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INACTIVATION OF ALPHA-CHYMOTRYPSIN AT THE ACTIVE SITE
BY A WATER SOLUBLE CARBODIMIDE

by

Betty Kenyon Blossey

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Biological Chemistry)
in The University of Michigan
1973

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To my teachers

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LIST OF ABBREVIATIONS

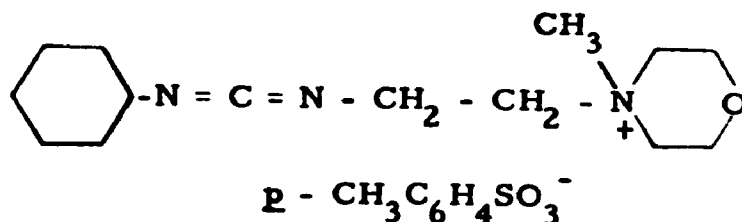
Ala	alanine
Arg	arginine
Asn	asparagine
Asp	aspartic acid
ATpNA	N-acetyl- <u>L</u> -tyrosine p-nitroanilide
BTPNA	N-benzoyl- <u>L</u> -tyrosine p-nitroanilide
CCT	<u>trans</u> -cinnamoyl-chymotrypsin
CDI	1-cyclohexyl-3-(2-morpholinyl-(4)-ethyl) carbodiimide
α-Chy	α-chymotrypsin
CI	N- <u>trans</u> -cinnamoyl imidazole
CMC	1-cyclohexyl-3-(2-morpholinyl-(4)-ethyl) carbodiimide metho-p-toluenesulfonate
¹⁴C-CMC	1-cyclohexyl-3-(2-morpholinyl-(4)-ethyl) carbodiimide metho- ¹⁴ C-p-toluenesulfonate
CMU	1-cyclohexyl-3-(2-morpholinyl-(4)-ethyl) ureylene metho-p-toluenesulfonate
CTgen	chymotrypsinogen-A
1/2 Cys	1/2 cystine
DFP	diisopropylfluorophosphate
DIP	diisopropylphosphoryl
DIP-CT	diisopropylphosphoryl-chymotrypsin
DNFB	dinitrophenylfluorobenzene
DNP	dinitrophenyl
EDC	1-ethyl-3-dimethylaminopropyl carbodiimide

LIST OF ABBREVIATIONS (Cont.)

Gln	glutamine
Glu	glutamic acid
Gly	glycine
His	histidine
I	indole
Ile	isoleucine
Leu	leucine
Lys	lysine
Met	methionine
Phe	phenylalanine
Ser	serine
TAME	<u>p</u> -toluenesulfonyl- <u>L</u> -arginine methyl ester
Thr	threonine
TPCK	1-tosylamido-2-phenylethyl chloromethyl ketone
Tris	tris-(hydroxymethyl)-aminomethane
Tyr	tyrosine
Val	valine

I. INTRODUCTION

Interest in further characterizing the structure and mechanism of action of α -chymotrypsin (from bovine pancreas) motivated this investigation of the reaction of 1-cyclohexyl-3-(2-morpholinyl-(4)-ethyl) carbodiimide metho-*p*-toluenesulfonate (CMC) with α -chymotrypsin in aqueous solution.



CMC

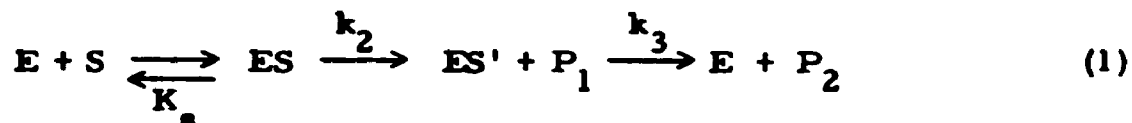
CMC was first synthesized by Sheehan and Hlavka¹ who used it as a condensing agent to form amide bonds between appropriately substituted amino acids. Sheehan and Hlavka² also used CMC to cross-link gelatin in aqueous solution, presumably via intermolecular amide bonds.

