty in partial
fulfillment of the requirements for the degree

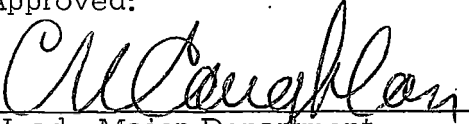
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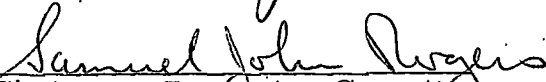
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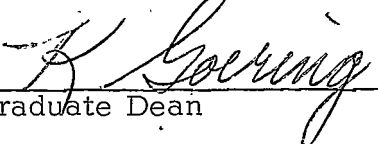
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ABSTRACT

Dye-sensitized photooxidation using either methylene blue or Rose Bengal as sensitizers rapidly diminishes both the peptidase and esterase activities of carboxypeptidase-A_γ. It is significant that both activities are simultaneously diminished by this chemical modification procedure, since heretofore a dual inactivation has been accomplished only by removal of the metal from the enzyme. All other chemical modifications employed on carboxypeptidase-A previously reported, including methylene blue sensitized photooxidation of carboxypeptidase-A_δ have resulted in an increase in esterase activity and a decrease in peptidase activity. There is a considerable difference in the mechanism of action of Rose Bengal and methylene blue. Rose Bengal functions as a very effective sensitizer for photooxidation of both carboxypeptidase-A_γ and apocarboxypeptidase-A_γ, while methylene blue sensitizes the photooxidation of only the metal free form of the enzyme. Both sensitizers show a marked pH dependence and give identical sigmoidal curves which are typical of the pH dependency of the dye-sensitized photooxidation of the imidazole moiety. Crystal violet, a sensitizer which is reported to be specific for cysteine residues, was found to be completely ineffective on apocarboxypeptidase-A_γ. Amino acid analysis of photooxidized enzyme revealed the loss of only histidine residues. Photosensitive amino acids such as cysteine, tyrosine, tryptophan, and methionine remained essentially unaltered. Significant protection against photooxidation was demonstrated using the substrates hippuryl-L-phenylalanine, glycyltyrosine, and the competitive inhibitor *g*-phenylpropionate. Kinetic studies indicate that Rose Bengal also functions as a competitive inhibitor of carboxypeptidase-A_γ and prevents the exchange of metal ions at the active center of the enzyme. The photolability of several metallo derivatives of carboxypeptidase-A_γ was investigated. Conformational changes subsequent to photooxidation and the photolability of the enzyme in various buffer systems were also investigated.

INTRODUCTION

Carboxypeptidase-A γ is a zinc metalloenzyme which exhibits peptidase and esterase activities.⁽¹⁾ The zinc atom can be reversibly removed, which results in the loss of both the peptidase and esterase activities.⁽²⁾ Since the removal of the metal ion from this metalloenzyme is reversible, it should be possible to identify the metal binding site by employing various specific chemical reagents or other procedures. Such chemical modification procedures have led to the suggestion that the metal ion is bound to a thiol group of a cysteine residue and to the α -amino group of the N-terminal amino acid of carboxypeptidase-A δ .⁽³⁾

Interpretation of the results of the methods used to detect the thiol group in this enzyme are subject to question. Reagents useful for thiol detection, such as iodo and bromoacetate, iodoacetamide and N-ethylmaleimide fail to react with the native or metal free enzyme.⁽³⁾ Sulfhydryl groups were, however, determined using silver ion, p-mecuri-benzoate, and ferricyanide.⁽³⁾ It is interesting that the only methods which were capable of demonstrating the presence of free sulfhydryl groups in this enzyme system were methods which depended upon metal binding. This fact becomes exceedingly important when one considers that these methods were applied to a system which is known to bind metals, that is carboxypeptidase-A γ . In addition, when carboxypeptidase was subject to reducing reagents such as sodium borohydride, or 2-mercaptoethanol, it would then form derivatives with iodoacetate and N-ethylmaleimide.⁽⁴⁾ These results imply that the enzyme contains in its structure a disulfide linkage, instead of two chemically unreactive sulfhydryl groups.⁽⁴⁾

Complexometric titration⁽⁵⁾ of apocarboxypeptidase with Zn^{2+} reveals two binding groups of apparent pK_a 's of 7.7 and 9.1. The pK_a of 9.1 represents the only substantial evidence of the involvement of sulfhydryl, but by no means confirms this hypothesis. The pK_a of 7.7 would correspond to either the titratable proton of imidazole or of the N-terminal α -amino group. The involvement of imidazole seems a better choice for the reasons suggested here. Carboxypeptidase

exists in three major forms and one minor form. These forms, the alpha, beta, gamma, and delta, have identical specificities, but differ in solubility, in chain length, and in their N-terminal residues. The N-terminal residues are alanine, serine, asparagine, and asparagine, respectively. Due to the heterogeneity of the N-terminal region of the molecule, conformational differences among the various forms would be expected if the binding of zinc atoms involved participation of the N-terminal residues. These conformational differences should result in altered specificity of the various enzymatic forms, which is not observed. Further, procarboxypeptidase has been shown to bind metal.⁽⁶⁾ Procarboxypeptidase consists of a single polypeptide chain of molecular weight 87,000, and yields one of the four carboxypeptidases-A, upon cleavage by the endopeptidases trypsin and chymotrypsin. This fact indicates that the binding site of the zinc is already established in the procarboxypeptidase, and that the N-terminus of carboxypeptidase is not involved in zinc binding. The possible involvement of imidazole in the catalytic activity of carboxypeptidase has been implied in several studies.

Since there was considerable evidence indicating that histidine could be involved in the activity of carboxypeptidase, a photochemical investigation was undertaken on the gamma form of the enzyme.

MATERIALS AND METHODS

The peptidase and esterase activities were determined in the following manner: The rate of hydrolysis of hippuryl-L-phenylalanine, (HPA), the peptide substrate, or of hippuryl-DL-phenyllactic acid, (HPLA), the esterase substrate, was measured by the increase in absorbance at 254 m μ at 25° C. A unit of activity is equal to one micromole of substrate hydrolyzed per minute under the conditions specified. The substrate solution was 0.001 M HPA or HPLA in 0.025 M Tris buffer, pH 7.5, containing 0.5 M NaCl. The change in optical density was recorded on a Gilford spectrophotometer. A full scale absorbance of 0.1 or 0.2 absorbance units was generally employed. The specific activity was determined according to the following equation:

$$\text{specific activity} = \frac{\Delta A_{254 \text{ m}\mu} / \text{min.}}{0.36 \times \text{mg enzyme/ml reaction mixture}}$$

where 0.36 equals the molar absorbance index of hippuric acid which is formed stoichiometrically.

The activity curves were obtained by pipetting a suitable aliquot of the experimental enzyme solution into 1.5 ml of substrate solution in a cuvette, mixing and placing into the spectrophotometer within 15 seconds. Carboxypeptidase-A γ was obtained from Worthington Biochemical Corp., Freehold, New Jersey, or was prepared by the method of Anson.⁽²⁷⁾ There was no detectable difference between the commercial preparation and the enzyme prepared in this laboratory. Stock solutions were prepared by dissolving suitable quantities of the commercial enzyme in three molar sodium chloride solution after washing the crystals several times with cold deionized water. The protein concentration was determined by measuring the optical density at 278 m μ and using an absorptivity constant of 6.42×10^4 liter mole.⁽²⁶⁾

Other chemicals were obtained from the following sources: Hippuryl-DL-phenyllactic acid (HPLA), Nutritional Biochemicals; Hippuryl-L-phenylalanine (HPA), Mann Research Laboratories; 1,10-phenanthroline (OP), J. T. Baker Chemical Co.; Cellex-T (TEAE),

Bio-Rad Laboratories; β -phenylpropionate, J. T. Baker Chemical Co.; Koshlands reagent, Cyclochemical Co.; Glycyltyrosine, Sigma Chemical; Rose Bengal and methylene blue, Fischer Scientific Co.; Crystal violet, Matheson, Coleman, and Bell; Thiopyronin, a gift from Dr. J. S. Bellin, Polytechnic Institute of Brooklyn.

The photooxidations were conducted in one of two ways. (A) A Sears 500 watt slide projector was focused at a distance of 18 to 20 cm. into the bottom of a plastic test tube which was supported at an angle in an ice bath. All manipulations of enzyme solutions containing dye were conducted in a darkened room. (B) The second method, which was found to be much more successful, was as follows: the projector was directed at a mirror held at a 45° angle to the projector lens. The projector lens was actually in contact with the mirror. Just below the mirror at a 90° angle to the projector lens was a Bell and Howell f 1.6 movie projector lens. The lens was positioned in such a manner as to allow the light to pass through in a condensing manner. This lens ensemble enabled the light to be condensed to a spot having a diameter of approximately 1.5 to 2.0 cm. The samples to be photooxidized were contained in small plastic cups which were inserted into holes in an aluminum block. The holes in the aluminum block were large enough so that the sample containing cups could be shut off from room light by means of rubber stoppers. The aluminum block was drilled to allow circulation of ice water during the determinations. The use of this block allowed consistent positioning of the sample in the light beam and permitted the use of very short exposure times. These two methods will be referred to as Method A and Method B in future discussion.

Buffer solutions were prepared with the aid of a Corning Model 12 research pH-meter. When metal ion contamination was to be avoided, all buffer solutions were extracted with a solution of dithizone in chloroform. The small amount of dissolved chloroform was removed from the buffer by bubbling purified air through the buffer at room temperature.

When plastic ware or glass ware was used to handle metalfree solutions, it was presoaked in an ethanol-EDTA bath for several hours, rinsed with ethanol, and air dried. Dialysis of solutions was conducted in special dialysis chambers. Teflon inserts were prepared to fit inside the dialysis tubing at each end. A neoprene O-ring was placed around the dialysis tubing and teflon insert to obtain a tight seal. One teflon insert was fitted with an O-ring sealed stopper which could be removed for introduction or removal of the sample. Stirring of the solution in the dialysis bag was accomplished by means of a small teflon stir bar. The entire apparatus was then suspended in the dialysis buffer, and both solutions stirred with a magnetic stirrer.

Hydrolysis of the protein mixtures were carried out in sealed Carius tubes for 20-24 hours at 110° C. Following hydrolysis an aliquot was removed and dried over KOH in vacuum. The residue was then dissolved in water and a suitable aliquot prepared and analyzed for amino acid content. Amino acid analyses were accomplished with a Technicon Amino Acid Analyzer.

PROCEDURE AND RESULTS

Photooxidation of carboxypeptidase-A_γ in the presence of 1,10-phenanthroline and methylene blue. This effect was determined by measuring the peptidase activity of the enzyme solution (0.023 mg/ml) before and after photooxidation, for both the apo and metal-containing enzyme. The apoenzyme was prepared by incubating, for up to fifteen minutes, the native enzyme at 0°, in 0.1 M Tris buffer, pH 7.0, containing 1.0 M NaCl and 5×10^{-4} M 1,10-phenanthroline. The measurement of peptidase activity in the apoenzyme system was accomplished by adding a slight molar excess of a 10^{-1} M ZnCl₂ solution to the apoenzyme system just prior to the assay. In the experimental solutions containing methylene blue the concentration of the dye was 0.002 mg/ml. Method A was used in the photooxidation. Table I summarizes the experimental composition and results of each determination.

The effect of photooxidation with methylene blue in the presence of 1,10-phenanthroline on esterase activity. Five microliters of a 3.58 mg/ml solution of carboxypeptidase-A_γ was diluted to 1.0 ml with 0.1 M Tris buffer, pH 7.0, containing 1.0 M NaCl. The final concentration of carboxypeptidase was 0.023 mg/ml. The reagents 1,10-phenanthroline and methylene blue, when included in the buffer, were at concentrations of 1×10^{-3} M and 0.002 mg/ml respectively. Photooxidation was by Method A. The esterase activity was determined as described in "Methods", using hippuryl-DL-phenyllactic acid as substrate. Table II summarizes the experimental composition and results of each determination.

The effect of methylene blue-1,10-phenanthroline photooxidation of carboxypeptidase-A_γ on esterase activity. Considerable care was taken to insure that each experimental solution of carboxypeptidase was handled in an identical manner, and for the same period of time. The experimental solutions were prepared so that the final concentrations of reagents were as follows: carboxypeptidase-A_γ,

TABLE I

Photooxidation of Carboxypeptidase-A_γ in the Presence
of 1,10-Phenanthroline and Methylene Blue

	μl enzyme	ml buffer	ml OP	ml MB	min hv	μl Zn ²⁺	spec. act. peptidase
A	5	1.0	—	—	—	—	56.6
B	5	0.75	0.25	—	—	—	5.5
C	5	0.75	0.25	—	—	5	50.2
D	5	0.25	0.25	0.5	—	5	44.8
G	5	0.25	0.25	0.5	30	5	24.6
H	5	0.5	—	0.5	30	—	50.2

The initial concentrations of reagents in 0.1 M Tris buffer, pH 7.0, containing 1.0 M NaCl were as follows: MB, 0.002 mg/ml; OP, 2×10^{-3} M; Zn²⁺, 10^{-1} M. Photooxidation was by Method A, of "Methods and Materials".

TABLE II

The effect of Photooxidation with Methylene Blue-1,10-phenanthroline on Esterase Activity

	μ l enzyme	ml buffer	ml OP	ml MB	min hr	μ l Zn^{2+}	spec. act. esterase	spec. act. peptidase
A	5	1.0	—	—	—	—	774	56.5
B	5	0.5	0.5	—	—	—	128	3.72
C	5	—	0.5	0.5	30	10	305	
F	5	0.5	—	0.5	30	—	650	
D'	5	—	0.5	0.5	60	10	228	14.1
F'	5	0.5	—	0.5	60	—	640	37.2

The initial concentrations of all reagents in 0.1 M Tris buffer, pH 7.0, containing 1.0 M NaCl were as follows: MB, 0.002 mg/ml; OP, 2×10^{-3} M; Zn^{2+} , 10^{-1} M. Photooxidation was by Method A.

0.023 mg/ml; OP, 1×10^{-3} M; methylene blue, 0.002 mg/ml. All components were dissolved in 0.1 M Tris buffer, pH 7.0, containing 1.0 M NaCl at 0° C. The esterase activity was determined as described in "Methods", using hippuryl-DL-phenyllactic acid as substrate. Photooxidation was done using Method A. Table III shows the experimental composition and results of each determination.

Photooxidation of carboxypeptidase-A_γ with increased methylene blue and Rose Bengal concentrations. In an attempt to increase the efficiency of the photooxidation process, the concentration of the sensitizing dye was increased. The photooxidation studies were expanded to include Rose Bengal sensitization as well as that of methylene blue. The effectiveness of Rose Bengal as a sensitizer was determined both in the presence and absence of the metal chelator 1,10-phenanthroline. The final concentrations of the components of each determination in 0.1 M Tris buffer, pH 7.0, containing 1.0 M NaCl were as follows: carboxypeptidase-A_γ, 0.023 mg/ml; 1,10-phenanthroline, 1×10^{-3} M; methylene blue, 0.095 mg/ml; Rose Bengal, 0.095 mg/ml. Photooxidation was accomplished by focusing a 500 watt projector on the sample from a distance of 18-20 cm. The esterase activity was measured as described in "Methods", using hippuryl-DL-phenyllactic acid as substrate. Table IV summarizes the experimental composition and experimental results of each determination.

Inhibition, reactivation, and photooxidation of carboxypeptidase-A_γ in the presence of EDTA. The dye sensitized photolability of carboxypeptidase-A_γ was determined in the presence of a second metal chelating agent, EDTA. The experimental conditions were arranged so that the final concentrations of the components were as follows: carboxypeptidase-A_γ, 0.023 mg/ml; EDTA, 5×10^{-4} M; methylene blue, 0.002 mg/ml. All components were dissolved in 0.1 M Tris buffer, pH 7.0, containing 1.0 M NaCl at 0° C. As in previous experiments, reversal of the inhibition resulting from the presence of

TABLE III

The effect of Methylene Blue-1,10-phenanthroline photooxidation of Carboxypeptidase-A_y on Esterase Activity

	μ l enzyme	ml buffer	ml O P	ml M B	min hr	μ l Zn ²⁺	spec. act. esterase
A	5	1.0	—	—	—	—	452
B	5	0.5	—	0.5	—	—	444
C	5	—	0.5	0.5	—	10	327
D	5	0.5	0.5	—	—	—	105
E	5	0.5	0.5	—	—	10	374
F	5	0.5	—	0.5	30	—	409
G	5	—	0.5	0.5	30	10	210

OP, 1,10-phenanthroline, 2×10^{-3} M; MB, methylene blue, 0.002 mg/ml; Zn²⁺, ZnCl₂, 0.1 M; were dissolved in 0.1 M Tris buffer, pH 7.0, containing 1.0 M NaCl. All data collected at 0° C. Photooxidation was by Method A.

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TABLE IV

Photooxidation of Carboxypeptidase-A_γ with increased
Methylene Blue and Rose Bengal Concentrations

	μ l enzyme	ml buffer	ml MB	ml OP	ml RB	min h ν	μ l Zn ²⁺	spec. act. esterase
A	5	—	0.5	0.5	—	30	10	141
B	5	—	0.5	0.5	—	—	10	433
C	5	—	—	0.5	0.5	30	10	49.4
D	5	—	—	0.5	0.5	—	10	374
E	5	0.5	0.5	—	—	30	—	360
F	5	0.5	—	—	0.5	30	—	42.3

The components: MB, methylene blue, 0.002 mg/ml; OP, 1,10-phenanthroline, 2×10^{-3} M; RB, Rose Bengal, 0.095 mg/ml; Zn²⁺, ZnCl₂, 0.1 M; were dissolved in 0.1 M Tris buffer, pH 7.0, containing 1.0 M NaCl. All data obtained at 0° C. Photooxidation was by Method A.

the metal chelator was accomplished by the addition of appropriate quantities of 10^{-1} M ZnCl_2 in 0.1 M Tris buffer, pH 7.0, containing 1.0 M NaCl. The peptidase activity was determined as described in "Methods", using hippuryl-L-phenylalanine as substrate. Table V summarizes the experimental composition and results of each determination.

