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Photooxidation of Crystalline Chymotrypsin in the Presence of Methylene Blue

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Introduction

The importance of the possible reactive groups in chymotrypsin has been brought into focus in recent years by investigations of the stoichiometric inactivation of this enzyme by phosphate esters. Progress in this field has been summarized recently by Balls and Jansen (1). It was of interest, therefore, to extend our studies on the photooxidation of proteins and enzymes (2–5) to chymotrypsin with the hope that these findings might shed some light on the chemistry of the reactive groups.

Experimental

Throughout the experiments, crystalline α-chymotrypsin, purchased from Armour and Company,[2] was used. Irradiation with visible light at 37° and the manometric measurement of oxygen uptake were carried out as described previously (6). All Warburg vessels contained concentrated KOH in the center well to absorb the simultaneously formed CO_2. The chymotrypsin concentration was calculated on the basis of 16.06% nitrogen content (7), which agreed well with our measurements. Although the pH optimum of oxygen uptake during the photooxidation of chymotrypsin was about 9.0, the same as for other proteins (4, 5), all experiments were carried out at pH 7.4 to reduce any possible enzyme inactivation at the higher pH.

Photochemical Action of Methylene Blue on the Amino Acid Composition of Chymotrypsin and on Its Enzymatic Activity

In our previous work (2), it was shown that the action of visible light on the various amino acids in the presence of traces of methylene blue

[1] One of the laboratories of the Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture.

[2] Mention of a product does not imply that it is endorsed or recommended by the U. S. Department of Agriculture over similar products not mentioned.

was confined to the oxidation of histidine, tryptophan, tyrosine, methionine and cystine. In view of these findings, the fate of these amino acids in the chymotrypsin molecule was investigated after various degrees of photooxidation. Analytical procedures for determination of these amino acids have been described in a previous paper (3). Calculation of the number of moles of oxygen uptake per mole of chymotrypsin was based on a molecular weight of 27,000 (8), inasmuch as our amino acid analyses fitted this value better than the 22,500 reported recently (9, 10). The following analytical values were obtained: histidine, 1.12 % (1.94 moles[3]); tryptophan, 5.68 % (7.54 moles[3]); tyrosine, 2.70 % (4.02 moles[3]); methionine, 1.09 % (2.04 moles[3]); and ½ cystine, 3.22 % (7.2 moles[3]). These values are in close agreement with those reported for chymotrypsinogen (7, 11, 12).

For determination of the amino acid content at any given level of oxygen uptake, the following procedure was used. To 1 ml. of solution containing 10 mg. chymotrypsin adjusted to pH 7.4, 0.5 ml. of 0.2 *M* phosphate buffer of pH 7.4 and 0.5 ml. of a 0.02 % methylene blue solution were added, and the mixture was irradiated for the period required. Aliquots were then taken for determination of the respective amino acids.

For the quantitative measurement of the proteinase activity of chymotrypsin, crystalline β-lactoglobulin was used as a substrate. Data for a calibration curve relating the enzyme concentration to α-amino groups liberated during the hydrolysis were obtained as follows. To 2 ml. of 0.1 *M* NaCl solution, containing 30 mg. of β-lactoglobulin at pH 7.4 were added 1 ml. of 0.2 *M* phosphate buffer of pH 7.4 and 1-ml. aliquots of chymotrypsin solution containing increasing amounts of the enzyme. After incubation for 1 hr. at 35°, the increase in α-amino groups was measured by the Van Slyke manometric procedure. The calibration curve obtained in this way (Fig. 1) was used for calculating the enzyme concentration.

Simultaneously with the determination of proteinase activity, the esterolytic activity of chymotrypsin (13) was measured by the continuous propotentiometric titration method of Jansen *et al.* (8), based on the procedure of Schwert *et al.* (13). L-Tyrosine ethyl ester was used as a substrate. Titration values obtained by this method with different enzyme concentrations at 25° resulted in a linear relationship up to 10 min. incubation time, and these values could be used, therefore, for the quanti-

[3] Calculated on the basis of 27,000 as the molecular weight of chymotrypsin.

tative evaluation of the esterolytic activity of the enzyme. Conditions for irradiation of chymotrypsin for determination of proteinase and esterolytic activities were the same as those for the estimation of amino acids described above. Aliquots containing 1 mg. of the enzyme were used.