The present work was begun with the goal of developing reactions between CMC and α -chymotrypsin into a general method for investigating the tertiary structure of proteins in solution. CMC was expected to mediate formation of intramolecular amide bonds between adjacent carboxyl and amino groups of α -chymotrypsin without inactivating the enzyme. It was expected that identification of the

linked groups would yield information about the spacing of amino and carboxyl groups in the enzyme. As the work progressed, it was decided to characterize an inactivation reaction in order to find out more about the interactions between CMC and enzymes and to further characterize the active site of α -chymotrypsin.

α -Chymotrypsin belongs to a class of enzymes called serine proteinases. These enzymes, which have no known prosthetic group, catalyze solvolyses of ester, amide, and peptide bonds. α -Chymotrypsin undergoes a 1:1 reaction with diisopropylfluorophosphate (DFP) that results in complete loss of enzymic activity³. The diisopropylphosphoryl (DIP) group is found on the hydroxyl group of a single, specific seryl residue after partial acid hydrolysis or enzymic digestion^{4,5}. This reaction with DFP characterizes α -chymotrypsin as a serine proteinase.

During the α -chymotrypsin-catalyzed hydrolysis of *p*-nitrophenyl acetate, an initial rapid release of one mole of *p*-nitrophenol per mole of enzyme, followed by a slow zero order reaction of the remaining substrate, is observed⁶. This observation is consistent with the reaction pathway depicted in equation 1. K_s is the dissociation constant of ES, the α -chymotrypsin-substrate complex.



ES' is acetyl-chymotrypsin⁷. The burst of one mole of *p*-nitrophenol per mole of enzyme is explained by a rapid reaction of essentially all

the α -chymotrypsin present to form acyl enzyme. Thereafter, the rate of reaction depends on the rate of deacylation, and zero order kinetics are observed.

The reaction pathway in equation 1 is also thought to be followed in all other solvolytic reactions catalyzed by serine proteases. Acetyl-chymotrypsin, one example of an acyl-chymotrypsin, has been prepared by reacting α -chymotrypsin with p-nitrophenyl acetate⁸. The acetyl group is linked to the same seryl residue (Ser-195) that was phosphorylated by DFP⁹. Several other acyl derivatives of α -chymotrypsin also esterified on Ser-195, have been prepared⁷. Shalitin and Brown¹⁰ showed that in the α -chymotrypsin-catalyzed hydrolysis of ethyl 3-nitrotyrosinate, the acyl portion of the substrate becomes covalently linked to the enzyme in the vicinity of Ser-195. Crystallographic studies of an N-formyl L-tryptophan complex show that the carboxyl group of N-formyl L-tryptophan lies close to Ser-195 and His-57¹¹. Acyl-enzyme intermediates in chymotrypsin's catalytic pathway have also been observed spectrophotometrically. For example, cinnamoyl-chymotrypsin from nitrophenyl cinnamates¹², cinnamoyl imidazole¹², and methyl cinnamate¹³; N-acetyl-L-tryptophanyl-chymotrypsin from methyl N-acetyl-L-tryptophanate¹⁴, N-acetyl-L-tryptophan¹⁴, and ethyl N-acetyl-L-tryptophanate¹⁵; and N-(2-furyl)-acryloyl-L-tryptophanyl-chymotrypsin from N-(2-furyl)-acryloyl-L-tryptophan¹⁶ have been observed.

In many cases where $k_3 \gg k_2$, and the acyl enzyme (ES') could not be directly observed, the existence of ES' could be inferred from studies with "trapping agents". For example, Inagami and Sturtevant¹⁷ compared the α -chymotrypsin-catalyzed hydrolysis and hydroxaminolysis of N-acetyl-L-tyrosine p-nitroanilide (ATpNA), and found that the rate of disappearance of ATpNA is independent of the concentration of hydroxylamine, whereas the yield of hydroxamic acid increases with increasing hydroxylamine concentration. These results imply that an acyl-enzyme intermediate is formed in a rate-limiting step.