Photosensitization with 1,10-phenanthroline. The photosensitizing effect of 1,10-phenanthroline was determined in the following manner: carboxypeptidase- A_γ was dissolved in 0.1 M Tris buffer, pH 7.0, containing 1.0 M NaCl at 0°C ., at a concentration of 0.61 mg/ml. Methylene blue and 1,10-phenanthroline, when used, were dissolved in 0.1 M Tris buffer, pH 7.0, containing 1.0 M NaCl at concentrations of 0.099 mg/ml and 1×10^{-3} M respectively. Ten microliter aliquots of a 0.1 M ZnCl_2 solution were used to reverse the effects of 1,10-phenanthroline. Peptidase activity was determined as described in "Methods", using hippuryl-L-phenylalanine as substrate. Photo-oxidation was by Method A. Table VI summarizes the experimental composition and results of these determinations.

Preparation of apoenzyme on Sephadex G-25 column. Apocarboxypeptidase- A_γ was prepared according to the methods of Vallee.⁽³⁾ A 10 ml hypodermic syringe was prepared as a chromatography column. A small filter paper disc was cut and placed in the end of the syringe to serve as a support for the Sephadex resin. The filter paper support was washed with 6 N HCl, and with deionized water. The Sephadex G-25 resin was then suspended in 0.1 M Tris buffer, pH 7.5, containing 1.0 M NaCl, and placed under a vacuum to remove air. This suspension then was poured into the syringe completing the preparation of the column. The column was fitted with a constant pressure reservoir. The capacity of the column to retain 1,10-phenanthroline was determined by placing 1.0 ml of a 0.1 M Tris buffer, pH 7.5, containing 1.0 M NaCl, saturated with 1,10-phenanthroline on the column and

TABLE V

Inhibition, Reactivation, and Photooxidation of
Carboxypeptidase-A_γ in the presence of EDTA

	μ l enzyme	ml buffer	ml EDTA	ml MB	min h ν	μ l Zn ²⁺	spec. act. peptidase
A	5	1.0	—	—	—	—	
B	5	0.75	0.25	—	—	5	45.2
C	5	0.75	0.25	—	—	5	54.2
D	5	0.25	0.25	0.5	30	5	22.6

The initial concentrations of reagents in 0.1 M Tris buffer, pH 7.0, containing 1.0 M NaCl were as follows: EDTA, 2×10^{-3} M; MB, 0.004 mg/ml; ZnCl₂, 0.1 M. Photooxidation Method A.

TABLE VI

Photosensitization of 1,10-Phenanthroline

	μ l enzyme	ml O P	ml MB	ml buffer	min hr	μ l Zn ²⁺	spec. act. peptidase
A	100	0.5	—	0.5	—	—	17.7
B	100	0.5	—	0.5	—	10	68.8
C	100	0.5	—	0.5	30	10	68.8
D	100	—	—	1.0	—	—	75.5
E	100	—	0.5	0.5	30	—	57.4

The initial concentrations of reagents dissolved in 0.1 M Tris buffer, pH 7.0, containing 1.0 M NaCl were as follows: OP, 2×10^{-3} M; MB, 0.018 mg/ml; ZnCl₂, 0.1 M. Photooxidation Method A.

eluting with 0.1 M Tris, 1.0 M NaCl, pH 7.5 buffer. the 1,10-phenanthroline did not appear until at least 200 drops of eluent had been collected.

The actual preparation of the apoenzyme was as follows: 10 μ l of a carboxypeptidase-A γ solution (4.03 mg/ml) was incubated for one hour at 0° C. with 1 ml of 0.1 M Tris buffer, pH 7.5, containing 1.0 M NaCl and saturated with 1,10-phenanthroline. The solution then was placed on the column described above and eluted with 0.1 M Tris buffer, pH 7.5 at 0° C., containing 1.0 M NaCl. The fraction containing drops 78 to 123 was collected in a plastic tube which had been carefully treated with a 95% ethanol-EDTA solution to reduce metal ion contamination. This enzyme-containing fraction then was assayed for peptidase activity. The presence of apoenzyme was demonstrated by the ability to increase the peptidase activity 60% upon the addition of a small quantity of Zn²⁺ as shown in Table VII.

Precautions were taken against metal ion contamination to obtain satisfactory results when the apoenzyme solution was assayed. All cuvettes and pipettes were treated beforehand in an H₂O-Ethanol (50:50, v:v) bath containing EDTA. Following this soaking, they were rinsed with deionized water. The substrate and all buffers which came in contact with the apoenzyme were extracted with dithizone before use. Only under these conditions could reliable assays be obtained.

Photooxidation of apocarboxypeptidase-A γ with methylene blue. Apocarboxypeptidase-A γ was prepared as previously described, and assayed for remaining and reactivatable peptidase activity. The results of these assays are given in Table VIII.

The photolability of this apoenzyme preparation was then determined in the following buffer solution; 0.1 M Tris, 1.0 M NaCl, pH 7.5 at 0° C. buffer and 0.1 M Tris buffer, pH 7.5 at 0° C., containing 1.0 M NaCl and 0.199 mg/ml methylene blue, and an identical methylene blue free buffer extracted with a carbontetrachloride dithizone

TABLE VII

Preparation of Apoenzyme
on Sephadex G-25 Column

μ l apoenzyme	μ l Zn^{2+}	$\Delta A / \text{min } 254 \text{ m}\mu$
100	—	0.0064
100	10	0.0156

The ZnCl_2 , 0.1 M, was dissolved in 0.1 M Tris buffer, pH 7.0, containing 1.0 M NaCl.

TABLE VIII

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Reactivation of Apocarbonylpeptidase-Ay
with Zinc Ions

μl apoenzyme	μl Zn^{2+}	$\Delta A / \text{min } 254 \text{ m}\mu$
100	0	0.0060
100	1	0.0180

ZnCl_2 concentration was 0.1 M in 0.1 M Tris buffer, pH 7.0, containing 1.0 M NaCl.

solution to remove contaminating metal ions. The trace quantities of carbontetrachloride dissolved in these buffers were removed by bubbling air which had been purified by passing through sulfuric acid and calcium hydroxide solutions through them. The buffers were then used to prepare the solutions described in Table IX. Precautions were taken against metal ion contamination where necessary as previously described.

Photooxidation of carboxypeptidase-A_γ and peptidase activity.

The effect of photooxidation on the peptidase activity of carboxypeptidase-A_γ was determined in the presence of Rose Bengal as well as the methylene blue 1,10-phenanthroline system. The final concentrations of all reagents in 0.1 M Tris buffer, pH 7.0 at 0° C., containing 1.0 M NaCl were as follows: carboxypeptidase-A_γ, 0.023 mg/ml, 1,10-phenanthroline, 1×10^{-3} M, methylene blue, 0.099 mg/ml, Rose Bengal, 0.095 mg/ml. The technique used in the photooxidation was that of Method A. The peptidase activity was determined as described in "Methods", using hippuryl-L-phenylalanine as substrate.

As in previous cases, 1,10-phenanthroline inhibition was reversed by the addition of a suitable aliquot of 0.1 M ZnCl₂ solution just prior to assay. Table X summarizes the experimental composition and results of each determination.

Rate of loss of peptidase activity of carboxypeptidase-A_γ when photooxidized in presence of Rose Bengal. Ten microliters of a carboxypeptidase-A_γ solution (2.8 mg/ml) was diluted to 1.0 ml with 0.1 M Tris buffer, pH 7.5 at 4° C., containing 1.0 M NaCl and 0.049 mg/ml Rose Bengal, giving a final enzyme concentration of 0.028 mg/ml. This solution was then exposed to several intervals of light, subsequent to which 50 μ l were removed and analyzed for peptidase activity. Photooxidation was done using Method B. Peptidase activity was determined as described in "Methods", using hippuryl-L-phenylalanine as substrate. The data in Table XI summarize the results of these

TABLE IX

Photooxidation of Apocarboxypeptidase-A_v
with Methylene Blue

μ l apoenz.	ml buffer	ml MB	min h ν	μ l Zn ²⁺	$\Delta A/\text{min}254$
100	0.5	0.5	30	—	0.0052
				1.5	0.0076
100	0.5	0.5	—	—	0.0074
				1.5	0.0162

The initial concentrations of reagents dissolved in 0.1 M Tris buffer, pH 7.0, containing 1.0 M NaCl were as follows: OP, 2×10^{-3} M; MB, 0.199 mg/ml; ZnCl₂, 0.1 M. $\Delta A/\text{min } 254 \text{ m}\mu$ are reported here because apoenzyme concentration was not determined. Photooxidation Method A.

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TABLE X

Photooxidation of Carboxypeptidase-A_v
and Peptidase Activity

	μ l enzyme	ml buffer	ml MB	ml OP	ml RB	min hv	μ l Zn ²⁺	spec. act. peptidase
A	5	1.0	—	—	—	—	—	50.4
B	5	—	0.5	0.5	—	30	10	5.7
C	5	—	0.5	0.5	—	—	10	42.1
D	5	—	—	0.5	0.5	30	10	—
E	5	—	—	0.5	0.5	—	10	19.5
F	5	0.5	0.5	—	—	30	—	51.5
G	5	0.5	—	—	0.5	30	—	43
H	5	0.5	—	—	0.5	—	—	44.2

The initial concentrations of reagents in 0.1 M Tris buffer, pH 7.0, containing 1.0 M NaCl were as follows: MB, 0.199 mg/ml; RB, 0.198 mg/ml; OP, 2×10^{-3} M; ZnCl₂, 0.1 M. Photooxidation Method A.

TABLE XI

Rate of loss of Peptidase Activity of Carboxypeptidase-A_y
when Photooxidized in presence of Rose Bengal

min $h\nu$	$\Delta A / \text{min } 254 \text{ m}\mu$	spec. act. peptidase
0	0.0200	61.5
1	0.0174	53.5
2	0.0080	24.6
3	0.0072	21.5
5	0.0052	15.4
7	0.0048	14.8
8	0.0032	9.8
9	0.0034	10.4
11	0.0028	8.6
12	0.0030	9.2
13	0.0026	8.0
15	0.0034	10.4

determinations. The data of Table XII summarize the results of an identical experiment in which esterase activity was determined.

Rose Bengal photooxidation, esterase activity. Ten microliters of a carboxypeptidase solution (2.8 mg/ml) in Tris buffer was diluted to 1.0 ml with 0.1 M Tris buffer, pH 7.5 at 4° C., containing 1.0 M NaCl and 0.049 mg/ml Rose Bengal. This solution then was divided among seven small plastic cups which were held in an aluminum block. The block was cooled by allowing it to stand in an ice bath. Each plastic cup was capable of being independently covered in such a manner as to exclude all light. Following the distribution of the enzyme solution, the light source was turned on and each cup covered after a certain exposure time. The data in Table XIII summarize the results of this determination. The esterase activity was determined as described in "Methods", using hippuryl-DL-phenyllactic acid as substrate. The data in Tables XIV and XV summarize the results of similar determinations. The light source employed was that described in Method B of "Methods and Materials". The data of Table XVI summarize an identical experiment in which peptidase activity was measured.

Photooxidation of carboxypeptidase-A_v with crystal violet sensitizer. A crystal violet solution was prepared by dissolving 3.9 mg of crystal violet dye in 38 ml of 0.1 M Tris buffer, pH 7.5 at 25° C., containing 1.0 M NaCl. Five microliters of a carboxypeptidase-A_v solution (9.21 mg/ml) was then diluted in the dark with 0.25 ml of a 2×10^{-3} M 1,10-phenanthroline solution in 0.1 M Tris, 1.0 M NaCl buffer and with 0.25 ml of the crystal violet buffer described above. The light source used in the photooxidation was that described in Method B. The peptidase activity was determined by the usual methods following the reversal of the inhibition caused by the 1,10-phenanthroline with the addition of 10 μ l of a Zn²⁺ solution. Table XVII summarizes the results of these determinations.

TABLE XII

Rate of loss of Esterase Activity of Carboxypeptidase-A_y
when photooxidized in presence of Rose Bengal

time min	ΔA min 254 m μ	spec. act. esterase
15 sec	0.1068	329
30 sec	0.0586	180
1	0.0550	169
2	0.0250	77
3	0.0228	70
4	0.0218	68
5	0.0248	74
6	0.0192	59
7	0.0182	56

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TABLE XIII

Rose Bengal Photooxidation, Esterase Activity

time sec	$\Delta A / \text{min } 254 \text{ m}\mu$	spec. act. esterase
0	0.1216	382
15	0.0932	277
45	0.0788	242
60	0.0776	239
120	0.0560	174
180	0.0388	119
360	0.0230	71

TABLE XIV

Rose Bengal Photooxidation, Esterase Activity

time sec	$\Delta A / \text{min } 254 \text{ m}\mu$	spec. act. esterase
0	0.0920	283
15	0.0760	234
30	0.0664	204
45	0.0660	203
60	0.0548	169
75	0.0516	158
90	0.0482	148
105	0.0332	102
120	0.0342	105
420	0.0112	34.5

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TABLE XV

Rose Bengal Photooxidation, Esterase Activity

time sec	$\Delta A / \text{min } 254 \text{ m}\mu$	spec. act. esterase
0	0.0880	271
15	0.0504	155
30	0.0356	110
45	0.0236	73
60	0.0212	65
75	0.0176	54
90	0.0144	44
105	0.0136	42
120	0.0104	32

TABLE XVI

Rose Bengal Photooxidation, Peptidase Activity

time hr sec	$\Delta A / \text{min } 254 m\mu$	spec. act. peptidase
0	0.0146	45.0
15	0.0080	24.5
30	0.0052	16.0
45	0.0042	13.0
60	0.0032	9.8
75	0.0032	9.8
90	0.0030	9.0

TABLE XVII

Photooxidation of Carboxypeptidase-A_γ with
Crystal Violet Sensitizer

	μl enzyme	ml OP	ml CV buffer	min hv	μl Zn ²⁺	spec. act. peptidase
A	5	0.25	0.25	—	10	67.4
B	5	0.25	0.25	—	—	10.0
C	5	0.25	0.25	5	10	61.9
D	5	0.25	0.25	10	10	63.9
E	5	0.25	0.25	15	10	63.6
F	5	0.25	0.25	30	10	57.3

The initial concentrations of reagents, dissolved in 0.1 M Tris buffer, pH 7.0, containing 1.0 M NaCl were as follows: OP, 2×10^{-3} M; CV, 1.02 mg/ml; ZnCl₂, 0.1 M. Photooxidation was by Method B.

pH Dependence of photooxidation of Carboxypeptidase-A_γ in the presence of 1,10-phenanthroline and methylene blue. Twenty microliters of a 2×10^{-3} M 1,10-phenanthroline solution in 0.1 M Tris buffer, containing 1.0 M NaCl, and 20 ml of a 0.198 mg/ml solution of methylene blue in the same buffer were mixed and the pH adjusted to the following values at 0° C.: 8.88, 8.47, 7.96, 7.55, 6.96, 6.52, 5.49. As each pH value was obtained, two 1 ml aliquots were removed, each aliquot placed in a separate tube, and kept at 0° C. The pair of aliquots, for each indicated pH value, were then treated in the following manner: five microliters of a solution of carboxypeptidase-A_γ (4.03 mg/ml) were introduced into each tube. One tube was kept in the dark, while the other was photooxidized for ten minutes. After this ten minute period, both tubes were treated with 10 μ l of a 10^{-1} M ZnCl₂ solution, and the peptidase activity determined. Photooxidation was accomplished by focusing a 500 watt projector on the sample from a distance of 18-20 cm. The peptidase activity was determined as previously described. Table XVIII summarizes the results of these determinations.

pH Dependence of photooxidation of carboxypeptidase-A_γ with Rose Bengal as sensitizer. Tris buffer, 0.1 M, containing 1.0 M NaCl was used to produce buffers having the pH values at 0° as shown in Table XIX. Concentrated HCl was added to the starting solution until each pH value was attained. During this procedure, 0.5 ml of each succeeding buffer was removed and placed in a test tube. Rose Bengal (0.1 mg/ml) dissolved in 0.1 M Tris buffer, containing 1.0 M NaCl, was likewise adjusted to the indicated pH values at 0° C. Again as each pH value was attained, 0.5 ml was removed and mixed with its counterpart above. Five microliters of a carboxypeptidase-A_γ solution (3.53 mg/ml) was then added in a darkened room to each tube, giving a final enzyme concentration of 0.023 mg/ml. One hundred microliters of each resulting enzyme solution was removed and assayed for peptidase activity. Following this initial activity determination, each solution was exposed for five minutes to a 500 watt light source, according to Method A.

TABLE XVIII

pH Dependence of Photooxidation of Carboxypeptidase-A_γ in the presence of 1,10-Phenanthroline and Methylene Blue

pH	specific activity unoxidized	specific activity oxidized
8.88	67.4	30.1
8.47	65.5	31.0
7.96	60.5	32.0
7.55	—	38.8
6.96	—	50.5
6.52	67.4	58.6
5.49	—	56.5

Subsequent to this exposure another one hundred microliter aliquot was removed, and the peptidase activity redetermined. The results of these determinations are represented in Table XIX.

Effect of peptidase substrate on rate of photooxidation with Rose Bengal. In order to determine if the photochemical deactivation of carboxypeptidase-A_γ was the result of an oxidation near or at the active center of the enzyme, a substrate protection experiment was undertaken. All reagents used in the experiment were dissolved in 0.1 M Tris buffer, pH 7.0 at 0° C., containing 1.0 M NaCl, at the following concentrations: carboxypeptidase-A_γ; 0.023 mg/ml; Rose Bengal, 0.095 mg/ml; hippuryl-L-phenylalanine, 5×10^{-4} M. Photooxidation was done according to Method A. The peptidase activity was determined as described in "Methods", using hippuryl-L-phenylalanine as substrate. The presence of partially hydrolyzed substrate did not affect the assay results. Table XX summarizes the experimental composition and results of each determination.

Substrate protection in the Rose Bengal system. Substrate protection was attempted by photooxidizing carboxypeptidase-A_γ in the presence of 5×10^{-4} M hippuryl-L-phenylalanine. The rates of deactivation in the presence and absence of substrate were then compared by preparing two experimental mixtures. The final concentrations of reagents in these mixtures were as follows: carboxypeptidase-A_γ, 0.023 mg/ml; Rose Bengal, 0.095 mg/ml; and hippuryl-L-phenylalanine 5×10^{-4} M. All reagents were dissolved in 0.1 M Tris buffer, pH 7.0 at 0° C., containing 1.0 M NaCl. These experimental mixtures were then exposed to three consecutive five minute intervals of light. Between each interval a suitable aliquot was removed and the peptidase activity determined in the usual manner. Method A was used to photooxidize the enzyme. Table XXI summarizes the experimental composition and results of these determinations.