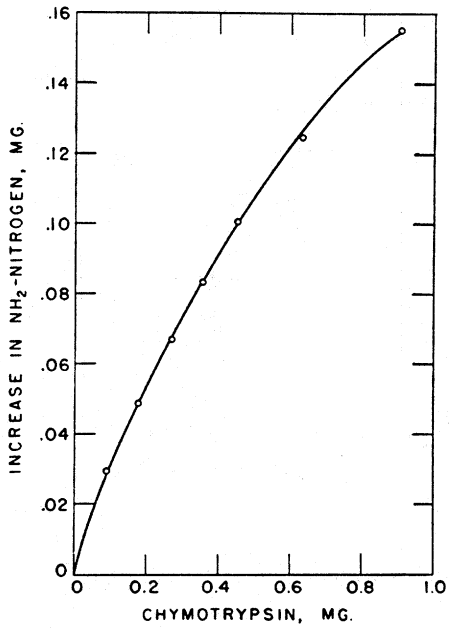

FIG. 1. Relationship between chymotrypsin concentration and α-amino groups liberated during the hydrolysis of β-lactoglobulin.

Figure 2 shows the changes produced in the amino acid composition of chymotrypsin and in its enzymatic activities by photooxidation. With progressive oxygen uptake, rates of inactivation for proteinase and esterolytic activities were the same; they reached a zero value after 4 moles of oxygen had been taken up per mole of enzyme. At 1.5 moles of oxygen uptake, 50 % decreases in both enzymatic activities were apparent as well as a 25 % decrease in histidine (0.48 mole) and a 15 % decrease in tryptophan (1.1 mole), whereas no changes in tyrosine, cystine, or methionine content were evident. At 4 moles of oxygen uptake per mole of enzyme, mentioned before, both enzymatic activities were gone, whereas a 53 % decrease in the histidine (1.02 moles) and a 40 % decrease

in tryptophan content (3.01 moles) were observed. Again at this point the tyrosine, cystine, and methionine contents of the enzyme had not been altered. The irradiation times required for 1.5 and 4 moles of oxygen uptake per mole of enzyme were 5 and 13 min., respectively, and the highest uptake of oxygen studied—16.5 moles—required 95 min. Control irradiation experiments, without the dye or with the dye but in a nitrogen atmosphere, showed no inactivation of the enzyme.

The experiments described above presupposed that the crystalline chymotrypsin used in these studies consisted essentially of the enzyme

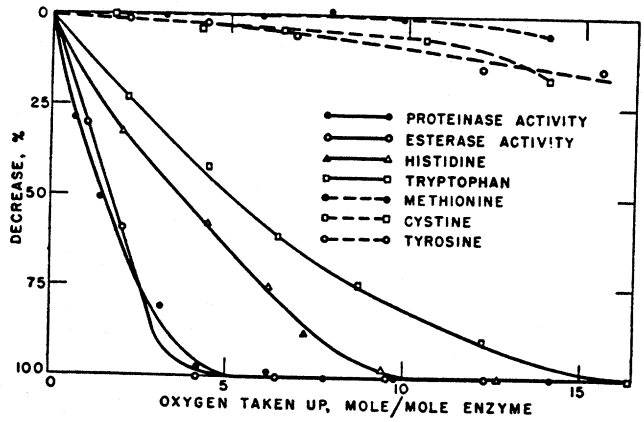

FIG. 2. Decrease of various amino acids and enzymatic activities produced by the photooxidation of chymotrypsin.

protein. In view of the work of Jansen *et al.* (8), there is a possibility that this enzyme contains impurities caused by the conversion of the chymotrypsinogen by traces of trypsin. Such impurities would affect our results in a quantitative aspect, since the photooxidation employed would involve any protein containing the photoreactive amino acids (2, 3) and would obscure our interpretation if the impurities were considerable.