The imidazolyl group of one of the two histidyl residues of α -chymotrypsin appears to also be necessary for catalytic activity. Photooxidation studies show that enzymic activity disappears when a histidyl residue is photooxidized^{18,19}. 1-Tosylamido-2-phenylethyl chloromethyl ketone (TPCK), which fits the specificity requirements of the enzyme and thus should bind at the active site, inactivates α -chymotrypsin by reacting with one histidyl residue in the enzyme²⁰. Studies using proteolytic enzymes to digest TPCK-inactivated α -chymotrypsin showed that N-3 of His-57 was alkylated by TPCK^{21,22}. His-57 was also shown to react with phenoxymethyl chloromethyl ketone²³, 2-phenyl-1,4-dibromoacetoin²⁴, and methyl p-nitrobenzenesulfonate²⁵ for form an inactive derivative of chymotrypsin. The possibility that these alkylating agents cause loss of enzymic activity by simply blocking the substrate binding site after alkylating

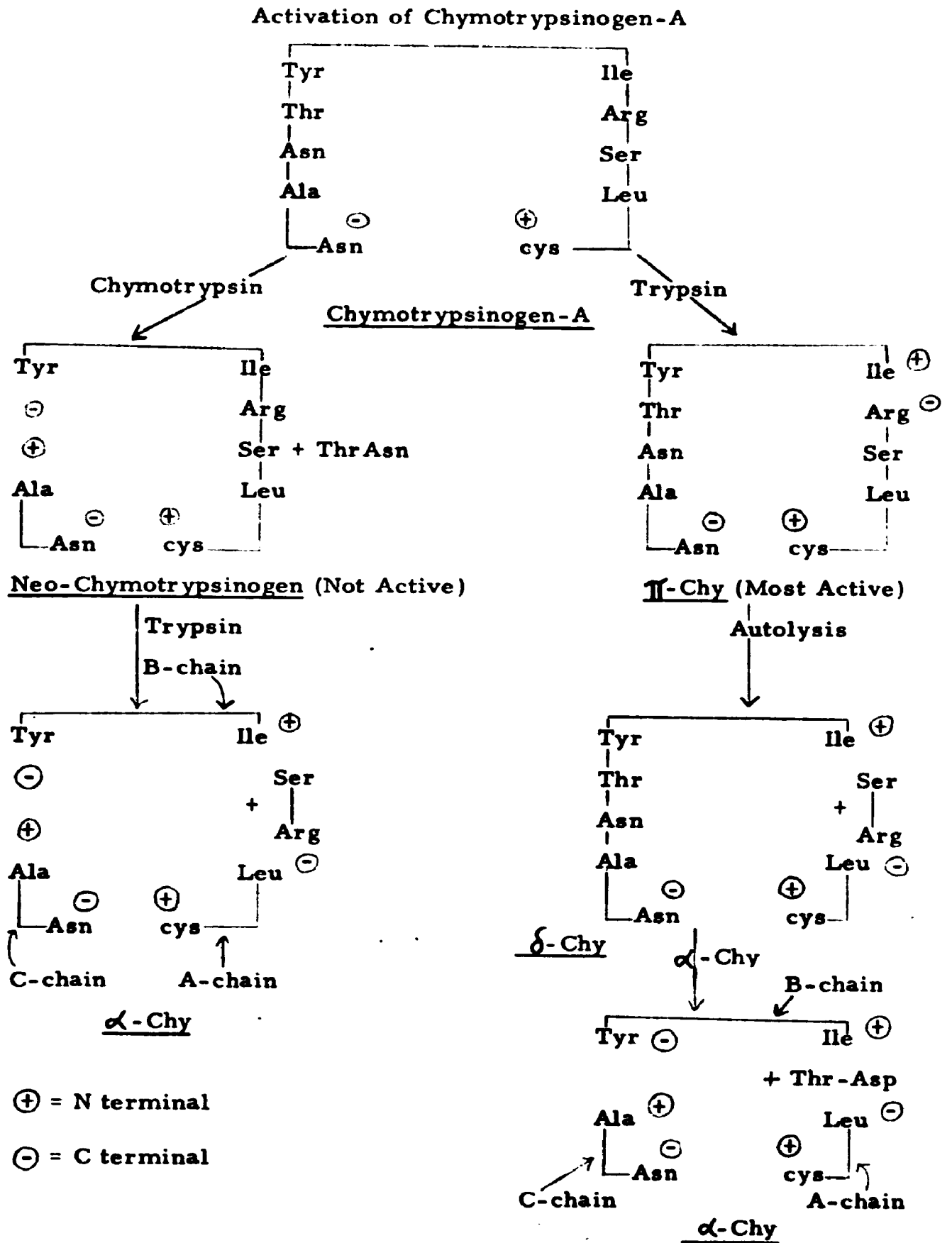
N-3 of His-57 seems unlikely, since methylation of N-3 of His-57 by methyl *p*-nitrobenzenesulfonate causes loss of enzymic activity. It is unlikely that the small methyl group in this catalytically inactive derivative of chymotrypsin interferes with binding of the substrates, since this derivative has an affinity for the substrate similar to that of the unmodified enzyme²⁶. The reactivity of N-3 of His-57 toward alkylating agents is in agreement with the three dimensional structure of α -chymotrypsin as determined by Blow *et al.*²⁷ N-1 of His-57 is not on the surface of the enzyme and is hydrogen bonded to Asp-102, whereas N-3 of His-57 lies on the surface of the enzyme and would be expected to be available for reaction with alkylating agents.