TABLE XIX

pH Dependence of Photooxidation of Carboxypeptidase-A γ
with Rose Bengal as sensitizer

pH	spec. act. unoxidized	spec. act. oxidized
9.0	30.8	2.37
8.5	30.0	1.89
8.0	30.8	3.31
7.5	30.8	4.73
7.0	30.8	6.15
6.5	27.9	7.10
6.0	30.8	7.56

TABLE XX

Effect of Peptidase Substrate on Rate of
Photooxidation with Rose Bengal

	μ l enzyme	ml buffer	ml sub.	ml RB	min hv	spec. act. peptidase
A	5	0.5	—	0.5	—	39.0
B	5	0.5	—	0.5	30	3.6
C	5	0.5	0.5	—	—	45.4
D	5	—	0.5	0.5	30	6.5

TABLE XXI

Substrate Protection, Rose Bengal System

	μ l enzyme	ml substrate	ml RB	min hr	$\Delta A/\text{min}$
A	5	0.5	0.5	5	0.0052
				10	0.0022
				15	—
B	5	—	0.5	5	0.0040
				10	0.0028
				15	—

Protection against the photooxidation by hippuryl-L-phenylalanine. A 0.1 M hippuryl-L-phenylalanine solution in 0.1 M Tris, 1.0 M NaCl, pH 7.5 was prepared by dissolving 327 mg of hippuryl-L-phenylalanine in enough of the buffer to give a total volume of 10 ml. Ortho-phenanthroline 2×10^{-3} M and methylene blue (0.199 mg/ml) were dissolved in 0.1 M Tris buffer, pH 7.5, containing 1.0 M NaCl, as previously described. As in previous experiments, the reversal of the inhibition due to the metal chelating agent was accomplished by the addition of a small aliquot of 0.1 M ZnCl_2 . The final enzyme concentration in all experimental solutions was 0.08 mg/ml. The assay was accomplished in the following manner: a buffered solution was prepared at the same concentration as that normally used in preparing the substrate solution, but the substrate was purposely omitted. Thus, when a 50 microliter aliquot of the experimental solution containing a large excess of substrate was introduced into this buffer, the final substrate concentration obtained by dilution was 10^{-3} M which is the normal concentration used in all assays. Photooxidation was accomplished by Method A. Table XXII summarizes the experimental composition and the results of each determination.

Substrate protection in the Rose Bengal system. A hippuryl-L-phenylalanine solution in 0.03 M Tris buffer, pH 7.4, containing 0.5 M NaCl buffer, was prepared as follows: 34.6 mg of hippuryl-L-phenylalanine was placed in a 10 ml volumetric flask and diluted to volume with a 10^{-3} M hippuryl-L-phenylalanine solution in 0.025 M Tris buffer, pH 7.5, containing 0.5 M NaCl. Rose Bengal was added to this solution so that the final concentration of dye was 0.04 mg/ml. Ten microliters of a carboxypeptidase- A_γ solution then was diluted to 1.0 ml with this buffered system and photooxidized for various periods of time. The peptidase activity was determined by removing a ten microliter aliquot and assaying in the normal manner. The photooxidation was done according to Method B. The results are summarized in Table XXIII.

TABLE XXII

Protection against the Photooxidation
by Hippuryl-L-Phenylalanine

	μ l enzyme	ml OP	ml sub.	ml MB	min hr	μ l Zn	ml buffer	spec. act. peptidase
A	20	0.36	—	—	—	10	1.64	25.3
B	20	0.36	—	—	—	—	1.64	2.5
C	20	0.36	—	1.0	30	—	0.64	4.1
D	20	0.36	—	1.0	30	10	0.64	7.7
E	20	0.36	0.64	1.0	—	10	—	22.9
F	20	0.36	0.64	1.0	30	10	—	16.9

The initial concentrations of reagents dissolved in 0.1 M Tris buffer, pH 7.0, containing 1.0 M NaCl were: OP 2×10^{-3} M; substrate, 0.01 M; 0.199 mg/ml; ZnCl_2 , 0.1 M.

TABLE XXIII

Substrate Protection Rose Bengal System

μ l enzyme	ml "sub" buffer	min hv	ml tris buffer	spec. act. peptidase
10	1.0	5	—	68.3
10	1.0	—	—	80.9
10	—	5	1.0	23.6

Photooxidation in the presence of β -phenylpropionate. Into a small plastic cup was placed 950 μ l of a 0.1 M Tris buffer, pH 7.0, containing 1.0 M NaCl and 0.03 mg/ml of Rose Bengal. Fifty microliters of a 0.1 M Tris buffer, pH 7.0, containing 2×10^{-2} M β -phenylpropionate and 1.0 M NaCl was mixed with the Rose Bengal buffer. A control buffer solution containing no β -phenylpropionate was prepared as above. To each solution 10 μ l of a carboxypeptidase-A γ solution (8.1 mg/ml) was added in the dark. Each solution was assayed for esterase activity in the usual manner and then exposed for various periods of time to a 500 watt light source. The enzyme was photooxidized according to Method B. Tables XXIV and XXV summarize the results of two separate determinations.

Glycyltyrosine protection. Glycyl-L-tyrosine, 6.3 mg, was dissolved in 0.1 M Tris buffer, pH 7.5, containing 1.0 M NaCl. The final concentration of glycyl-L-tyrosine was 0.001 M. Nine hundred eighty microliters of this buffered solution was then added to a small plastic cup and 10 μ l of a Rose Bengal water solution (300 mg/100 ml) was added. Ten microliters of a carboxypeptidase-A γ solution (8.1 mg/ml) was added, and the resulting solution kept in the dark. The peptidase activity was determined and then the solution was exposed to light for various periods of time. The light source was that described in Method B. The peptidase activity was measured as described in "Methods", using hippuryl-L-phenylalanine as substrate. Table XXVI summarizes the results of these determinations.

Aggregation subsequent to photooxidation of carboxypeptidase-A γ . A special column was prepared in an attempt to remove the sensitizing dye and also to determine if any conformational change occurred subsequent to photooxidation of carboxypeptidase-A γ . The column was prepared as follows: a 5 mm x 40 cm column was packed with 27 cm of Sephadex G-75, which had been slurried in 0.1 M Tris buffer, pH 7.5, containing 1.0 M NaCl. This resin then was capped with a 3.5 cm section of TEAE cellulose resin also suspended in 0.1 M Tris buffer.

TABLE XXIV

Photooxidation in the presence of β -Phenylpropionate

min hv	protected	control
0	941	994
5	1570	765
10	1000	610
20	980	302
35	823	126

TABLE XXV

Photooxidation in the presence
of β -Phenylpropionate

min hv	spec. act. esterase
0	1150
0	1515
0	1470
1	1340
5	1300
30	568

TABLE XXVI

Glycyltyrosine Protection

min hv	spec. act. peptidase
0	84.8 , 84.4
20	74.0
30	67.4
130	54.6

The entire column was jacketed with circulating water at 0° C. The elution of protein was followed by employing the flow cell of the Gilford spectrophotometer which was set to measure the absorbance at 278 m μ . The elution patterns of oxidized and unoxidized carboxypeptidase-A γ are shown in Figures 1, 2, and 3 respectively.

Chromatography of oxidized and unoxidized carboxypeptidase on TEAE cellulose. A column (31 x 0.5 cm) was packed with a slurry of TEAE cellulose in 0.1 M Tris buffer, pH 7.5, containing 1.0 M NaCl. Fifty microliters of a carboxypeptidase-A γ solution (9.21 mg/ml) was diluted with 200 μ l of either 0.1 M Tris buffer, pH 7.5, containing 1.0 M NaCl, or 0.1 M Tris buffer, pH 7.5, containing 1.0 M NaCl and 0.049 mg/ml of Rose Bengal. The curves of Figures 4, 5, 6, and 7 show the elution patterns and activity profiles of oxidized and unoxidized enzyme.

Chromatography of photooxidized carboxypeptidase-A γ on Sephadex G-75 column. A Sephadex G-75 column (1 cm x 25 cm) was prepared with 0.1 M Tris, 1.0 M NaCl, pH 7.5 buffer. The column was jacketed so that it could be cooled to near 0° C. when used in a normal temperature room. The flow rate was controlled by pumping the buffer and was determined before and after each run on the column. Samples were prepared in the following manner: 50 μ l of a carboxypeptidase-A γ solution (9.21 mg/ml) were diluted with 200 μ l of 0.1 M Tris buffer containing 0.049 mg/ml Rose Bengal, pH 7.5. The samples were then assayed for peptidase activity, photooxidized, reassayed, and placed on the column. The results of these determinations are shown in the elution patterns of Figures 8, 9, and 10.

Co $^{2+}$ activation of photooxidized carboxypeptidase-A γ . Co $^{2+}$ has been shown to activate carboxypeptidase-A γ , resulting in a twofold increase in peptidase activity, as compared to the native Zn $^{2+}$ containing enzyme. The inactivation of the enzyme with the metal chelator 1,10-phenanthroline has been demonstrated. This inactivation is almost completely reversible upon the addition of metal ion. Generally the

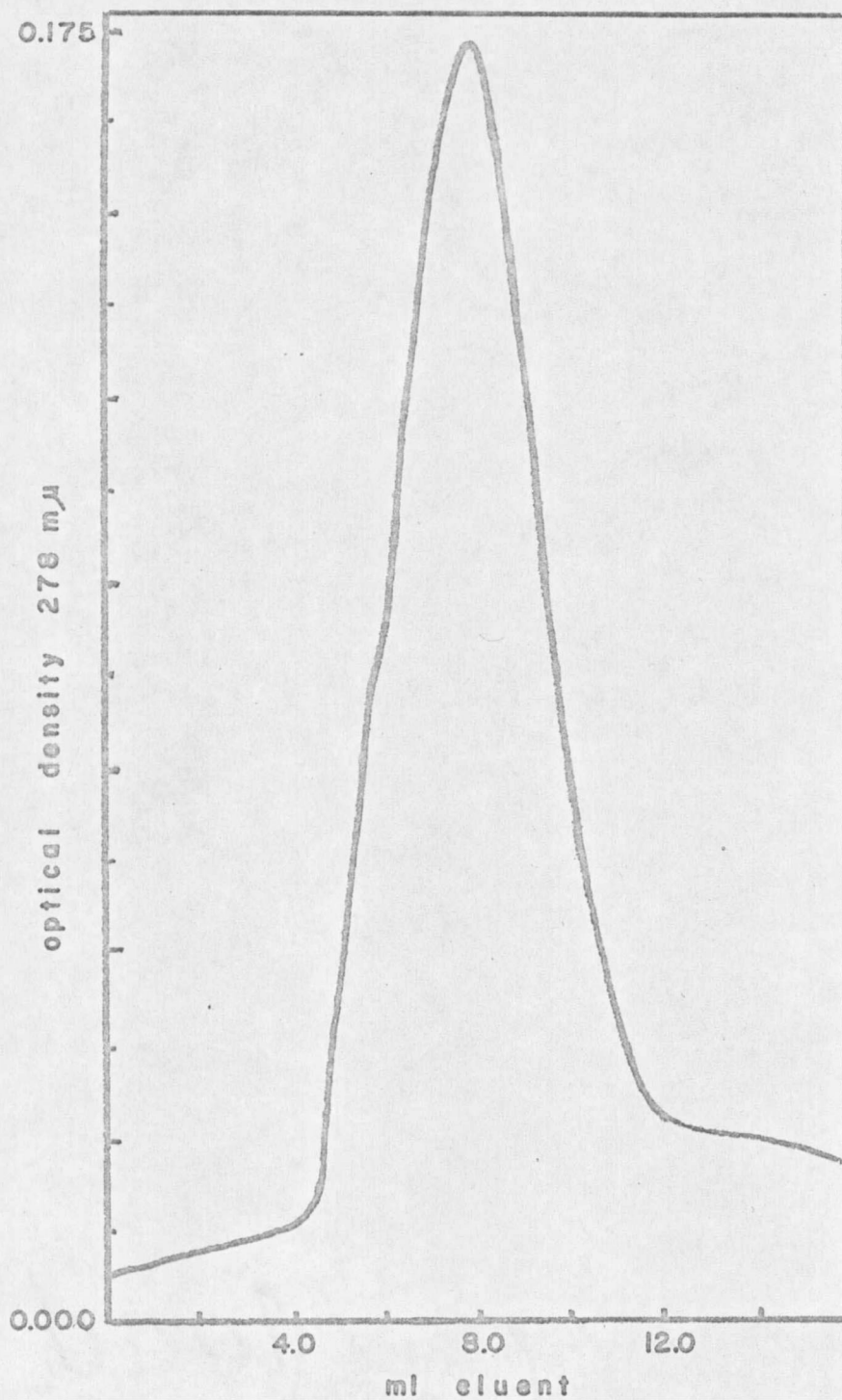


Figure 1. Sephadex G-75 chromatography of carboxypeptidase-A γ photooxidized twenty minutes.

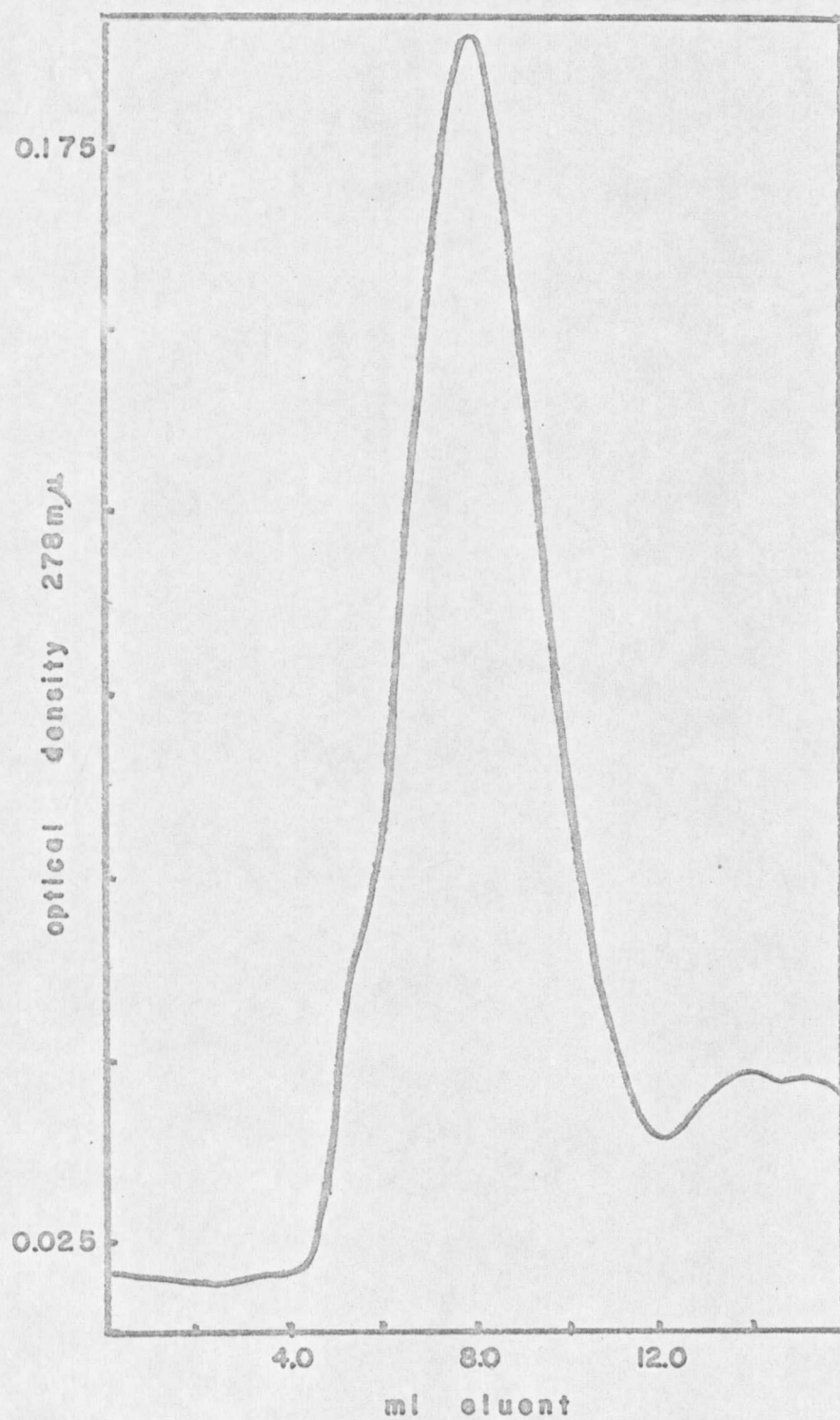


Figure 2. Sephadex G-75 chromatography of carboxypeptidase-A γ photooxidized sixty minutes.

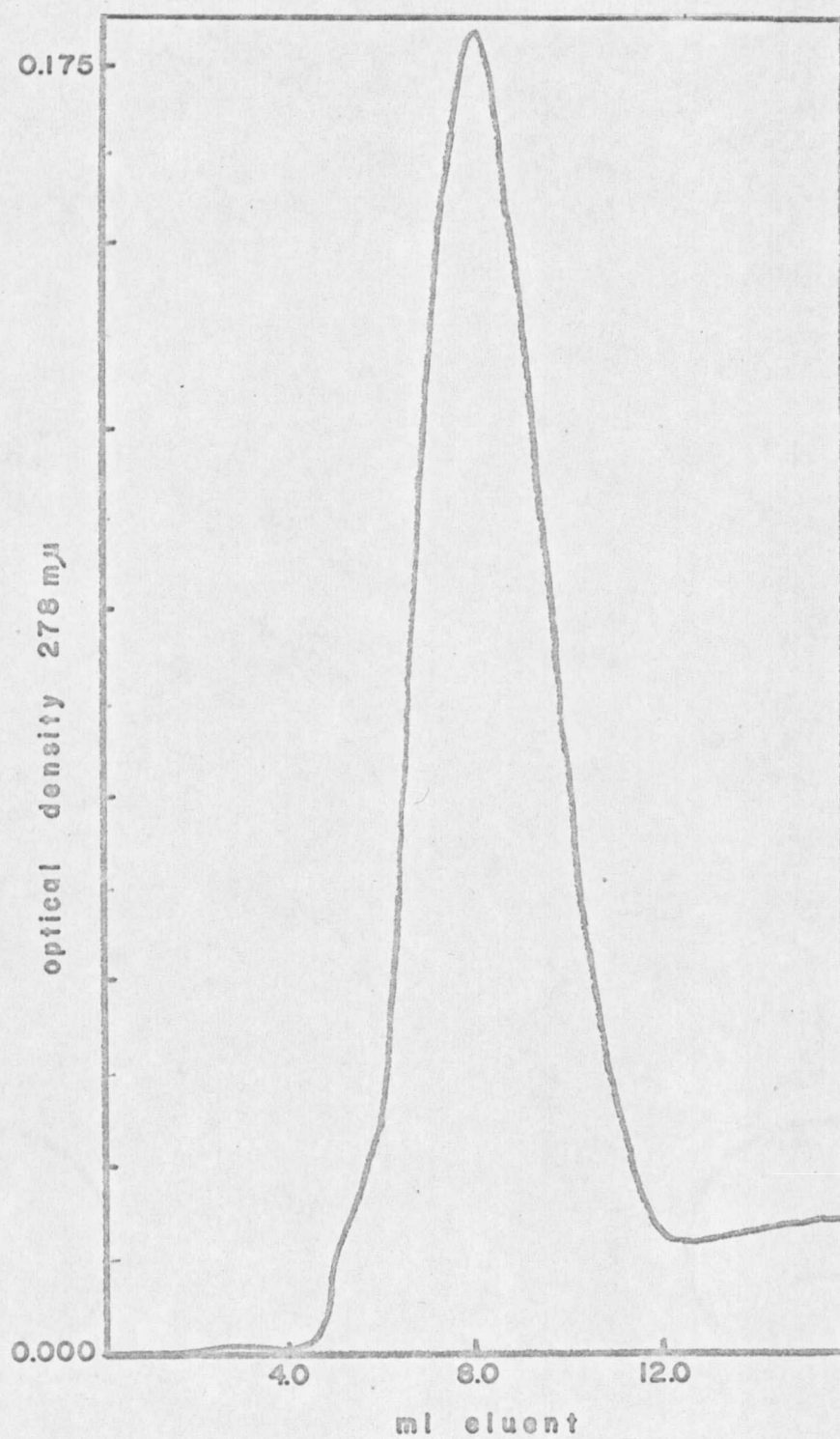


Figure 3. Sephadex G-75 chromatography of carboxypeptidase-A_γ unoxidized.