Since nonenzymatic impurities of chymotrypsin can be removed only with great difficulty, irradiation experiments were carried out on chymotrypsinogen, which can be easily obtained in pure form. The experiments described below were made on a crystalline chymotrypsinogen sample purchased from Armour and Company, which was recrystallized three times more according to Kunitz and Northrop (14). This preparation contained about 1.5% of the active enzyme. The oxygen uptake was based on a molecular weight of 27,000 for chymotrypsinogen, and the

chymotrypsinogen concentration was calculated on the basis of 16.18% nitrogen content (8). The degree of photooxidation was determined, at which this precursor loses its ability to be converted into an active enzyme by the addition of traces of crystalline trypsin. For this purpose, aliquots containing 10 mg. chymotrypsinogen were irradiated to various degrees of oxygen uptake, under the same conditions as those described for chymotrypsin (Fig. 2). After the desired stage of photooxidation was reached, aliquots containing 5 mg. chymotrypsinogen were withdrawn from the Warburg vessels. To these samples were added aliquots containing 20 μg. of crystalline trypsin, respectively, and the solutions were adjusted to a volume of 5 ml. The reaction mixture then was incubated at 5°, and the conversion to chymotrypsin was measured at various time intervals. It was found that incubation for 5.5 hr. was sufficient to reach maximal activity.

The activation of chymotrypsinogen by its conversion into the enzyme was measured in the following manner: To 1-ml. aliquots containing 8 μg. of the original chymotrypsinogen was added 1 ml. of a 1% casein solution containing 0.1 *M* phosphate buffer of pH 7.4. After incubation for 20 min. at 35°, the mixture was precipitated with 3 ml. of a 5% solution of trichloroacetic acid and filtered, and the hydrolytic products present in 1 ml. of the filtrate were determined colorimetrically at 650 mμ by Herriott's procedure (15).

For the quantitative measurement of the degree of activation of the precursor, a calibration curve representing the relation between chymotrypsin concentrations and activities was established. Under the above described conditions with enzyme concentrations of 1–10 μg., the optical densities obtained at 650 mμ showed a linear relationship (Fig. 4), and could be used, therefore, for the quantitative measurement of the enzymatic activity.

The results (Fig. 3) closely resemble the results obtained on chymotrypsin (Fig. 2), namely, 4 moles of oxygen uptake per mole of protein was sufficient to eliminate the ability of the precursor to be converted into the active enzyme. In addition, they demonstrated the close similarity in the rate of photo-inactivation of the two proteins.

The essentially identical results obtained on the photo-inactivation of chymotrypsin and its precursor indicate that the observed chemical changes on chymotrypsin (Fig. 2) were not due in any measurable extent to impurities, but were chiefly caused by the modification of the enzyme itself.

In support of this contention, the enzymatic activities of various concentrations of chymotrypsin were compared with activities obtained with the same concentrations of recrystallized chymotrypsinogen after con-

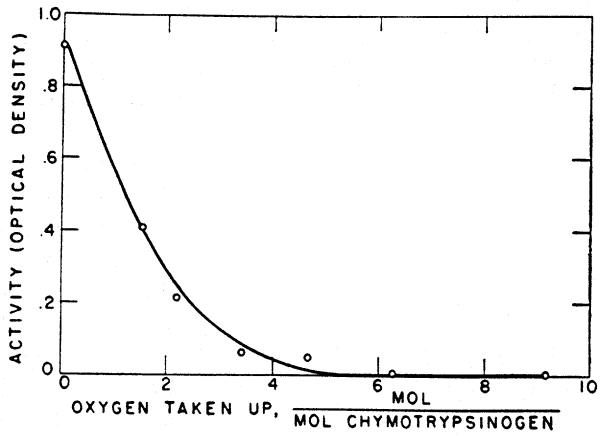

FIG. 3. Effect of photooxidation on chymotrypsinogen and on its conversion to chymotrypsin by trypsin.

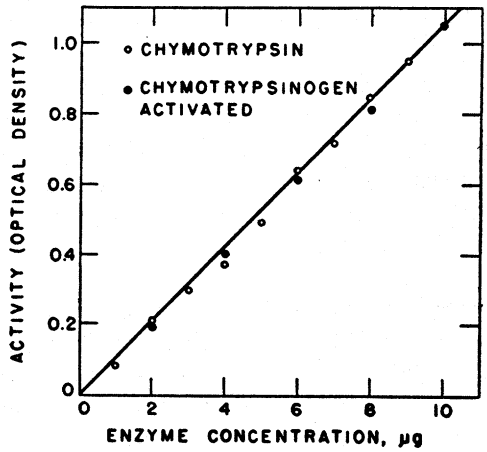

FIG. 4. Relationship between enzymatic activities and concentration of enzymes.

version to the active enzyme. The experimental conditions used were the same as those described for the experiments presented in Fig. 3. The results in Fig. 4 show that the activities of chymotrypsin at any

given concentration were equal to that obtained with the same amount of chymotrypsinogen after conversion to the active enzyme.