Henderson²⁶ has shown that this methyl derivative of His-57 has a small activity (less than 1/5000 that of native α -chymotrypsin. This activity is attributed to a small amount of the methylated α -chymotrypsin being in a conformation in which the imidazolyl ring is rotated so that the N-1 nitrogen occupies a position close to that which the N-3 occupies in the native enzyme²⁶. In the native enzyme the N-3 nitrogen forms a hydrogen bond with Ser-195, which is believed to be important for catalytic activity. The inability of the N-1 nitrogen in the methylated derivative to form a good hydrogen bond with Ser-195 is thought to be responsible for the low catalytic activity of the methylated derivative²⁶.

There is also evidence that the amino group of the N-terminal isoleucyl residue of α -chymotrypsin has a role in maintaining the

conformation of the active site. It is the trypsin-catalyzed cleavage of an arginyl-isoleucyl bond in chymotrypsinogen-A which yields π -chymotrypsin, the most active form of chymotrypsin (Figure 1)²⁸. Pronounced changes in tertiary structure accompany the cleavage of that arginyl-isoleucyl bond and the resulting formation of catalytically active protein²⁸. This result in itself suggests that the isoleucyl amino group (or arginyl carboxyl group) has an important role in establishing the geometry at the active site. More conclusive evidence for the involvement of the N-terminal isoleucyl amino group was presented by Oppenheimer et al.²⁹ These workers activated fully acetylated chymotrypsinogen-A to produce active acetylated δ -chymotrypsin (see Fig. 1) in which the only free amino group is that of the N-terminal isoleucyl residue. Deprotonation of this N-terminal isoleucyl residue is accompanied by changes in the tertiary structure of the enzyme, as indicated by the pH-dependence of the specific rotation of acetylated δ -chymotrypsin²⁹. The specific rotation of the deprotonated enzyme is the same as that of chymotrypsinogen-A, suggesting that on deprotonation of the N-terminal isoleucyl residue, the enzyme reverts back to a conformation characteristic of the zymogen. Kinetic studies indicate that protonation of a basic residue ($pK_a \sim 8.5$) is required for effective binding between enzyme and substrate in the α -chymotrypsin-, δ -chymotrypsin-, and acetylated δ -chymotrypsin-catalyzed hydrolysis of amide substrates^{30, 31}.