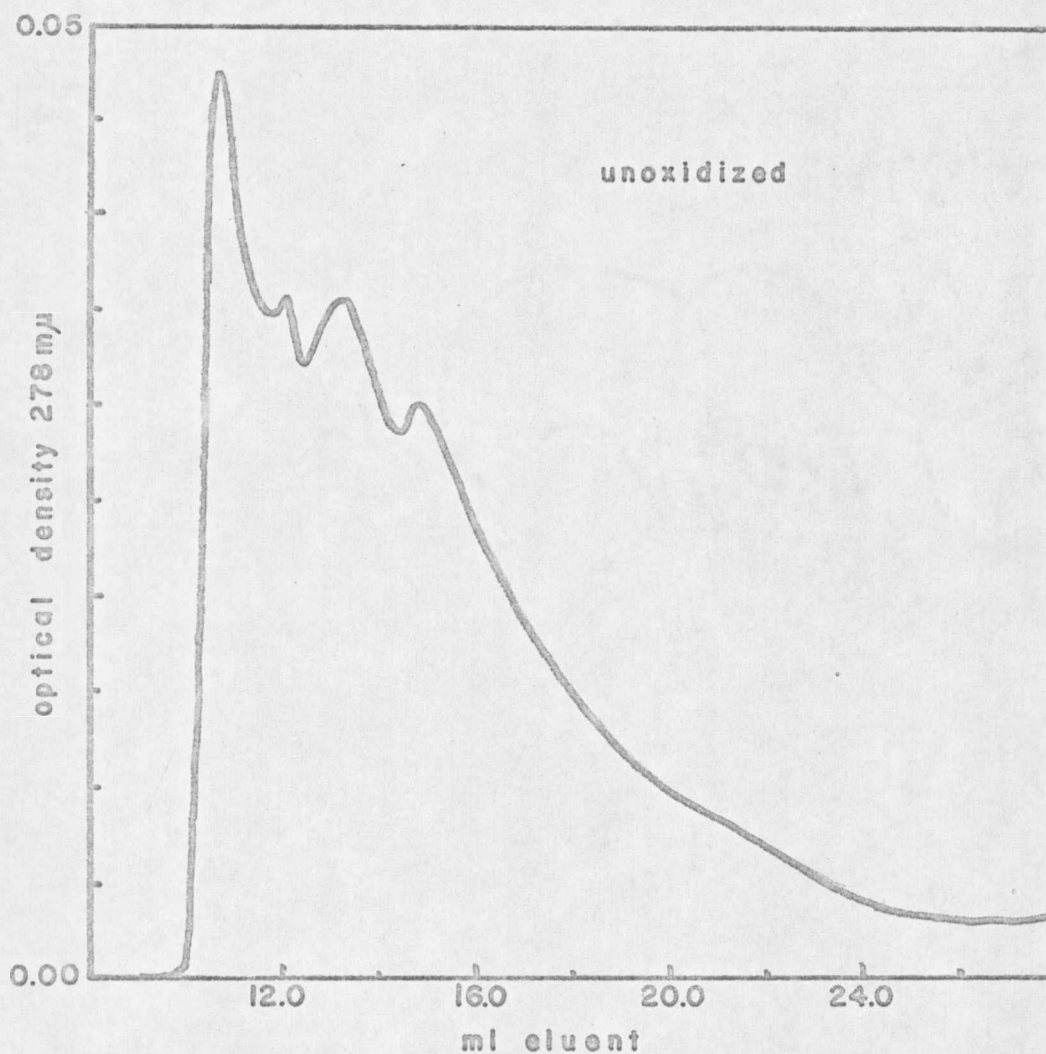


Figure 4. Chromatography of unoxidized carboxypeptidase-A γ on TEAE cellulose.

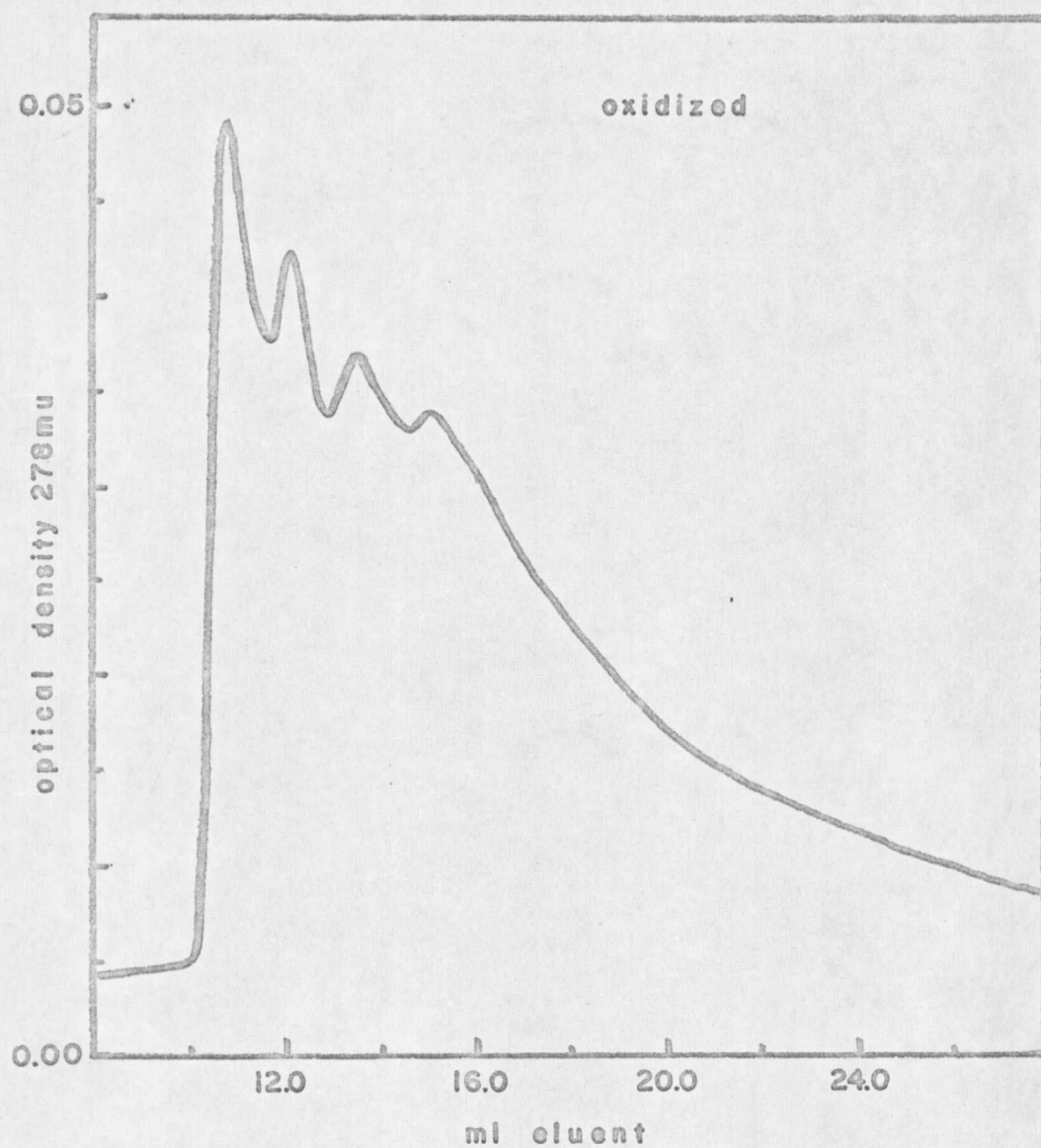


Figure 5. Chromatography of oxidized carboxypeptidase on TEAE cellulose, photooxidized twenty minutes.

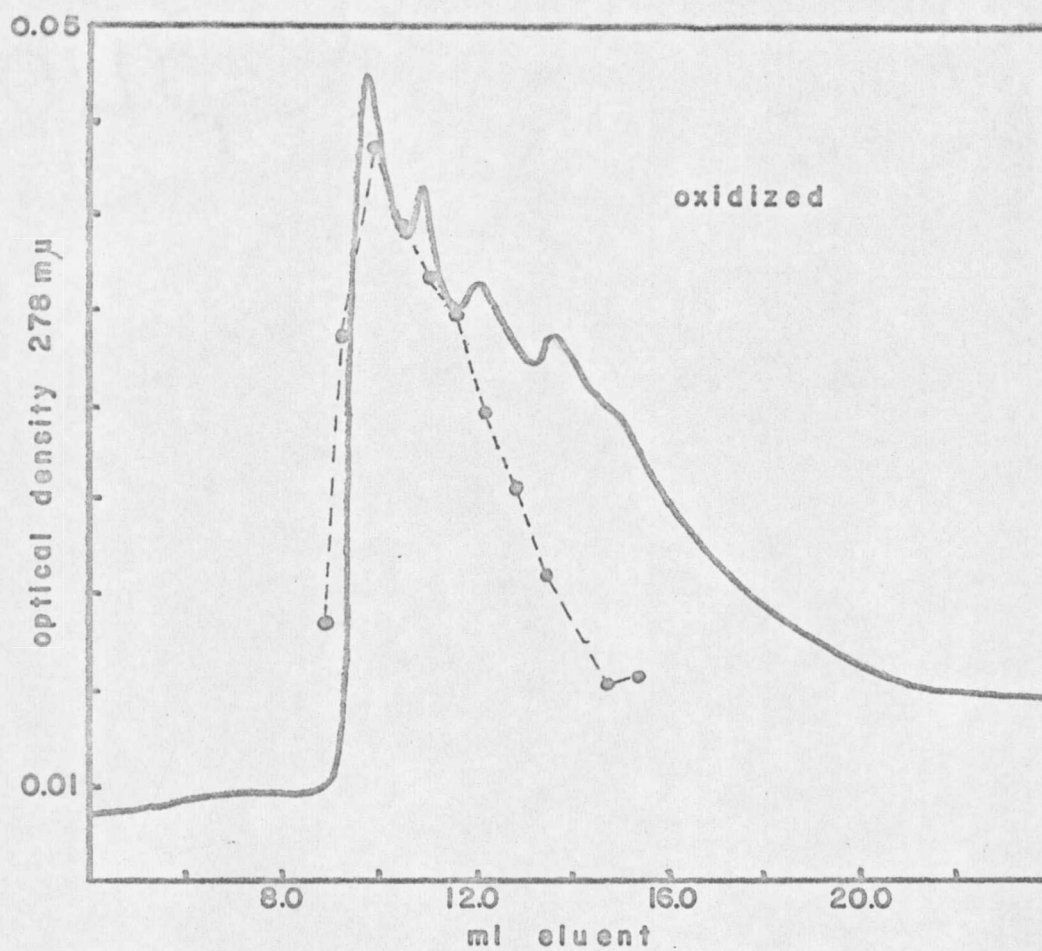


Figure 6. Chromatography of oxidized carboxypeptidase on TEAE cellulose. Photooxidized thirty minutes. (o-o) indicates activity profile of peaks.

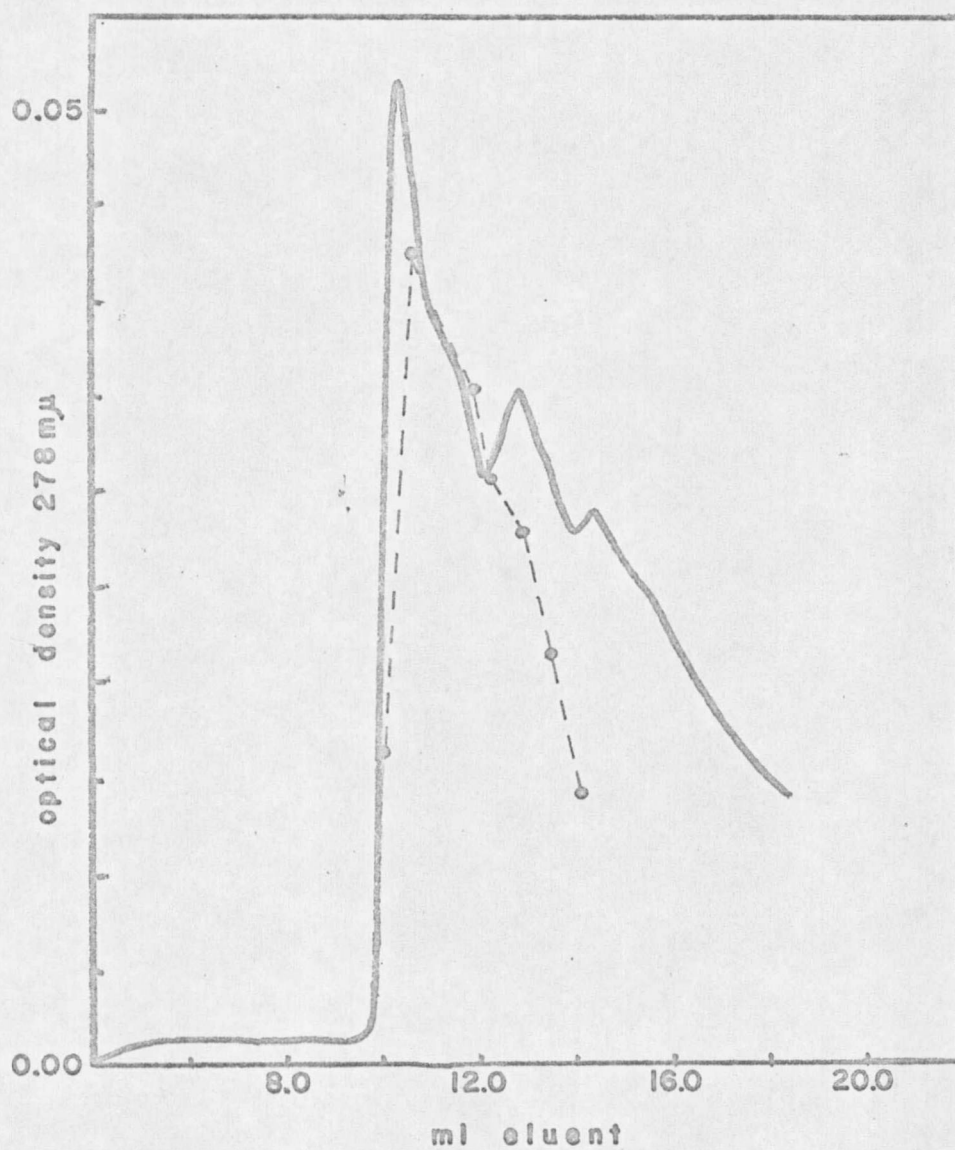


Figure 7. Chromatography of unoxidized carboxypeptidase-A γ on TEAE cellulose. (o-o) indicates activity profile of peaks.

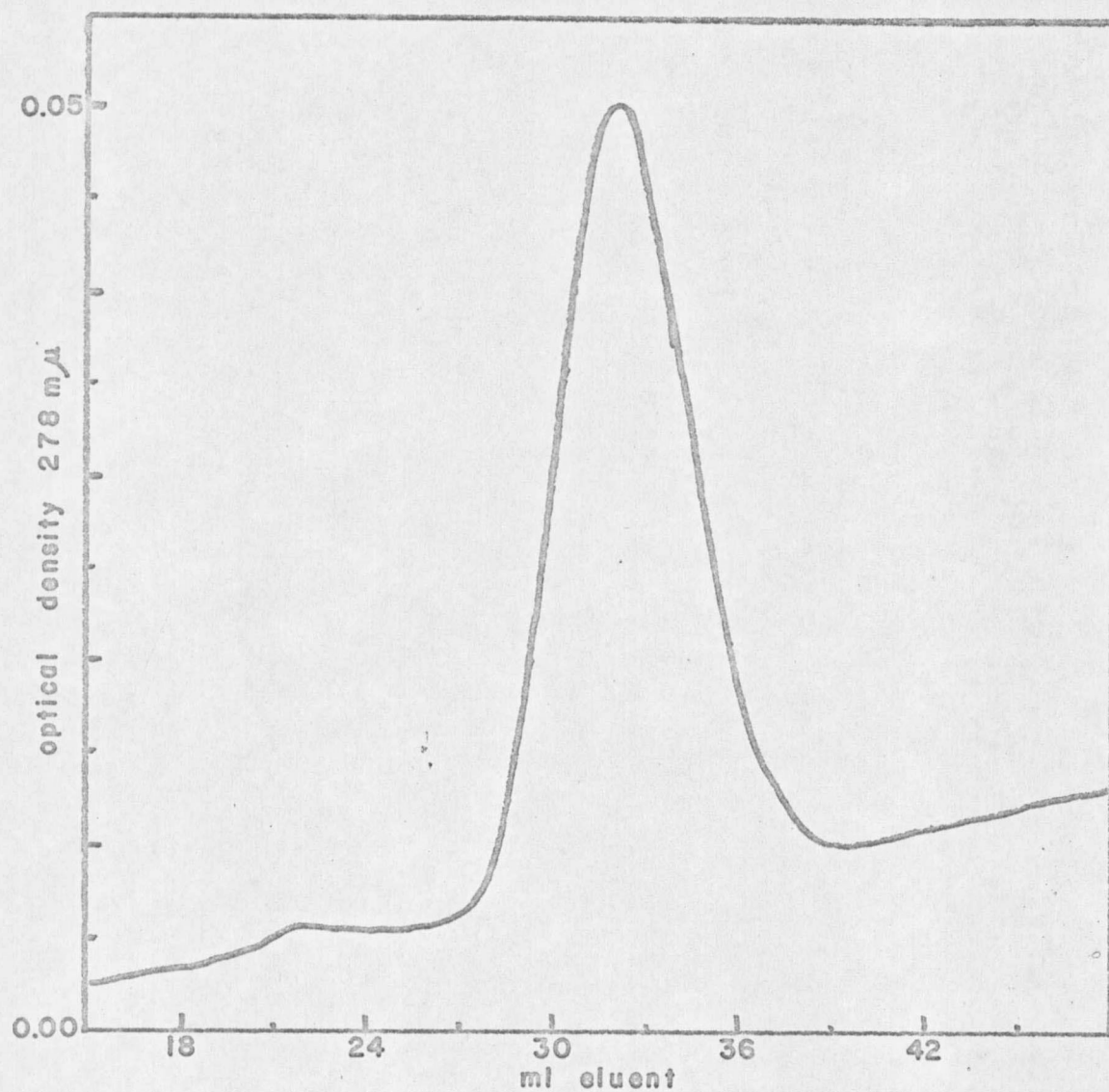


Figure 8. Chromatography of native carboxypeptidase-A γ on Sephadex G-75 column.