On the basis of this evidence, it is concluded that the crystalline chymotrypsin used in our experiments consisted essentially of the enzyme protein and that any impurities present were too low to affect our measurements.

Photochemical Action of Methylene Blue on the Ultraviolet Absorption Spectrum of Chymotrypsin

The photooxidation of chymotrypsin was carried out under the same condition described for the experiments presented in Fig. 2. After removal of the dye by a small amount of charcoal, the absorption spectra were measured at various degrees of oxygen uptake, as described in our previous work (3). The results (Fig. 5) show that at 1.9 moles of oxygen uptake per mole of enzyme a marked decrease of the absorption maximum at 280 mμ and a decrease and slight shift in the minimum from 250 to 253 mμ took place. At 3.9 moles of oxygen uptake, at which point (Fig. 2) the enzyme is completely inactivated, only a slight maximum at 270 mμ and a minimum at 256 mμ could be observed. At 12 moles of oxygen uptake, only an inflection in the curve could be observed. The decrease of the extinction coefficient at 280 mμ from the original of $E_{1\,cm.}^{1\%} =$ 17.1 to 12.9 at 1.9 moles of oxygen uptake per mole of enzyme was due entirely to the photooxidation of tryptophan, inasmuch as tyrosine had not been affected at this point, and the photooxidation of histidine does not yield products that would influence the extinction coefficient at 280 mμ. In view of these facts, the decrease of tryptophan was calculated on the basis of the decrease of $E_{1\,cm.}^{1\%}$ at 280 mμ as being about 25 %. Our actual analytical figure at this point was 21 %, a reasonably good agreement between the two methods. At higher oxygen-uptake levels, values could not be similarly calculated from the optical data because of interfering reactions, which resulted in an over-all increase in absorption.

Photochemical Action of Methylene Blue on the Solubility and Viscosity of Chymotrypsin

Inasmuch as denaturation of the enzyme itself could account for the observed photo-inactivation of chymotrypsin, particular attention was given to this possibility. In our first series of experiments, the method of Kunitz and Northrop (14) was used for the measurement of denatura-

tion. Two milliliters of chymotrypsin solution at pH 7.4 containing 10 mg. of the enzyme was placed in the main chamber of the Warburg vessel, and 0.5 ml. of a 0.02 % solution of methylene blue was put in the side arm. After temperature equilibrium had been reached, the two were mixed, and irradiation was carried out to the extent of 1.9, 4.0, 5.8, 7.9, 11.5 and 16.9 moles of oxygen uptake per mole of enzyme. The irradia-

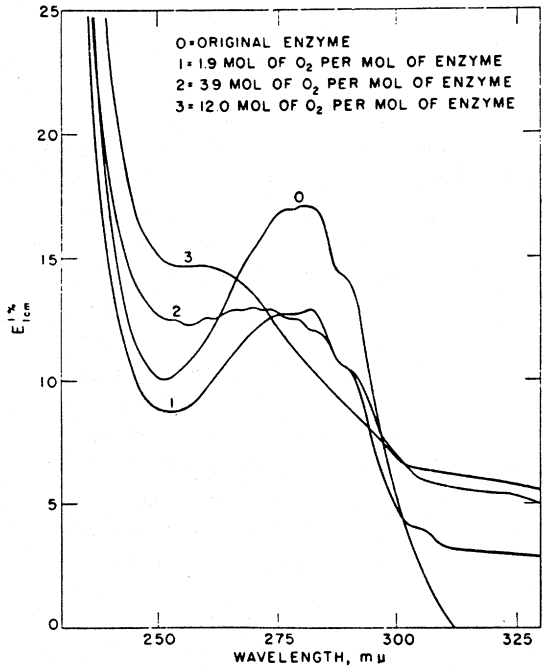

FIG. 5. Changes in the ultraviolet absorption spectrum of chymotrypsin produced by photooxidation.

tion times for these oxygen uptakes were: 4, 10, 16, 32, 62, and 90 min., respectively. After the desired oxygen uptake had been reached, 2 ml. of a 2 *M* NaCl solution in 0.005 *M* HCl was added to the contents of the Warburg vessel. Any denatured enzyme should form a precipitate under these conditions. No such precipitation was observed, however, even at 16.9 moles of oxygen uptake.