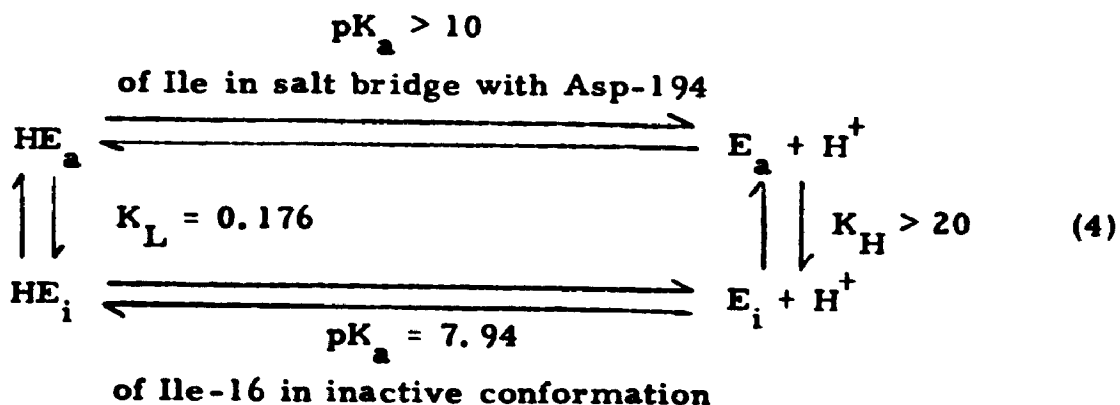
Figure 1.



Fersht and Requena³² have shown that the dye proflavin, which is a competitive inhibitor in α -chymotrypsin-catalyzed reactions, only binds to the active form of the enzyme (E_a). These workers were able to characterize the pH-dependence of the equilibrium depicted in eq. 2 by taking advantage of the fact that binding of the dye to the enzyme (eq. 3) is much faster than conversion of the inactive conformer to the active conformer. Thus, addition of proflavin to the enzyme is accompanied by a rapid burst in absorbance corresponding to the binding of the dye to the active conformer (E_a) followed by a slow change in absorbance corresponding to the re-establishment of the conformational equilibrium and the concomitant binding of proflavin to the newly formed active conformer.



The pH-dependence of the equilibrium depicted in eq. 2 indicated that deprotonation of Ile-16 causes the equilibrium depicted in eq. 2 to shift to the left. This interpretation is quantitatively represented in eq. 4.