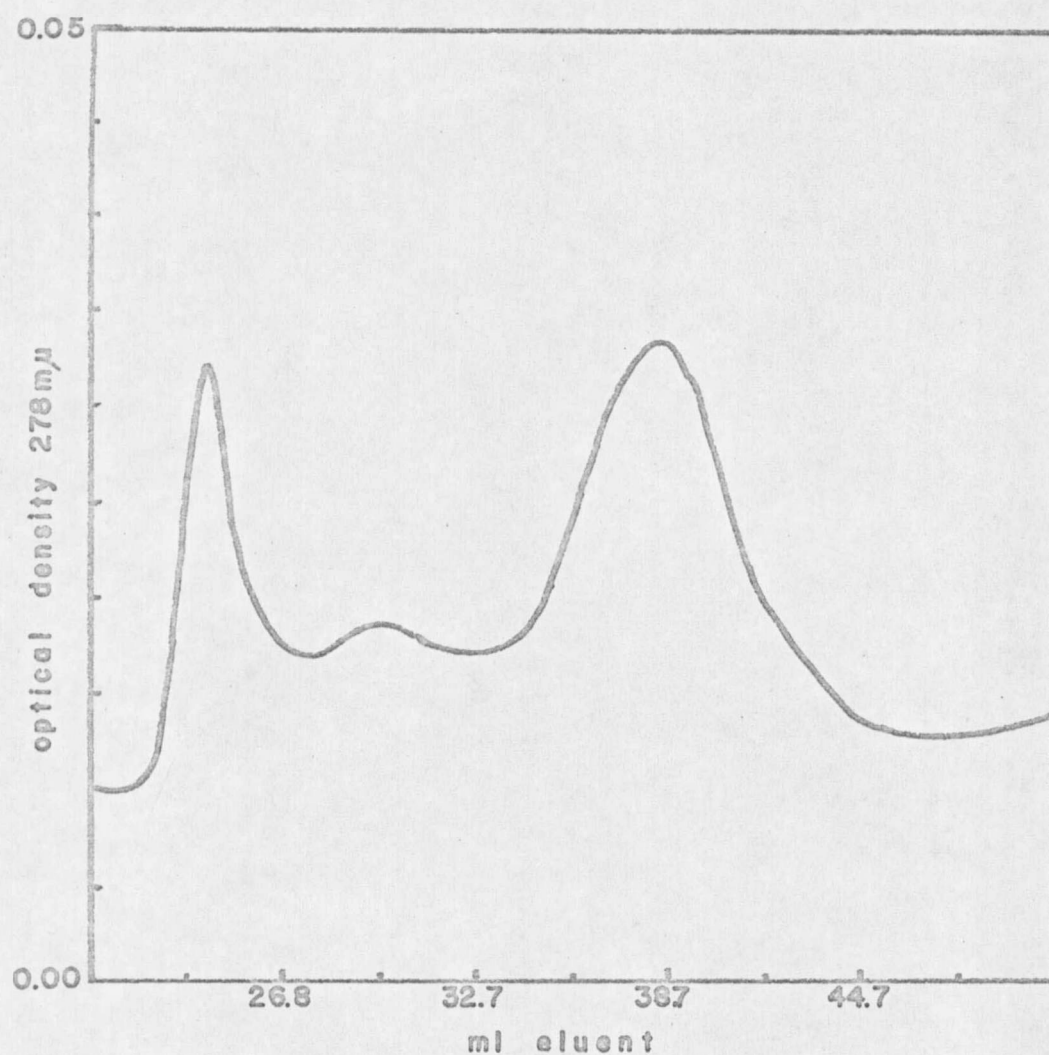


Figure 9. Chromatography of carboxypeptidase-A_v on Sephadex G-75 column. Photooxidized for seventy minutes before chromatography.

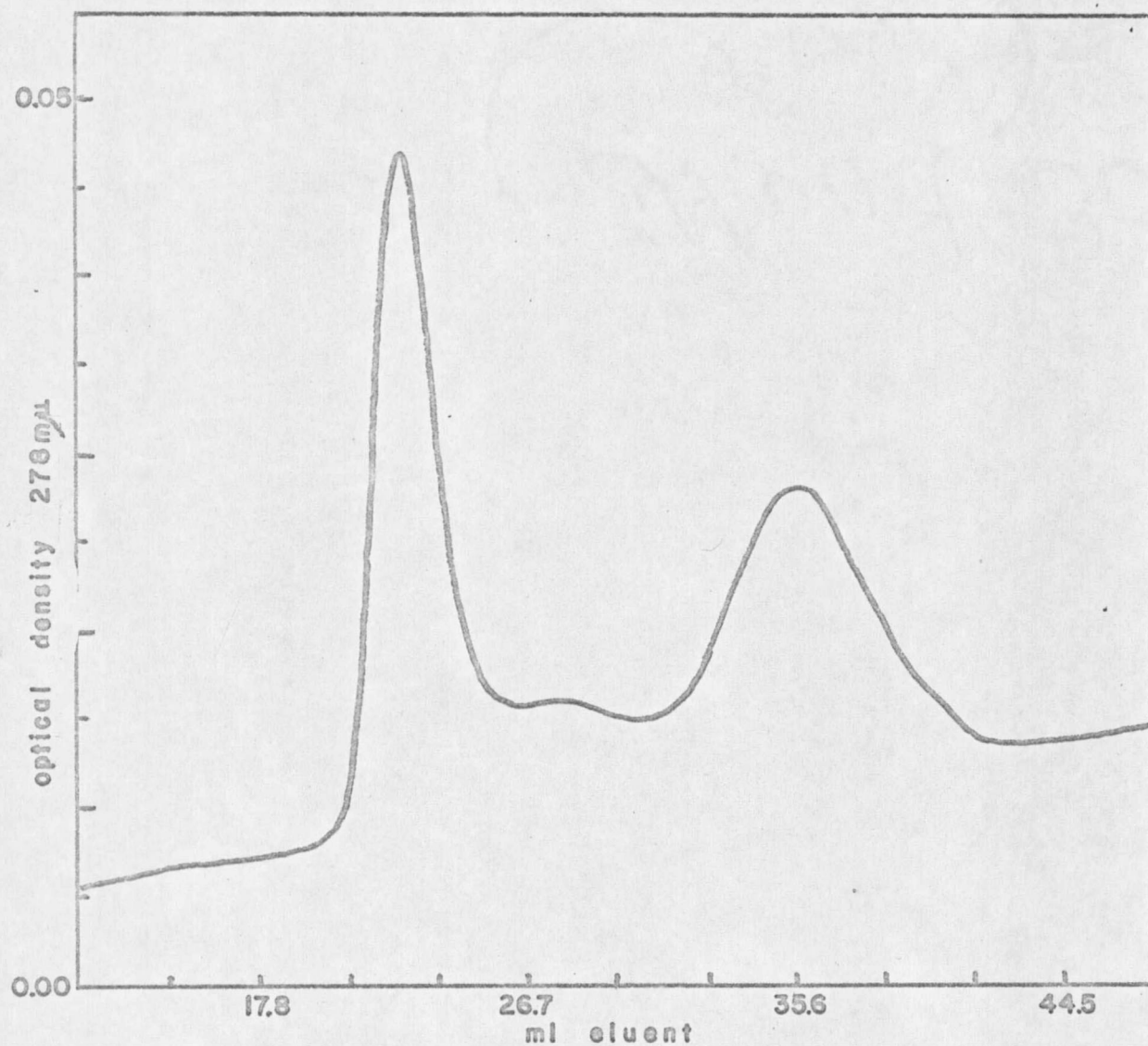


Figure 10. Chromatography of carboxypeptidase-A γ on Sephadex G-75 column. Photooxidized for one hundred twenty minutes before being placed on column.

reactivation has been carried out with the addition of sufficient Zn^{2+} in 0.1 M Tris, 1.0 M NaCl, pH 7.0, to the inactive system. In this series of experiments, Co^{2+} in 0.1 M Tris, 1.0 M NaCl, pH 7.0 was used to reactivate the system subsequent to photooxidation. As in previous experiments, methylene blue and 1,10-phenanthroline were used in final concentrations of 0.002 mg/ml and 5×10^{-4} M respectively. The technique used in the photooxidation was that of Method A. All reactions were carried out at 0°C . Peptidase activity was determined as described in "Methods", using hippuryl-L-phenylalanine as substrate. Table XXVII summarizes the experimental composition and results of each determination.

Preparation of cobalt-carboxypeptidase- A_γ by dialysis. A solution of carboxypeptidase- A_γ (3.50 mg/ml) dissolved in 0.05 M Tris, 0.05 M sodium acetate, 1.0 M NaCl, pH 7.95 at 8°C . was placed in a dialysis bag which had been previously treated with hot water, soaked in deionized water and in 0.05 M Tris, 0.05 M sodium acetate, 1.0 M NaCl, pH 8.0 buffer containing 10^{-2} M CoCl_2 . Dialysis was continued in this buffer for 48 hours, after which Co^{2+} free buffer was substituted. Following seven hours in the metal free buffer, a second change of metal free buffer was introduced and dialysis continued an additional 24 hours. At this point the protein concentration was determined by measuring the optical density at 278 m μ . Assuming the same absorptivity as the native zinc enzyme, the concentration was 2.76 mg/ml. A 5 μl aliquot of this preparation was then diluted to 1.0 ml and a 50 μl portion assayed for peptidase activity in the usual manner. The specific activity was determined to be 99.4 units/mg as compared to the 69.5 units/mg of the native enzyme, or a 1.43 fold increase in peptidase activity.

Photooxidation of cobalt-carboxypeptidase- A_γ and native carboxypeptidase- A_γ in same buffer system. Cobalt-carboxypeptidase- A_γ was prepared by dialysis against 0.1 M Tris, 1.0 M NaCl, buffer containing 10^{-2} M CoCl_2 for 48 hours. The activity of the cobalt-carboxypeptidase- A_γ was determined and found to be 1.5 times that of the native

TABLE XXVII

Co^{2+} Activation of Photooxidized Carboxypeptidase-A γ

	μl enzyme	ml buffer	ml OP	ml MB	min $h\nu$	μl Zn^{2+}	μl Co^{2+}	spec. act. peptidase
A	5	1.0	—	—	—	—	—	61.8
B	5	0.75	0.25	—	—	—	—	4.0
C	5	0.75	0.25	—	—	5	—	47.9
D	5	0.75	0.25	—	—	—	10	105.5
E	5	0.25	0.25	0.5	30	5	—	27.9
F	5	0.25	0.25	0.5	30	—	10	53.8

The reagents were dissolved in 0.1 M Tris buffer, pH 7.0, containing 1.0 M NaCl at initial concentrations of: OP, 2×10^{-3} M; MB, 0.004 mg/ml; ZnCl_2 , 0.1 M; CoCl_2 0.1 M.

enzyme. This cobalt enzyme preparation was not freed of adventitious metal ions. Photooxidation of the cobalt-carboxypeptidase-A_γ was as follows: 10 μl of the cobalt and carboxypeptidase-A_γ (4.03 mg/ml) was diluted with 1.0 ml of 0.1 M Tris, 1.0 M NaCl, pH 7.5 buffer at 4° C. Fifty microliters of a methylene blue water solution (approximately 3.0 mg/ml) was added to the enzyme solution. The mixture was divided, and one half stored in the dark, while the second half was exposed to thirty minutes of light. The photooxidation was done using Method A. Peptidase activity was determined as described in "Methods", using hippuryl-L-phenylalanine as substrate. The results of this determination are summarized in Table XXVIII, part A. Part B of Table XXVIII summarizes the results of an identical treatment of a native carboxypeptidase solution.

Preparation and photooxidation of nickel-carboxypeptidase-A. A 1.5 ml aliquot of a carboxypeptidase-A_γ solution (2.67 mg/ml) in 0.1 M Tris, 1.0 M NaCl, pH 7.5 buffer was placed in a pretreated dialysis bag. The dialysis bag then was immersed in one hundred ml of 0.1 M Tris, 1.0 M NaCl, 10⁻² M NiCl₂, pH 7.59 buffer and dialysis continued 24 hours. Photooxidation studies on this solution were as follows: 50 μl of the nickel-carboxypeptidase-A_γ solution were diluted with 1 ml of 0.1 M Tris, 1.0 M NaCl, buffer and 50 μl of a methylene blue-water solution (approximately 4.0 mg/ml) was added. The solution was divided, and one half exposed to 30 minutes of light. The second half was stored in the dark for a similar length of time. The photooxidation was by Method A. Peptidase activity was determined as described in "Methods", using hippuryl-L-phenylalanine as substrate. The results of this photooxidation are summarized in Table XXIX parts A and B. Parts C and D of Table XXIX summarize the data collected when nickel-carboxypeptidase-A_γ is photooxidized under identical conditions, but with the sensitizer Rose Bengal.

Copper Carboxypeptidase-A_γ. Copper-carboxypeptidase-A_γ was prepared by dialysis of native zinc carboxypeptidase-A_γ (2.67 mg/ml)

TABLE XXVIII

Photooxidation of Cobalt-Carboxypeptidase-A_γ and
Native Carboxypeptidase-A_γ in same buffer system

part	half	min $h\nu$	$\Delta A / \text{min } 254 m\mu$
A	1	30	0.0214
	2	—	0.0334
B	1	30	0.0202
	2	—	0.0256

TABLE XXIX

Preparation and Photooxidation of Nickel-Carboxypeptidase

part	half	min $h\nu$	$\Delta A / \text{min } 254 \text{ m}\mu$
A	1	30	0.0164
B	2	—	0.0210
C	1	30	0.0038
D	2	—	0.0200

in 0.1 M Tris, 1.0 M NaCl, pH 7.5 buffer against 100 ml of the same buffer, containing 10^{-2} M CoCl_2 , for 24 hours, and was followed by two changes of metal free buffer.

Fifty microliters of the copper-carboxypeptidase were removed from the dialysis bag and diluted to 1.0 ml with Tris buffer pH 7.5. The specific peptidase activity of this solution was 6.2. When this solution was further diluted with one ml of Tris buffer, pH 7.5, containing 2×10^{-3} M 1,10-phenanthroline and 20 μl of 10^{-2} M ZnCl_2 solution added, the specific activity increased to 61.0 units/mg. This procedure was then used in assaying the copper-carboxypeptidase- A_γ subsequent to photo-oxidation or other chemical modifications.

Photooxidation of copper-carboxypeptidase- A_γ was done as follows: 50 μl of copper carboxypeptidase- A_γ prepared by dialysis was diluted to 1.0 ml with 0.1 M Tris, 1.0 M NaCl, pH 7.5 buffer. Fifty microliters of a Rose Bengal/water solution, (approximately 3 mg/ml) was added to the diluted enzyme solution and the sample divided. One half was exposed to 30 minutes of light, and the other stored in the dark. Method A was used to photooxidize the enzyme. The assay for peptidase activity was done as described above, using hippuryl-L-phenylalanine as substrate. The data in Table XXX-A summarize the results of this experiment. The data in Table XXX-B summarize the results of a repeat of the above experiment.

Effect of Rose Bengal on the exchange of Cu^{2+} and Zn^{2+} ions in carboxypeptidase- A_γ . Fifty microliters of copper-carboxypeptidase- A_γ which had been prepared by dialysis was diluted to a concentration of 0.134 mg/ml with 0.1 M Tris, 1.0 M NaCl, pH 7.5 buffer. A water solution of Rose Bengal (3 mg/ml) was prepared. Aliquots of this Rose Bengal solution were added to several of the enzyme solutions, and the ability of Zn^{2+} ions to reactivate the enzyme was determined. Reactivation was accomplished by adding 1.0 ml of a 2×10^{-3} M 1,10-phenanthroline solution in 0.1 M Tris, 1.0 M NaCl, pH 7.5 buffer, followed by

TABLE XXX-A

Copper Carboxypeptidase-A_γ

μl enzyme	μl R B	ml buffer	min hr	OP, Zn ²⁺ added	spec. act. peptidase
50	50	1.0	30	yes	15.2
			—	yes	28.4
50	—	1.0	—	yes	61.0
50	—	1.0	—	no	6.2

TABLE XXX-B

μl enzyme	μl R B	ml buffer	min hr	OP, Zn added	spec. act. peptidase
50	50	1.0	30	yes	14.9
			—	yes	22.4
50	—	1.0	—	no	4.36

20 μ l of a 0.1 M ZnCl_2 solution in Tris buffer. Peptidase activity was determined as described in "Methods", using hippuryl-L-phenylalanine as substrate. Table XXXI summarizes the results of these determinations.