In a second series of experiments, the photooxidation of chymotrypsin was carried out at the isoelectric point of the enzyme, where the lowest solubility would be expected, should denaturation take place. The iso-

electric points of chymotrypsin reported in the literature show great divergence. Kunitz (14, 16) observed a value of pH 5.4 whereas others (17, 18) obtained values of pH 8.1–8.6. The higher values might be ascribed to the dimerization of the enzyme (19). In view of these observations, two series of experiments were carried out as above, only the enzyme solution was adjusted to pH 5.4 in the first series and to pH 8.4 in the second. The photooxidation in both series was carried out to 2.1, 3.9, 5.7, 7.8, 9.5, and 11.9 moles of oxygen per mole of enzyme, respectively. In no instance could we observe any precipitate. Thus the three series of experiments gave no indication of denaturation of chymotrypsin during photooxidation.

Since denaturation of a protein in general causes an increase in viscosity, the relative viscosity of chymotrypsin during photooxidation was measured as described previously (3). To 1.5 ml. of chymotrypsin solution containing 30 mg. of enzyme, adjusted to pH 7.4, was added 0.5 ml. of a 0.02% methylene blue solution, and the mixture was irradiated to the extent of 3.0, 4.1, 5.8, and 9.8 moles of oxygen uptake per mole of enzyme. No increase in relative viscosity was observed ($\eta_r = 1.046$ at 25°), thus confirming our solubility studies, which demonstrated the absence of denaturation during photooxidation.

Is the Peptide Bond Hydrolyzed During the Photooxidation of Chymotrypsin?

Chymotrypsin (30 mg.) was irradiated as described for experiments presented in Fig. 2 to the extent of 2.1, 4.06, 7.0, 9.8, and 11.9 moles of oxygen per mole of enzyme. α-Amino nitrogen was measured by the manometric Van Slyke procedure. No increase in α-amino groups could be observed during the photooxidation, indicating the absence of hydrolysis of the peptide bond.

Reactivity of Native and Photo-Inactivated Chymotrypsin with Diisopropyl Fluorophosphate

Previous studies (1, 8, 20, 21) on the inactivation of chymotrypsin with diisopropyl fluorophosphate (DFP) demonstrated the stoichiometric nature of this reaction. In addition, they showed that the blocking of a single group of the enzyme by DFP results in the elimination of catalytic activity. In view of these facts it was of interest to compare the reactivity of native and photo-inactivated chymotrypsin with DFP. To determine this reaction manometrically, CO_2 liberated from $NaHCO_3$

media by the HF formed during the reaction between DFP and chymotrypsin was measured. Aliquots (1.5 ml.) of 0.1 *M* $NaHCO_3$ solutions containing 30, 40, and 50 mg. of chymotrypsin, respectively, were placed in the main chambers of Warburg vessels, and 0.5-ml. portions of 0.1 *M* $NaHCO_3$ solution containing two equivalents of DFP were put in the side arms. The vessels were attached to the manometers, placed in a water bath of 25°, and while being shaken were flushed for 10 min. with a gas mixture of 95% N_2 and 5% CO_2. The contents of each vessel were then mixed, and the CO_2 evolution was measured. Appropriate blank determinations without the enzyme were also made, and the CO_2 values obtained in this way were subtracted from the main determinations. The values calculated from the observed CO_2 evolution were: 1.07, 1.11, and 1.17 moles of CO_2 per mole of enzyme, respectively, which is in fairly good agreement with the stoichiometric nature of this reaction. Identical experiments were carried out as above with chymotrypsin that had been photooxidized to the extent of 4 moles of oxygen per mole of enzyme, at which point (Fig. 2) the activities of the enzyme were completely eliminated. Under these conditions, no CO_2 evolution could be observed upon addition of DFP, indicating that photooxidation destroys the configuration required for attachment of DFP to the enzyme molecule.