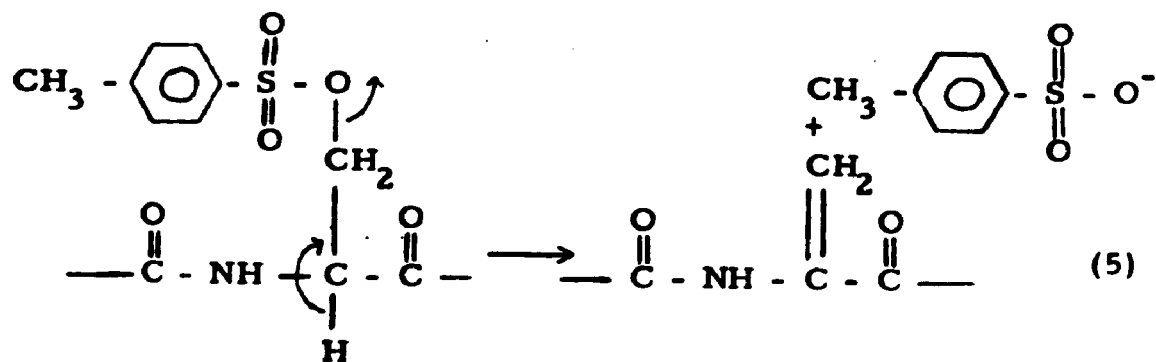


α -Chymotrypsin is one of the few enzymes whose structure has been studied in detail. Hartley³³ and Meloun et al.³⁴ independently determined the sequence of amino acyl residues in chymotrypsin-A. In 1967 Matthews et al.³⁵ presented the results of their x-ray crystallographic work on the tertiary structure of α -chymotrypsin. The sequence of amino acyl residues around the "active serines" of α -chymotrypsin, trypsin, and elastase are very similar, as are the sequences in the region of the two histidyl residues of the same enzymes³⁶. Since the catalytic functions of these enzymes are similar, a definite relationship between structure and function is suggested. Steitz et al.¹¹ have been able to rationalize specificity differences in these structurally similar serine proteinases. Ser-189 in α -chymotrypsin is replaced by an aspartyl residue in trypsin (Asp-177 of trypsinogen). According to Steitz et al.¹¹, replacement of Ser-189, which is at the bottom of the apolar substrate binding site, with an anionic group is responsible for trypsin's specificity toward substrates containing the cationic side chains of Lys and Arg. Consistent with this proposal is the chemical evidence presented by Eyl and Inagami³⁷ that Asp-177 is at the substrate binding site of trypsin. These workers showed that the water soluble carbodiimide, 1-ethyl-3-dimethylaminopropyl carbodiimide (EDC) mediated the condensation between carboxyl groups of trypsin and the amino group of glycylglycyl-L-phenylalanine. Formation of this amide derivative of trypsin resulted in almost complete loss of enzymatic activity. In the presence of the

cationic competitive inhibitor, benzamidine, the extent of carboxyl group modification and the extent of loss of activity decreased, indicating that benzamidine prevented reaction of certain carboxyl groups essential to the catalytic ability of the enzyme. By removing the inhibitor and reacting the carboxyl groups shielded by the inhibitor with EDC and ^{14}C -glycinamide, Eyl and Inagami³⁷ were able to identify the carboxyl group of Asp-177 as the carboxyl group at trypsin's substrate binding site.

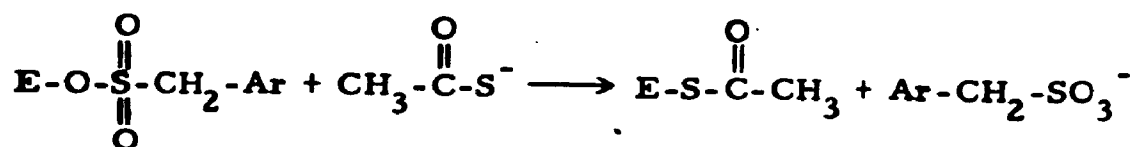
The inability of elastase to efficiently hydrolyze chymotrypsin substrates is thought to be caused by partial filling of the substrate binding site by side chains of amino acyl residues¹¹. Gly-216 and Gly-226 at the binding site in α -chymotrypsin are changed to Val and Thr in elastase. It should be possible to obtain additional information for correlations of structure and function of enzymes by specifically altering amino acyl residues at the active site of enzymes.

Tosyl and phenylmethanesulfonyl derivatives of α -chymotrypsin have been prepared. β -Elimination of the tosyl group from inactive α -chymotrypsin monotosylated on the "active serine" (serine-195) produced anhydrochymotrypsin (eq. 5), which was also inactive^{38, 39}. This experiment suggests that the inactivity of DIP or tosyl-chymotrypsin is not caused simply by steric hindrance due to the bulky DIP or tosyl group blocking the active site, since the conditions for conversion of tosyl-chymotrypsin to anhydrochymotrypsin do not inactivate native α -chymotrypsin.



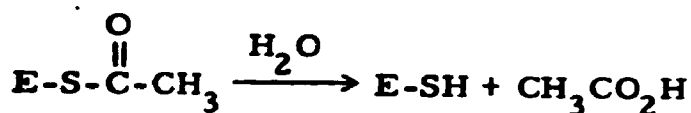
Ser-195

Polgar and Bender⁴⁰ and Neet and Koshland⁴¹, converted the seryl residue at the active site of subtilisin to a cysteinyl residue.



Phenylmethanesulfonyl-subtilisin

(6)



Thiol-subtilisin

Surprisingly, this derivative (thiol-subtilisin) does not catalyze the hydrolysis of the amide and alkyl ester substrates of subtilisin⁴⁰.

On the other hand, thiol-subtilisin does catalyze the hydrolysis of substrates with good leaving groups such as p-nitrophenyl acetate⁴⁰.

Polgar and Bender⁴² observed a pronounced difference in chromatographic behavior (on carboxymethylcellulose) between thiol-subtilisin and subtilisin. This observation supports Polgar and Bender's⁴² proposal that the low catalytic activity of thiol-subtilisin is caused by

distortion of the active site in thiol-subtilisin. The possibility that the inactivity of thiol-subtilisin is the result of some intrinsic difference in chemical reactivity between thiol and hydroxyl groups has not been completely excluded, however. Further studies of enzymes in which amino acyl residues at the active site have been altered should lead to the development of methods for changing the type of chemical reaction a particular enzyme catalyzes.