Sephadex G-75 chromatography of carboxypeptidase-A γ in the presence of 10^{-3} M Cu^{2+} ions. Tris buffer, 0.1 M, pH 7.0, containing 1.0 M NaCl, and 10^{-3} M Cu^{2+} ions, was prepared. 3.9 mg of Rose Bengal was added to this buffer, and the photolability of carboxypeptidase-A γ in this buffer determined. The loss of peptidase activity and esterase activity as a result of photooxidation in this buffer is shown in Table XXXII. This data was obtained when 10 μ l of a carboxypeptidase-A γ solution (9.21 mg/ml) was diluted with 500 μ l of the Cu^{2+} ion containing buffer. Photooxidation was done as described in "Methods". The Sephadex G-75 chromatography was accomplished in a 1 x 64 cm column at 0° c. One hundred microliters of a carboxypeptidase-A γ solution (9.21 mg/ml) was diluted with 500 μ l of the Rose Bengal containing buffer above and placed on the column. The enzyme was then eluted from the column with 0.1 M Tris buffer, pH 7.0, containing 1.0 M NaCl. The elution pattern of oxidized and unoxidized carboxypeptidase-A γ in this buffer system are shown in Figures 11 and 12.

Photooxidation of cobalt-carboxypeptidase-A γ . Cobalt-carboxypeptidase-A γ prepared by dialysis in 0.05 M Tris buffer, pH 8.0, containing 0.05 M sodium acetate and 1.0 M NaCl, was photooxidized with methylene blue sensitizer. Five microliters of the cobalt enzyme were diluted to 1.0 ml with 0.5 ml of 0.05 M Tris buffer, pH 7.95, 0.05 M sodium acetate and 1.0 M NaCl, and 0.5 ml of 0.1 M Tris, buffer, pH 7.5, containing 1.0 M NaCl, and 0.198 mg/ml of methylene blue. The resulting solution was then divided, and one half kept in the dark for thirty minutes, while the other half was exposed to thirty minutes of light. Photooxidation was accomplished by Method A.

TABLE XXXI

Effect of Rose Bengal on the Exchange of Cu^{2+} and Zn^{2+} ion in Carboxypeptidase- A_γ

μl CuCPD	μl RB	ml buffer	spec. peptidase
50	0	1.0	72.3
50	5	1.0	68.5
50	10	1.0	59.3
50	20	1.0	49.2
50	50	1.0	26.1
50	100	1.0	29.3
50	—	1.0	14.3**

Assay was performed without the addition of buffer containing OP and 20 μl of Zn^{2+} buffer.

TABLE XXXII

Photooxidation of Carboxypeptidase-A γ in the
Presence of 10^{-3} M Cu $^{2+}$ ions

min hv	spec. act. peptidase	spec. act. esterase
0	58.9	734
6	49.8	—
10	41.9	—
15	—	486
20	31.8	—
30	—	386
36	20.8	—
50	17.7	—
60	—	264
65	10.0	—

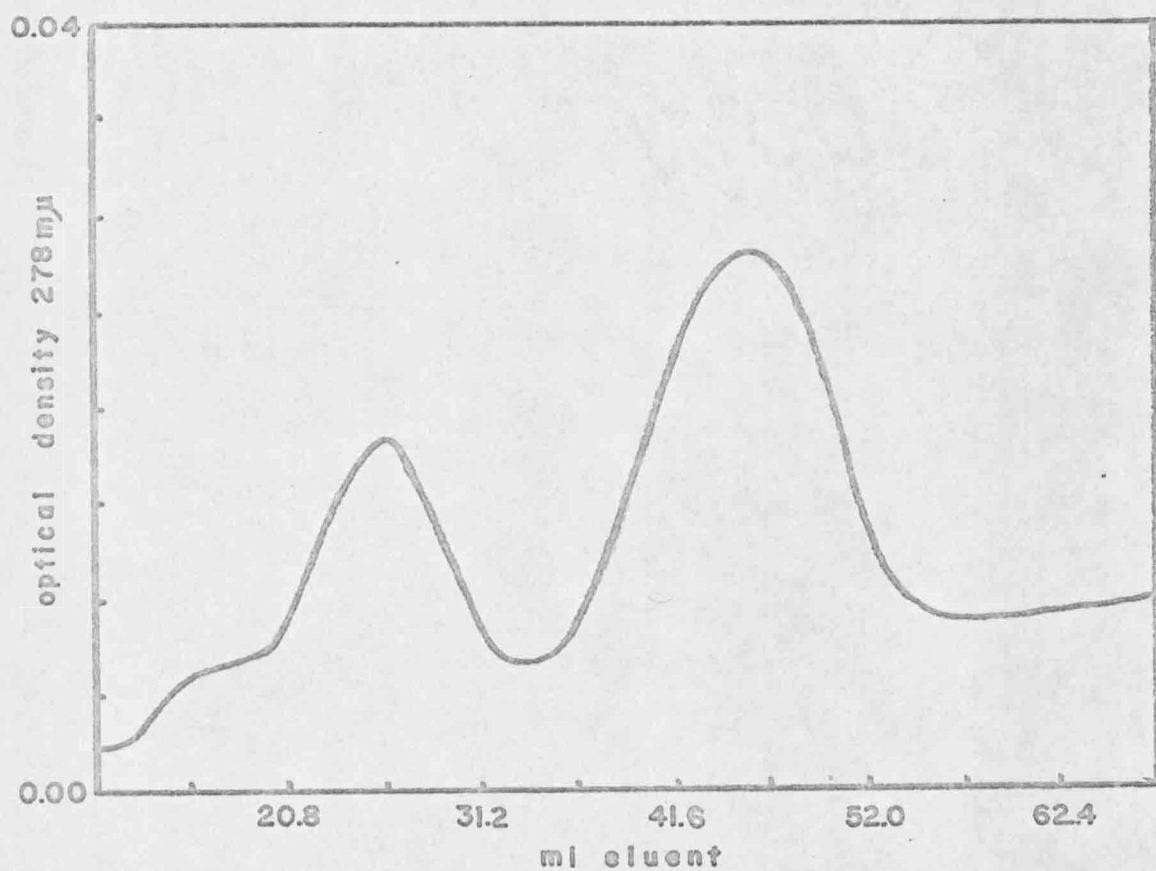


Figure 11. Sephadex G-75 chromatography of carboxypeptidase-A_γ in the presence of 10^{-3} M Cu^{2+} ions, photooxidized for six hours.

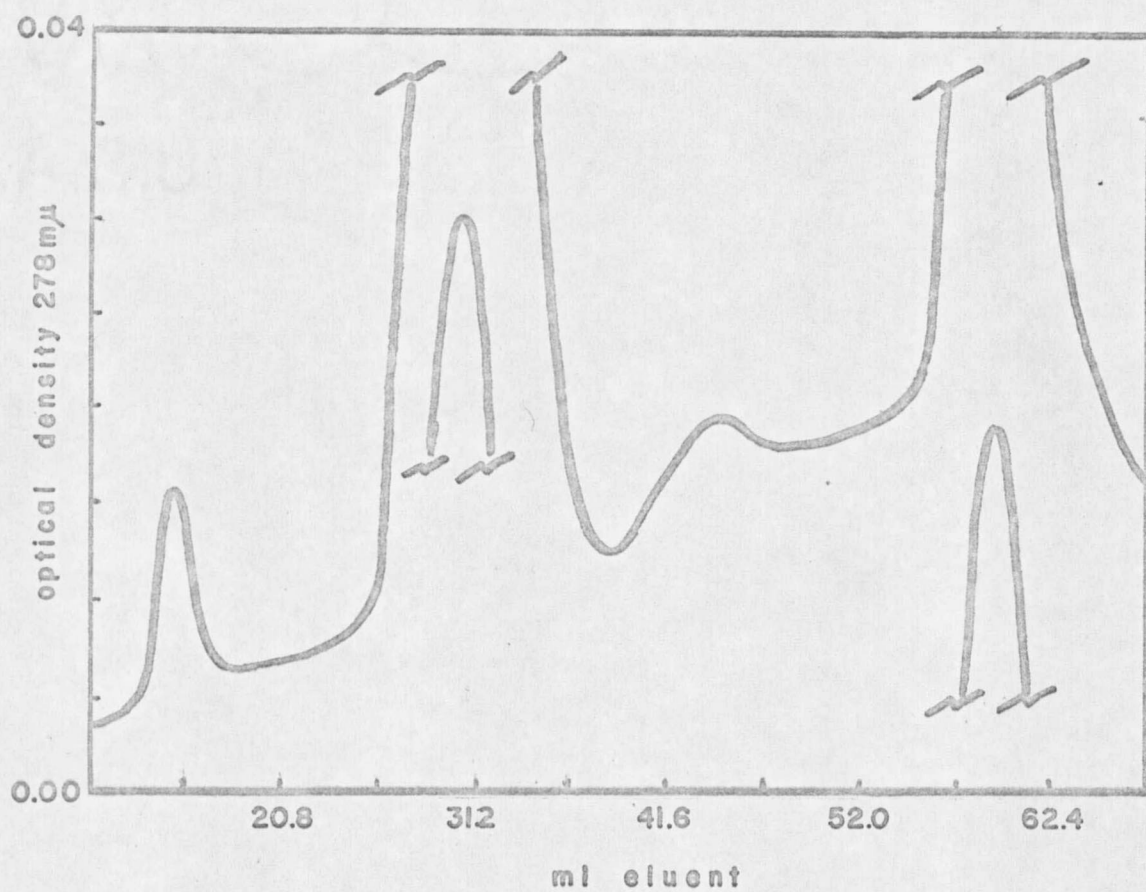


Figure 12. Sephadex G-75 chromatography of carboxypeptidase-A_γ in the presence of 10^{-3} M Cu^{2+} ions.

The peptidase activity was determined as described in "Methods", using hippuryl-L-phenylalanine as substrate. The results of these determinations are given in Table XXXIII.

Photooxidation of carboxypeptidase-A_γ in 0.05 M sodium acetate, 0.05 M Tris, 1.0 M NaCl buffer. Because of the rather large decrease in activity of the cobalt-carboxypeptidase when photooxidized in 0.05 M sodium acetate, 0.05 M Tris, 1.0 M NaCl, and 0.1 M Tris, 1.0 M NaCl, 0.199 mg/ml methylene blue, pH 7.5 buffer mixture, it was felt that the decrease in the activity of the native carboxypeptidase in this buffer system should be determined for comparison. Thus, 5 μl of a carboxypeptidase-A_γ solution, 4.03 mg/ml, was diluted with 0.5 ml of each of the above described buffers, and the sample divided. One half was stored in the dark at 0° C., while the other half was exposed to 30 minutes of light. The light source and assay conditions were as previously described in Method A. The results are summarized in Table XXXIV-A.

In a similar fashion, 10 μl of carboxypeptidase was diluted with 0.5 ml of the methylene blue buffer, as described above, and 0.5 ml of 0.1 M Tris, 1.0 M NaCl, pH 7.5 at 0° C. buffer. This solution likewise was divided, and one half oxidized 30 minutes. The results of this determination are shown in Table XXXIV-B.

Photooxidation of carboxypeptidase in acetate ion buffer. Ten microliters of a carboxypeptidase-A_γ solution (4.03 mg/ml) was diluted to 1.0 ml by the addition of 0.5 ml of 0.05 M Tris buffer, pH 7.95, containing 1.0 M NaCl and 0.05 M sodium acetate, and 0.5 ml of 0.1 M Tris buffer, pH 7.0 at 0° C., containing 1.0 M NaCl and 0.2 mg/ml methylene blue. The mixture then was divided, and one half exposed to 30 minutes of light, while the second half was stored in the dark. Photooxidation was done according to Method A. Peptidase activity was determined as described in "Methods", using hippuryl-L-phenylalanine as substrate. The results of this experiment are summarized in Table XXXV.

TABLE XXXIII

Photooxidation of Cobalt-Carboxypeptidase-A_γ

half	min hr	spec. act. peptidase
1	—	84.8
2	30	41.2

TABLE XXXIV

Photooxidation of Carboxypeptidase-A_v in 0.05 M sodium acetate, 0.05 M Tris, 1.0 M NaCl buffer

half	min hv	$\Delta A/\text{min } 254 \text{ m}\mu$
1	30	0.0126
2	—	0.0242

half	min hv	$\Delta A/\text{min } 254 \text{ m}\mu$
1	30	0.0170
2	—	0.0210

TABLE XXXV

Photooxidation of Carboxypeptidase
in Acetate Ion Buffer

half	min $h\nu$	$\Delta A / \text{min } 254 \text{ m}\mu$
1	30	0.0118
2	—	0.0238

Photooxidation of carboxypeptidase-A_γ with Rose Bengal and determination of sedimentation velocity. A 200 μ l portion of a carboxypeptidase-A_γ solution (28.14 mg/ml) dissolved in 3 M NaCl was diluted with 0.8 ml of 0.1 M Tris buffer, pH 7.5 at 4° C., containing 0.77 M NaCl. A small quantity of Rose Bengal (unmeasured) was added to the solution. The peptidase activity of this solution was then determined in the usual manner by removing a 10 μ l aliquot and diluting it to 1.0 ml with 0.1 M Tris buffer. A 50 μ l aliquot of this solution was assayed for peptidase activity. The remainder of the enzyme solution was photooxidized for 2 1/2 hours. The enzyme was photooxidized according to Method A. The initial specific activity of the enzyme solution was determined to be 53.4 units/mg, and following 2 1/2 hours of photooxidation was 22.6 units/mg. A 0.6 ml portion of the photooxidized enzyme solution was then placed in a single sector cell and the sedimentation velocity determined at 60,000 rpm in the Beckman model E ultra-centrifuge. The most significant observation made from this study was that the photooxidized enzyme did not sediment as a single species, but rather as a completely heterogeneous system, in which none of the components were of sufficient concentration to appear as a single sedimenting boundary.

Photooxidation of carboxypeptidase-A_γ in various buffer systems. The rate of inactivation as a result of photooxidation in various buffer systems was studied. Fifty microliters of a stock carboxypeptidase-A_γ solution (8.1 mg/ml) was diluted with 6 ml of the following buffer solutions: 0.1 M sodium citrate buffer, pH 7.0, containing 1.0 M NaCl; 0.1 M Tris buffer, pH 7.0, containing 1.0 M NaCl; 0.1 M Tris buffer, pH 7.0, containing 1.0 M Na₂SO₄. Each buffer solution was divided into six parts, and each part exposed to various periods of photooxidation. The light source was that described in Method B. Table XXXVI summarizes the loss of esterase activity in the various buffer systems. The esterase activity was determined as described in "Methods",

TABLE XXXVI

Photooxidation of Carboxypeptidase-A γ
in Various Buffer Systems

min hv	spec. act. sulfate	spec. act. tris	spec. act. citrate
0	710	908	755
1	525	797	643
2	463	729	584
3	456	564	564
4	352	570	518
5	290	518	278

DISCUSSION AND CONCLUSIONS

The experimental data discussed in this section were selected from those experiments which were performed after the experimental methods had been perfected. Many of the experiments in the previous section were of a probing nature, and subsequently the results obtained were inferior to those results obtained later. The reader should be aware that two methods of photooxidation were used in obtaining data, and should not quantitatively compare data obtained by different methods. In addition, attention should be paid to the enzyme concentrations of the various experiments, since the rate of oxidation is concentration dependent.

Since it is necessary to photooxidize both the native and the apoenzyme in order to determine if histidine was involved in binding zinc, a convenient method for the preparation of the apoenzyme had to be found. Vallee has previously described the preparation of apocarboxypeptidase-A γ on a Sephadex G-25 column.⁽³⁾ However, this preparation is stable only over short periods of time. In addition to the instability of the apoenzyme prepared by the column method, other inconveniences are encountered with this method. The apoenzyme is reactivated rapidly by the metal ions which are present in minute quantity in the plastic ware and glass ware. Extreme precautions must be employed to prevent this reactivation, and they were found to be too inconvenient to employ on a regular basis. Therefore the apoenzyme was prepared by simply incubating the native enzyme in the presence of 1,10-phenanthroline. The apoenzyme prepared in this manner can be fully reactivated by the addition of a suitable quantity of zinc ions. Having now a system in which one can rapidly prepare the apoenzyme, photooxidation was undertaken using the dye methylene blue. The data in Figure 13 shows the effect of the photooxidation on the peptidase activity. The loss of peptidase activity following a similar photooxidation of the native enzyme is not appreciable. These results suggest that zinc binding has a protective effect. The results in Figure 14 reveal a striking difference between the effect of photooxidation on the gamma

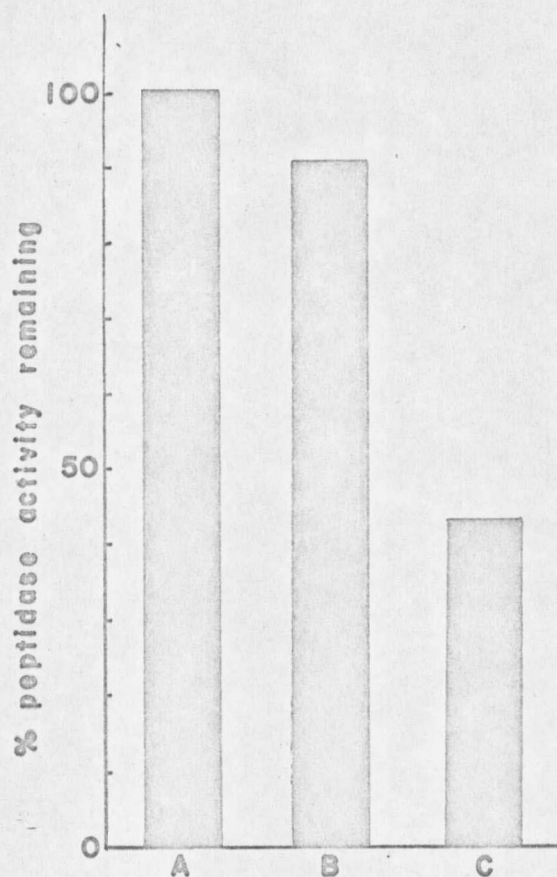


Figure 13. Bar A represents the native activity of carboxypeptidase-A_γ at a concentration of 0.023 mg/ml. Bar B represents the activity remaining after photooxidation of the native enzyme with methylene blue. Bar C represents the activity remaining after photooxidation of the enzyme with methylene blue in the presence of 5×10^{-4} M 1,10-phenanthroline subsequent to reactivation with 10^{-1} M ZnCl₂ buffer. The methylene blue concentration was 0.002 mg/ml in all cases. Photooxidation was carried out according to Method A.