Discussion

The great advances made in the field of the proteolytic enzymes are chiefly due to the work of Northrop and his school. These enzymes were shown to be proteins, and, because of their catalytic properties, one must assume that they have some configuration that sets them apart from inert proteins. The comparatively slight changes required for conversion of inactive trypsinogen (22) and chymotrypsinogen (14, 23, 24) into active trypsin and chymotrypsin illustrate this point. In searching for the possible reactive group in enzymes, the method employed must be of sufficient mildness to prevent alteration in the essential characteristics of the native protein. Such a requirement has been met in the chemical modification of proteolytic enzymes (8, 25) when the reaction products can be isolated in crystalline form. When chemical modification of the enzyme does not involve the loss of activity (26, 27), the retention of activity alone suffices to justify the method.

In view of these considerations, we extended our work on the photooxidation of proteins and enzymes (2–5) to chymotrypsin. Our experiments showed that if chymotrypsin is photooxidized by visible light in

the presence of traces of methylene blue, rapid inactivation of the enzyme takes place. A 50 % inactivation of both proteinase and esterase activities was observed when 1.5 moles of oxygen was taken up per mole of enzyme. At this point, a decrease of only 0.48 mole of histidine of the total of 2 moles, and a decrease of 1.1 moles of tryptophan of the total of 7 moles had occurred. Complete inactivation of both activities was achieved at 4 moles of oxygen uptake, at which point the decrease in histidine content amounted to 1 mole and that of the tryptophan content to 3 moles. The identical inactivation rates of proteolytic and esterolytic activities confirm the conclusion of previous workers (13, 28) that the reactive groups responsible for these activities are the same.

The absence of denaturation as shown by our solubility and viscometric studies indicate that the photo-inactivation of chymotrypsin is related to alteration in the amino acid structure within the framework of the chymotrypsin molecule. The changes at the point of complete inactivation of the enzyme amounted to the alteration of 1 mole of histidine and 3 moles of tryptophan. On this basis alone, no preference can be given to either of the two amino acids as a possible contributing factor to the catalytic activity.

The observation, however, that photo-inactivated chymotrypsin lost its ability to react with DFP (in spite of the absence of denaturation) indicates that the single group required for the attachment of this inhibitor to the enzyme was eliminated by the photooxidation. One might assume, therefore, that the photooxidation of only 1 mole of amino acid of the observed 4 moles (1 mole of histidine plus 3 moles of tryptophan) was sufficient to bring about complete inactivation and that the photooxidation of the remaining three units was just coincidental and was not involved in the catalytic activities. With this assumption, the problem would narrow down to a choice between 1 mole of histidine and 1 mole of tryptophan as a possible point of attachment for DFP. The first possibility appears to be supported by the work of Hackley and Wagner-Jauregg (29), which showed that imidazole derivatives can act as catalysts for phosphorylation by DFP. This might also explain the isolation of serinephosphoric acid from a hydrolyzate of DFP-chymotrypsin by Schaffer, May, and Summerson (30).

In support of histidine as a possible contributing factor for chymotrypsin activity, one might mention the proportionality between the rate of enzyme inactivation and the photooxidation of this amino acid in the chymotrypsin molecule. The possible significance of the imidazole

group for enzymatic esterase activity was emphasized by Wilson (31, 32) in his cholinesterase studies. In view of the studies (19, 33–35) which have advanced the conception that chymotrypsin possesses three catalytically active sites, histidine might represent one of the essential active centers. The alteration of this one site alone would upset the union between enzyme and substrate and result in the complete inactivation of the enzyme. These assumptions are suggestive, but further investigations are required to clarify this important problem.

Summary

Photooxidation of chymotrypsin with visible light in the presence of traces of methylene blue resulted in rapid inactivation of the enzyme.

Both the proteolytic and esterolytic activities were inactivated at the same rate. A 100 % inactivation of the enzyme took place at 4.0 moles of oxygen uptake per mole of enzyme, at which point 1 mole of histidine, of the total of 2 moles, and 3 moles of tryptophan, of the total of 7 moles, were photooxidized.

Solubility and viscosity measurements indicated the absence of denaturation during the photooxidation of the enzyme. No hydrolysis of the peptide bonds of chymotrypsin could be observed during the photooxidation of this enzyme.

Photooxidation of chymotrypsinogen to the extent of 4 moles of oxygen per mole of the precursor eliminated its ability to be converted into the active enzyme by traces of trypsin.