Although carbodiimides have been known for over ninety years, it was the synthesis of water soluble carbodiimides in 1956 by Sheehan and Hlavka¹, that made these reagents available for general use on proteins. Figures 2 and 3 show reactions which can occur between a water soluble carbodiimide and functional groups found in proteins.

Carbodiimides react with carboxyl groups to form unstable O-acylisoureyne intermediates^{43,44}. This reaction is the basis for many of the uses of carbodiimides in protein chemistry. The very reactive O-acylisoureyne intermediate can, as shown in Figure 2, either rearrange to form the stable N-acylureylene derivative or react with a suitable nucleophile such as water or an amine. The rearrangement to the N-acylureylene derivative probably occurred when Riehm and Scheraga⁴⁵ reacted ribonuclease with an excess of CMC at pH 4.75 and modified some of the carboxyl groups in the enzyme. The modified carboxyl groups appeared to be N-acylureylene derivatives.

Figure 2

Reactions of Carbodiimides Involving Formation of an
O-Acylisoureyene Intermediate

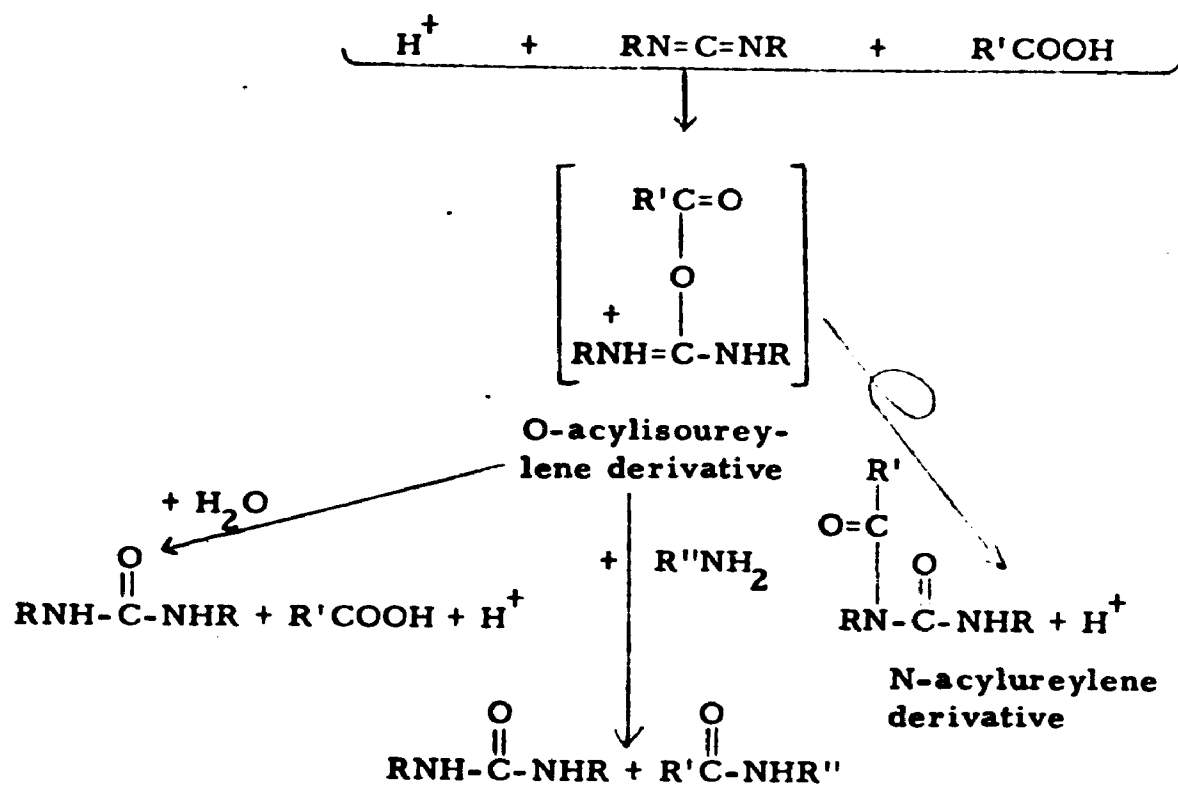
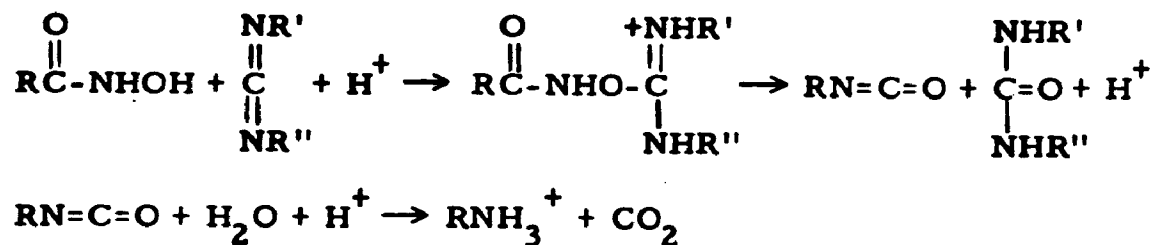