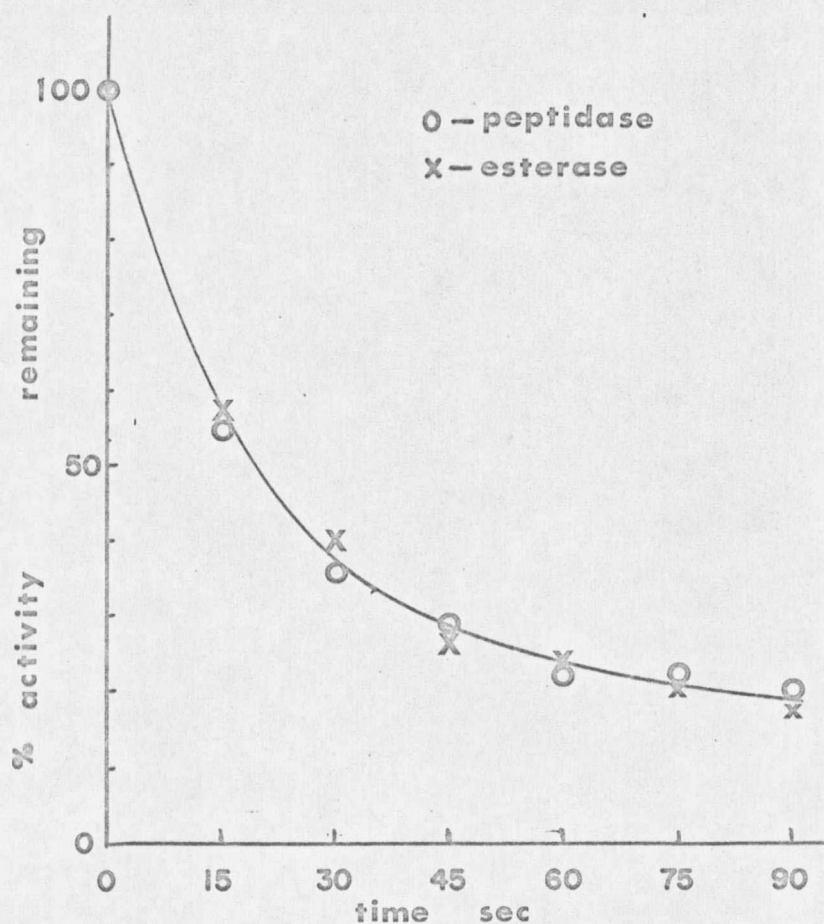


Figure 14. Photooxidation of carboxypeptidase-A γ in 1.0 M NaCl, 0.1 M Tris, 0.10 mg/ml Rose Bengal, pH 7.5, at 3 $^{\circ}$ C. Protein concentration 0.028 mg/ml. 500 watt light source focused through an f 1.6 lens was used. Samples were placed 4.3 cm from the lens. Esterase activity (x-x) and peptidase activity (o-o) were determined spectrophotometrically as described in "Methods".

form of the enzyme when compared to the delta form.⁽³⁾ Photooxidation of δ -carboxypeptidase-A results in a loss of peptidase activity, and in a two-fold increase in the esterase activity. The data in Figure 14 indicates that photooxidation with methylene blue of apocarboxypeptidase-A γ reduces the esterase activity at the same rate as the peptidase activity is lost, which indicates a significant difference between the two enzymatic forms. It was necessary to determine if the photooxidation was the result of methylene blue sensitization, or the result of the 1,10-phenanthroline zinc complex. Experiments were carried out in which sensitization by 1,10-phenanthroline was tested by exposing the enzyme to light in the presence of OP but absence of methylene blue. These experiments show that 1,10-phenanthroline had no photochemical effect on the enzyme, and demonstrate that the loss of peptidase and esterase activity is solely the result of the methylene blue sensitization. In order to completely eliminate the possibility of metal chelator or chelated complex involvement, apocarboxypeptidase-A γ was prepared by the methods of Vallee⁽³⁾ on a Sephadex G-25 column. The resulting enzyme was photooxidized in the presence of methylene blue, and the results were found to be completely analogous to the photooxidations in the presence of the metal chelator 1,10-phenanthroline. Photooxidation of carboxypeptidase-A γ in the presence of the second chelator, EDTA, was investigated, and was found to have the same general effect on the peptidase and esterase activity, as shown in Table V. It should be noted that the incubation of carboxypeptidase-A γ in the presence of EDTA resulted in a decreased ability of the apoenzyme to be reactivated with zinc, which was not observed with 1,10-phenanthroline. No other chelating agents were tried.

Several enzymes have been photooxidized using the dye Rose Bengal.^(14,15,16) Westhead has described the results of Rose Bengal sensitized photooxidation on the enzyme enolase, and has indicated that the Rose Bengal may be quite specific for histidine destruction.⁽¹⁴⁾ Therefore, in an attempt to eliminate some of the possibilities of residues being attacked in the photooxidation of carboxypeptidase-A γ ,

Rose Bengal was used under the same experimental conditions as the methylene blue. The results of Figure 15 clearly indicate that Rose Bengal rapidly decreases both the peptidase and esterase activity of carboxypeptidase-A_γ. In contrast to the methylene blue sensitized photooxidation, it was found that Rose Bengal did not require the presence of the metal chelator. The dye was equally effective in reducing the peptidase and esterase activity of the native enzyme, which indicates that the mechanism of action of the dyes are different. This difference could be explained in the manner in which they bind to the protein. Rose Bengal is an anionic compound of aromatic nature, and contains a free carboxyl group. It should be noted that the substrates of carboxypeptidase are likewise aromatic and contain free carboxyl groups. Thus, one might expect that Rose Bengal could bind to the enzyme in a fashion similar to that of the substrate. In this case, one would expect that Rose Bengal would be concentrated more in the area of the active center of the enzyme than methylene blue, which is cationic. The fact that methylene blue requires a metal chelator can be explained in the following manner. Methylene blue, which is cationic, would be expected to interact with areas of negative charge on the enzyme surface. Thus, when the zinc ion is present, the dye will be attracted to areas on the enzyme surface having a negative charge density. Since the enzyme is not rapidly photooxidized under these conditions, one can assume that these interaction sites, and the subsequent oxidations at these sites are not critical to the catalytical activity. Removal of the zinc, however, which is a positive species, will expose a new negatively charged binding site. This new site, which is known to be catalytically functional, will also attract the methylene blue, and subsequent photooxidation at this site would be expected to have gross effects on the activity of the enzyme. While the above explanation seems to satisfactorily explain the data, alternate explanations can also be given. Metals have been known to induce conformational changes in proteins. It is possible that the binding of zinc to the apoenzyme and

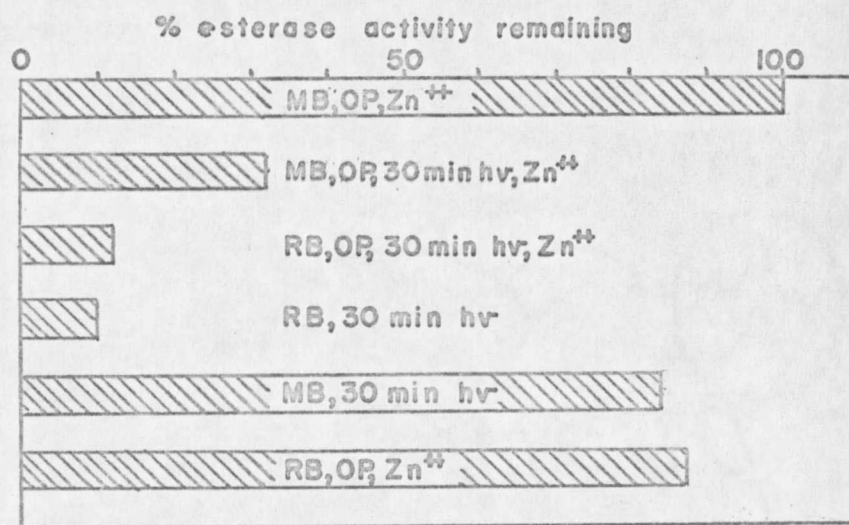


Figure 15. Comparison of the dyes Rose Bengal and methylene blue as effective sensitizers in the presence and absence of 1,10-phenanthroline. Methylene blue and Rose Bengal concentrations were 0.095 mg/ml. Protein concentration was 0.023 mg/ml. ZnCl₂ was added where indicated to reverse inactivation by 1,10-phenanthroline. The esterase activity was determined as described in "Methods".

the removal of zinc from the native enzyme are accompanied by changes in conformation. These changes might allow the methylene blue to be effective on the apoenzyme, and not on the native enzyme. Previous studies on carboxypeptidase-A_γ have shown; however, that removal of the metal produced an apoenzyme which could not be distinguished from native carboxypeptidase-A_γ by any of the physical criteria which relate to the gross configuration of the molecule.⁽¹⁷⁾

The hypothesis of the binding of Rose Bengal to the active site can be tested in several ways. It has been demonstrated previously that peptidase and esterase substrates form metallo enzyme-substrate complexes which prevent the dissociation of the metal ion, and therefore the exchange of one metal for another.^(18, 19) In addition, the competitive inhibitor β-phenylpropionate markedly retards the exchange of $^{65}\text{Zn}^{2+}$ > Zn^{2+} at the active site of the enzyme.⁽²⁰⁾ This phenomenon was also shown for the Cd^{2+} and Co^{2+} carboxypeptidases.⁽²⁰⁾ The results shown in Figure 16 establish that Rose Bengal interferes with the exchange of zinc for copper in copper carboxypeptidase. The reactivation was accomplished by the addition of 1,10-phenanthroline to the inactive copper enzyme system, in the presence of Rose Bengal, followed by the addition of zinc ions. As the concentration of Rose Bengal was increased, the ability to reactivate the enzyme with the addition of zinc ions was decreased, indicating that the Rose Bengal was, in effect, interfering with either the rebinding of the zinc ion, or the release of the copper ion from the enzyme. The latter would be consistent with the results observed by Vallee with the substrate HPLA and β-phenylpropionate. Secondly, if Rose Bengal is binding at the active site, it should be a competitive inhibitor of the enzyme.

The ability of Rose Bengal to function as a competitive inhibitor was investigated, and the results are shown in Figure 17. It appears that Rose Bengal is indeed a competitive inhibitor of carboxypeptidase-A_γ. As mentioned previously, Rose Bengal and the substrates of carboxypeptidase are anionic. Thus, it would not be too surprising that Rose

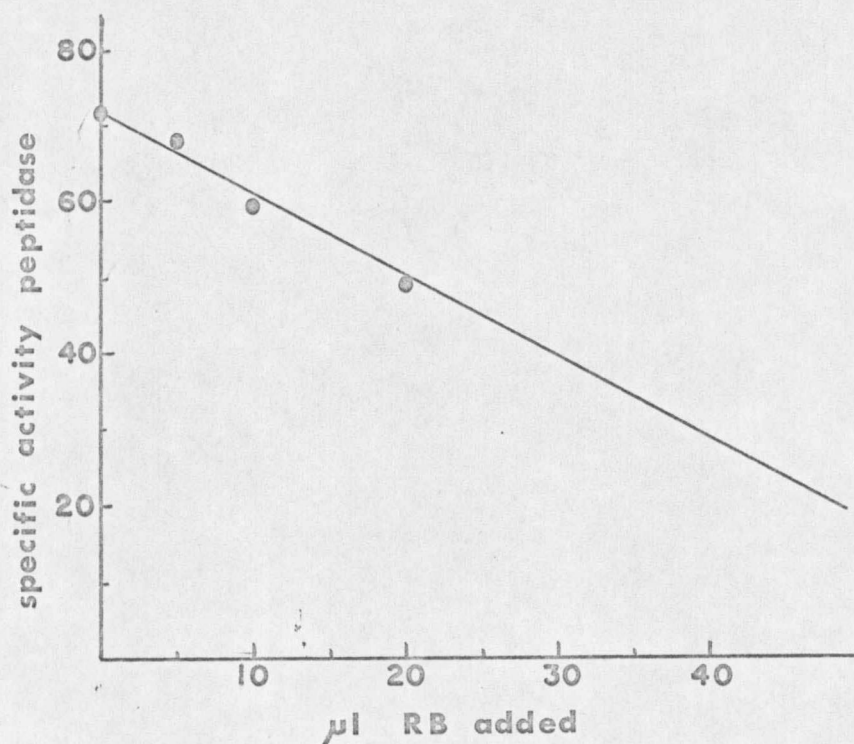


Figure 16. The effect of Rose Bengal on the exchange of Cu^{2+} and Zn^{2+} ions in carboxypeptidase- A_γ . Protein concentration was 0.134 mg/ml in all determinations. Reactivation of the inactive copper carboxypeptidase was accomplished by incubating the copper enzyme, in the presence of varying concentrations of Rose Bengal buffer, with 2×10^{-3} M, 1,10-phenanthroline and then adding a suitable aliquot of 10^{-1} M ZnCl_2 buffer. The Rose Bengal buffer used had a concentration of 3 mg/ml.

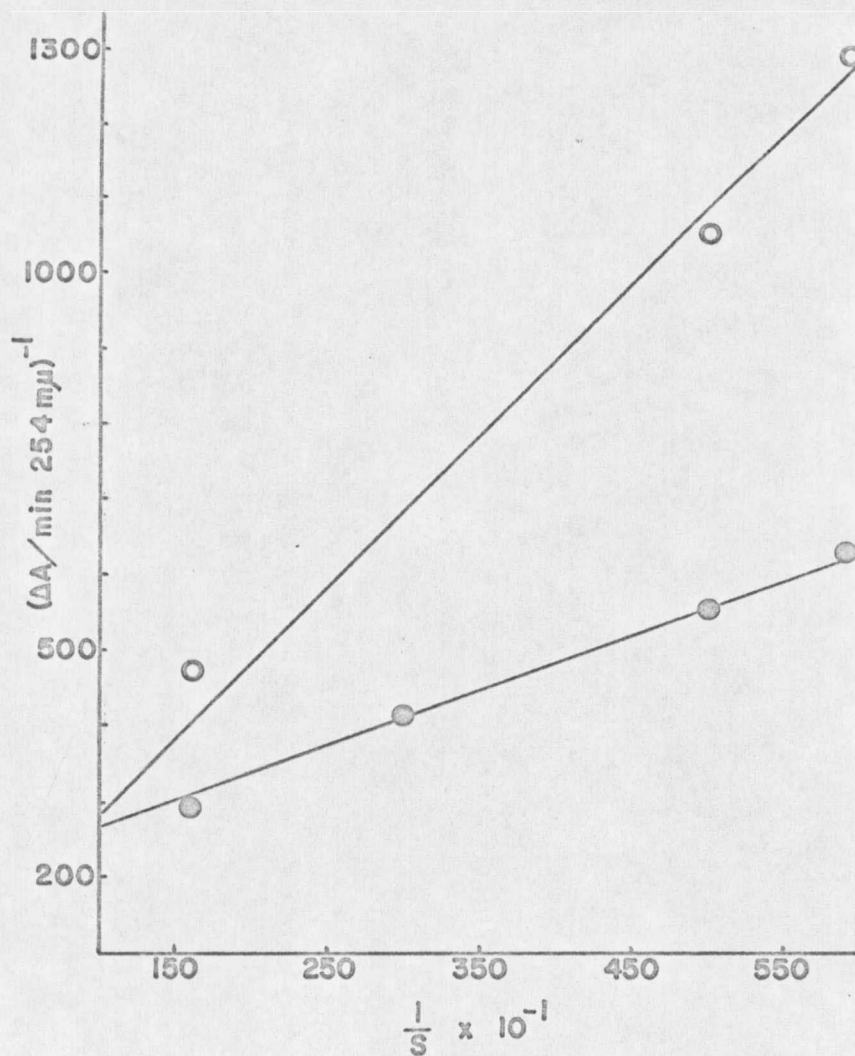


Figure 17. Lineweaver-Burk plot of carboxypeptidase activity in the presence and absence of Rose Bengal. Rose Bengal concentration was 0.001 mg/ml. Protein concentration was 0.0054 mg/ml. The esterase activity in the presence of Rose Bengal, (o-o), and in the absence of Rose Bengal (●-●), was determined at the appropriate substrate concentrations as described in "Methods".

Bengal would bind at the active center of the enzyme. This binding is not the only instance of apparent active site binding of Rose Bengal. Studies on liver alcohol dehydrogenase, which also binds anionic substrates, indicate that Rose Bengal is bound to the active site of this enzyme and functions as well as a competitive inhibitor.⁽¹⁶⁾

In addition, another kinetic study was undertaken, in which the loss of peptidase and esterase activities were plotted as a function of time. A semi-logarithmic plot of these data gives the curve in Figure 18 with two independent slopes. The fact that such a kinetic curve was obtained might indicate that more than one reaction is occurring. The initial more rapid reaction can be associated with the initial loss of the peptidase and esterase activity, the result of photooxidation of the active site. The slower reaction can be rationalized. It is likely that the initial oxidation occurs at the active site of the enzyme and manifests itself in the loss of peptidase and esterase activity. If the oxidized residue had in addition to its catalytic function, a structural role, or if oxidation of a residue in the active site exposed to oxidation a second residue of structural importance, the tertiary structure of the enzyme would be altered. As the denaturation of the protein molecules progressed there would be more and more photo-oxidizable residues exposed, and these would compete for the dye molecules and singlet oxygen, thus causing an apparent decrease in rate of loss of peptidase and esterase activity with time.

The dye, crystal violet, has been shown to be specific for the photooxidation of cysteine residues.⁽²¹⁾ Therefore, crystal violet sensitized photooxidation of the carboxypeptidase-A_γ was undertaken, and it was found that the enzyme was not affected under these conditions, even in the presence of the chelator, 1,10-phenanthroline.