Photo-inactivation of chymotrypsin eliminated the reactive site for the attachement of diisopropyl fluorophosphate. The possible significance of these findings in relation to the catalytic property of chymotrypsin is discussed.

References

1. Balls, A. K., and Jansen, E. F., *Advances in Enzymol.* **13,** 321 (1952).
2. Weil, L., Gordon, W. G., and Buchert, A. R., *Arch. Biochem. and Biophys.* **33,** 90 (1951).
3. Weil, L., and Buchert, A. R., *Arch Biochem. and Biophys.* **34,** 1 (1951).
4. Weil, L., Buchert, A. R., and Maher, J., *Arch. Biochem. and Biophys.* **40,** 245 (1952).
5. Weil, L., and Buchert, A. R., *Federation Proc.* **11,** 307 (1952).
6. Weil, L., and Maher, J., *Arch. Biochem.* **29,** 241 (1950).
7. Northrop, J. H., Kunitz, M., and Herriott, R. M., Crystalline Enzymes, p. 26. Columbia University Press, New York, 1948.

8. Jansen, E. F., Nutting, M. D. F., Jang, R., and Balls, A. K., *J. Biol. Chem.* **179**, 189 (1949).
9. Smith, E. L., Brown, D. M., and Laskowski, M., *J. Biol. Chem.* **191**, 639 (1951).
10. Smith, E. L., and Brown, D. M., *J. Biol. Chem.* **195**, 525 (1952).
11. Lewis, J. C., Snell, N. S., Hirschman, D. J., and Fraenkel-Conrat, H., *J. Biol. Chem.* **186**, 23 (1950).
12. Tristram, G. R., *Advances in Protein Chem.* **5**, 145 (1949).
13. Schwert, G. W., Neurath, H., Kaufman, S., and Snoke, J. E., *J. Biol. Chem.*, **172**, 221, (1948).
14. Kunitz, M., and Northrop, J. H., *J. Gen. Physiol.* **18**, 433 (1935).
15. Herriott, R. M., *Proc. Soc. Exptl. Biol. Med.* **46**, 642 (1942).
16. Kunitz, M., *J. Gen. Physiol.* **22**, 207 (1938).
17. Anderson, E. A., and Alberty, R. A., *J. Phys. & Colloid Chem.* **52**, 1345 (1948).
18. Kubacki, N., Brown, K. D., and Laskowski, M., *J. Biol. Chem.* **180**, 73 (1949).
19. Neurath, H., and Schwert, G. W., *Chem. Revs.* **46**, 69 (1950).
20. Jansen, E. F., and Balls, A. K., *J. Biol. Chem.* **194**, 721 (1952).
21. Jansen, E. F., Nutting, M. D. F., and Balls, A. K., *J. Biol. Chem.* **179**, 201 (1949).
22. Kunitz, M., *Enzymologia* **7**, 1 (1939).
23. Desnuelle,P ., Rovery, M., and Fabre, C., *Biochim. et Biophys. Acta* **9**, 109 (1952).
24. Gladner, J. A., and Neurath, H., *Biochim. et Biophys. Acta* **9**, 335 (1952).
25. Herriott, R. M., *J. Gen. Physiol.* **19**, 283 (1935).
26. Sizer, I. W., *J. Biol. Chem.* **169**, 303 (1947).
27. Fraenkel-Conrat, H., Bean, R. S., and Lineweaver, H., *J. Biol. Chem.* **177**, 385 (1949).
28. Jansen, E. F., Nutting, M. D. F., and Balls, A. K., *J. Biol. Chem.* **179**, 201 (1949).
29. Hackley, B. E., and Wagner-Jauregg, T., *Federation Proc.* **11**, 224 (1952).
30. Schaffer, N. K., May, S. C., and Summerson, W. H., *Federation Proc.* **11**, 282 (1952).
31. Wilson, I. B., and Bergmann, F., *J. Biol. Chem.* **186**, 683 (1950).
32. Wilson, I. B., *J. Biol. Chem.* **199**, 113 (1952).
33. Shine, H. J., and Niemann, C., *J. Am. Chem. Soc.* **74**, 97 (1952).
34. Huang, H. T., Foster, R. J., and Niemann, C., *J. Am. Chem. Soc.* **74**, 105 (1952).
35. Huang, H. T., and Niemann, C., *J. Am. Chem. Soc.* **75**, 1395 (1953).