The photooxidation of histidine has been shown to be pH-dependent.⁽¹⁴⁾ The relative rates of oxidation of histidine as a function of pH was sigmoidal, and had an inflection near pH 7.3. The amino acids tryptophan, tyrosine, and methionine, which are also

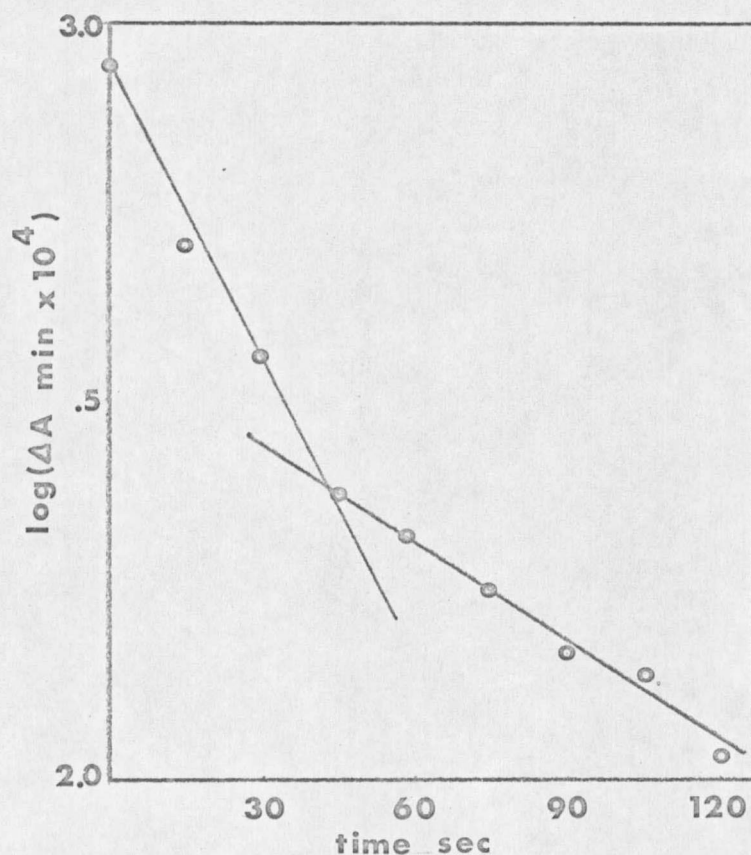


Figure 18. Semi-logarithmic plot of loss of esterase activity as a function of time. Protein concentration was 0.028 mg/ml. Rose Bengal concentration was 0.10 mg/ml. Photooxidations were conducted in 0.1 M Tris buffer, pH 7.5 at 3° C., containing 1.0 M NaCl. 500 watt light source was focused through an f 1.6 lens. Samples were 4.3 cm from the lens. The esterase activity was determined as described in "Methods".

susceptible to photooxidation do not show this pH dependence.⁽¹⁴⁾ Significantly, when carboxypeptidase-A_γ is photooxidized in the presence of Rose Bengal, or in the methylene blue 1,10-phenanthroline system, (Figure 19), a sigmoidal pH profile is obtained which is almost identical to that exhibited for the enzyme enolase.⁽¹⁴⁾ This pH dependence of the photooxidation of carboxypeptidase-A_γ suggests that the rate limiting step is controlled by the oxidation of a histidine residue.

If substrate or competitive inhibitors were to protect the enzyme from photooxidation, substantial evidence that the initial oxidation was occurring at the active site of the enzyme would be obtained. Thus, studies were undertaken to determine if the substrate would protect. The substrate chosen was hippuryl-L-phenylalanine, the peptidase substrate. Substantial substrate protection was observed in the Rose Bengal system (Figure 20). The competitive inhibitor, β-phenylpropionate was also tried as a protector against the photooxidation. Previous studies have shown that β-phenylpropionate binds to the active site of the enzyme.^(22, 23) It is obvious from the data in Figure 21 that β-phenylpropionate does protect the enzyme against photooxidation. Likewise, X-ray studies have shown that glycylytyrosine, a substrate of the enzyme, binds only at the metal binding site. Therefore, if the photooxidation were occurring on the surface of the enzyme at random, one would not expect glycylytyrosine to protect. Glycylytyrosine substrate protection was undertaken, and it was found to significantly hinder the photooxidation of the enzyme (Figure 22). This result establishes that the photooxidation leading to inactivation of the enzyme is occurring at the active site of the enzyme. It was of interest to determine if any gross conformational changes occurred subsequent to the photooxidation of carboxypeptidase. It was felt that these conformational changes might be detected through Sephadex chromatography. A series of experiments were undertaken where carboxypeptidase was photooxidized with Rose

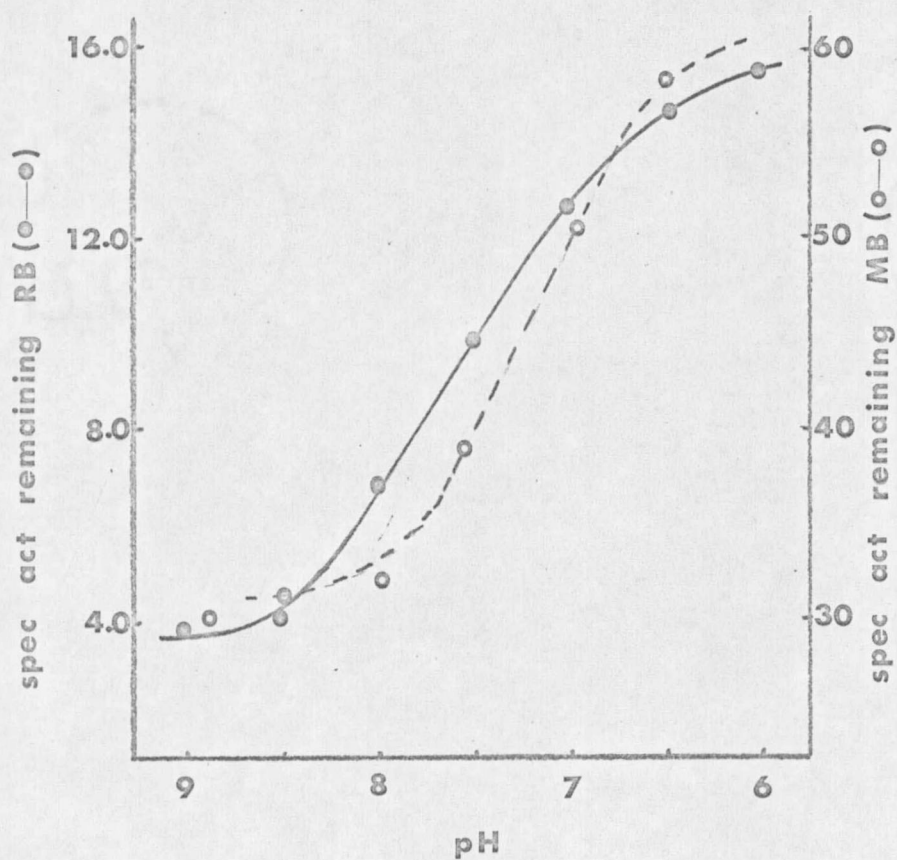


Figure 19. The pH dependence of the methylene blue-1,10-phenanthroline sensitized photooxidation (o-o) and the Rose Bengal sensitized photooxidation (●-●) are shown. Protein concentration was 0.02 mg/ml. The concentrations of methylene blue and Rose Bengal were 0.19 mg/ml and 0.10 mg/ml respectively.

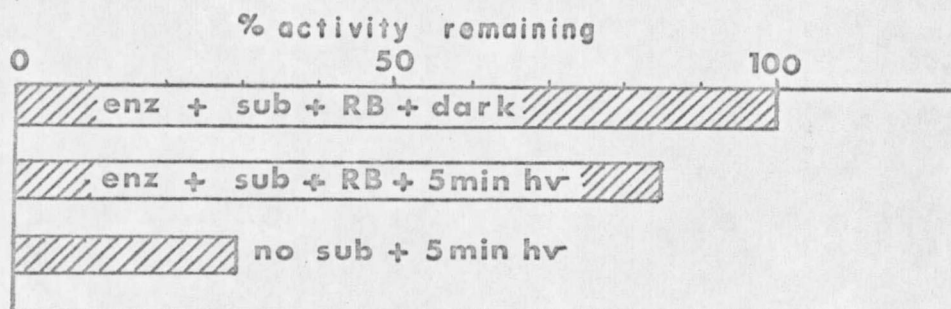


Figure 20. Protection against Rose Bengal photooxidation of carboxypeptidase-A_v with the substrate hippuryl-L-phenylalanine. Hippuryl-L-phenylalanine concentration was 10^{-2} M. Rose Bengal concentration was 0.04 mg/ml and the protein concentration was 0.04 mg/ml. Peptidase activity was determined by removing a 10 μ l aliquot and assaying in the normal manner.

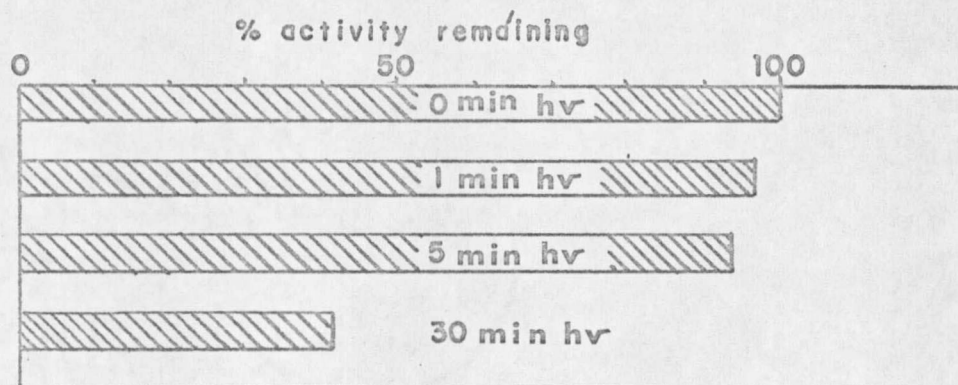


Figure 21. Protection against Rose Bengal photooxidation with β -phenylpropionate. Rose Bengal concentration was 0.03 mg/ml. β -phenylpropionate concentration was 2×10^{-2} M. Protein concentration was 0.08 mg/ml. The esterase activity was determined as described in "Methods".

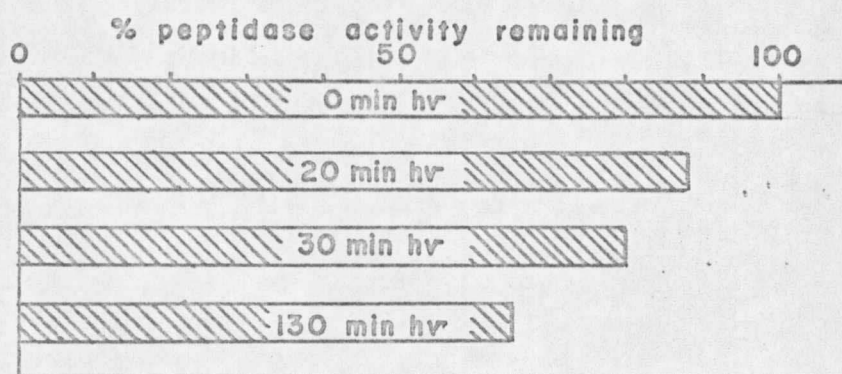


Figure 22. Protection against Rose Bengal photooxidation with glycylytyrosine. Final concentrations of components were as follows: Rose Bengal, 0.03 mg/ml; carboxypeptidase, 0.08 mg/ml; glycylytyrosine, 0.001 M. The peptidase activity was determined as described in "Methods".

Bengal, and then placed on a G-75 column. The elution patterns in the experimental section (Figures 1, 2, and 3), indicate that as photooxidation time increases, a rapidly moving peak appears in the elution pattern of the enzyme, which suggests that possibly some aggregation is occurring. This aggregation was subsequently confirmed when it was found that upon prolonged photooxidation the protein actually precipitated from solution. This precipitate was collected by centrifugation, washed with water, and then subjected to amino acid analysis, and no histidine could be detected in the samples. Chromatography on TEAE cellulose produced a very interesting and somewhat surprising result. When the unoxidized enzyme was passed through the TEAE column, five peaks were observed in the eluent pattern. When fractions were collected as the various peaks emerged from the column, and the peptidase activity determined, it was found that each peak contained enzymatic activity. Carboxypeptidase-A γ apparently exists in at least five active conformations as it is purchased, or as prepared by the method of Anson. These various forms have likewise been observed by Dr. Neurath and coworkers at the University of Washington, who have been able to separate these subforms by chromatography on DEAE cellulose in the presence of the inhibitor β -phenylpropionate.⁽²⁴⁾ It appears that TEAE is capable of partial separation of these forms without the presence of the inhibitor. If the photooxidized enzyme is placed on the TEAE column, a similar pattern is observed, as shown in Figures 4, 5, and 6 in the experimental section. One should note, however, that as photooxidation time increases, the amount of enzyme eluted from the column is decreased, but there is no change in the activity profile of the elution pattern, which suggests that the photooxidized enzyme is tightly bound to the column and is not eluted. Attempts were made to elute the oxidized enzyme from the TEAE column by increasing the salt concentration of the eluent. The sodium chloride concentration was increased to four molar, without elution of protein from the column. Since high salt concentrations failed to elute the

oxidized enzyme from the column, it was concluded that irreversible aggregation had occurred following the photooxidation. No further attempts were made to use ion exchange columns to separate the oxidized enzyme from unoxidized enzyme. Photooxidized enzyme was subjected to Sephadex G-75 column chromatography, and the results support the hypothesis that photooxidation is leading to aggregation. Figures 8, 9, and 10 of the experimental section show the elution patterns of photooxidized enzyme on Sephadex G-75. The fast running peak appears with photooxidation, and increases in intensity with increased time of photooxidation. One concludes from this that photooxidation does result in aggregation of the enzyme molecules. This aggregation may result by several mechanisms. The photooxidation of the native protein may alter the surface charge distribution so that electrostatic interaction leads to aggregation. If the aggregation is of this type, the charge interactions must be extremely strong, since 4 M NaCl does not break it up. A second possibility is that photooxidation leads to conformational change of the native molecule, and that these conformers then aggregate. This possibility is consistent with the rate data, the amino acid analysis data, and the sedimentation study. As previously mentioned, two distinct rates are observed (Figure 18). These rates could correspond to (a) the rate of oxidation of native enzyme, and (b) photooxidation of newly formed conformers. Amino acid analysis of this aggregated material, collected by precipitation, shows that all the histidine has been destroyed. It is very tempting to suggest that the complete destruction of the remaining histidines is occurring after the newly formed conformers are produced. If conformational changes are occurring, this would be the first such change observed in an enzymatic system which was photooxidized.

The effects of various other metal ions subsequent to photooxidation of the enzyme were investigated. It has been shown that if the native zinc enzyme is incubated with ortho-phenanthroline, and the system then flooded with cobalt ion, the activity is increased

roughly two-fold from that of the native enzyme. If the enzyme is photooxidized following the incubation with 1,10-phenanthroline, and then cobalt is added to the system, the activity is decreased proportionately with the time of photooxidation as was observed with zinc. In other words, if it is observed that in a certain period of time fifty percent of the activity was lost, with the readdition of zinc, it would also be observed that fifty percent of the activity is lost on the addition of cobalt. This indicates that the photooxidation not only destroys the capacity of zinc to reactivate the enzyme, but also the capacity of cobalt to reactivate the enzyme. A cobalt carboxypeptidase was prepared by dialysis of the zinc enzyme against a cobalt solution.⁽²⁵⁾ This cobalt enzyme was photooxidized and found to have essentially the same properties as that of the zinc enzyme. That is, photooxidation of the cobalt enzyme leads to the destruction of both peptidase and esterase activities. Other metalloenzymes were prepared as described in the experimental section, and their properties subsequent to photooxidation determined. The results of these photooxidations were similar to those obtained with the zinc enzyme. These experiments support the hypothesis that the activating metal ions bind at the same site in the enzyme.

The rate of photooxidation seemed to depend to some extent upon the buffer system employed during the photooxidation. It would be interesting to further investigate the effect of various buffers on the photooxidation of other enzymes as well as carboxypeptidase-A_v. I have observed that the rate of photooxidation changes with different buffer systems, but the general appearance of the semi-log rate plot remains the same. It is possible that the differing rates may reflect minor changes in the conformation of the total protein molecule.

A typical amino acid analysis subsequent to photooxidation is shown in Figure 23. The most important aspect of this analysis is the almost total destruction of histidine in photooxidized molecules. Histidine is the principal amino acid affected, the analysis showing little loss of any other amino acid.

	his	tyr	meth	cys-SH	val
μ mole*	0.046	0.306	0.034	0.016	0.225
μ mole**	0.134	0.372	0.041	0.024	0.272
$\frac{\mu \text{ mole}^*}{\mu \text{ mole}^{**}}$	0.343	0.824	0.829	0.667	0.828
% loss	58	0.5	0	19.5	0
residue loss	4.64	0.09	0	0.39	0

Figure 23. A typical amino acid analysis of oxidized (μ mole**) and unoxidized (μ mole*) carboxypeptidase-A_v. The loss of cysteine was variable depending on the oxygen content of the hydrolysis mixture.

The photooxidation of carboxypeptidase-A₈ is worthy of further comment.⁽³⁾ This photooxidation results in increased esterase activity, and decreased peptidase activity. At least two explanations can be given for this apparent discrepancy. Either the conformation of the apo-₈ form in the zinc binding site is such that it inhibits the dye binding, or the metal binding site does not involve histidine.

SUMMARY

It was found that dye-sensitized photooxidation using either methylene blue or Rose Bengal as sensitizers rapidly diminishes both the peptidase and esterase activities of carboxypeptidase- A_{γ} . It is significant that both activities are simultaneously diminished by this chemical modification procedure, since heretofore a dual inactivation has been accomplished only by removal of the metal from the enzyme. All other chemical modifications employed on carboxypeptidase-A previously reported, including methylene blue sensitized photooxidation of carboxypeptidase- A_{δ} have resulted in an increase in esterase activity and a decrease in peptidase activity.

A difference was found in the mechanism of action between Rose Bengal and methylene blue. Rose Bengal functions as a very effective sensitizer for both carboxypeptidase- A_{γ} and apocarboxypeptidase- A_{γ} , while methylene blue sensitizes the photooxidation of only the metal-free form of the enzyme. Both sensitizers show a marked pH dependence and give identical sigmoidal curves which are typical of the pH dependency of the dye-sensitized photooxidation of the imidazole moiety. Crystal violet, a sensitizer which is reported to be specific for cysteine residues, was found to be completely ineffective on apocarboxypeptidase- A_{γ} .

Amino acid analysis of photooxidized enzyme revealed the loss of only histidine residues. Photosensitive amino acids such as cysteine, tyrosine, and methionine remained essentially unaltered. Significant protection against this photooxidation was demonstrated using the substrates hippuryl-L-phenylalanine, glycyltyrosine, and the competitive inhibitor β -phenylpropionate. Kinetic studies indicate that Rose Bengal also functions as a competitive inhibitor of carboxypeptidase- A_{γ} and prevents the exchange of metal ions at the active center of the enzyme. The photolability of several metallo derivatives of carboxypeptidase- A_{γ} were investigated. Conformational changes subsequent to photooxidation and the photolability of the enzyme in various buffer systems were also investigated.

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