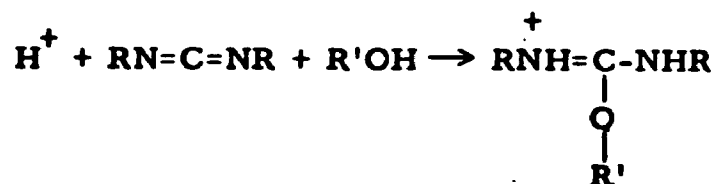
Figure 3

Reactions of Carbodiimides with Nucleophiles

1. Reaction with hydroxamic acids

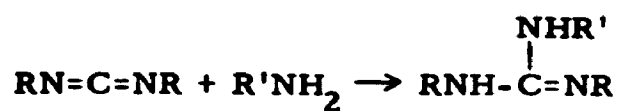


2. Reaction with alcohols and phenols



O-alkyl- (or O-aryl-) isoureyene
derivative

3. Reaction with amino groups



substituted guanidine

Several workers used water soluble carbodiimides to mediate condensations between carboxyl groups of proteins or polypeptides and various nucleophiles. Franzblau et al.⁴⁶ used CMC and hydroxylamine to convert some of the carboxyl groups of gelatin to hydroxamic acid groups. Marfey et al.⁴⁷ formed intramolecular crosslinks in two polypeptides using a water soluble carbodiimide to mediate condensation between neighboring lysyl and glutamyl residues.

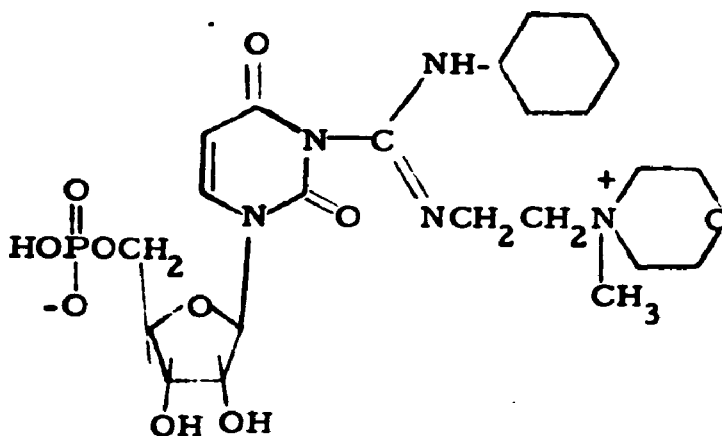
In 1966, Hoare and Koshland⁴⁸ used a carbodiimide to modify most of the carboxyl groups of several proteins, including α -chymotrypsin, by condensing them with glycine methyl ester at pH 4.75. This reaction is the basis for a general method for the quantitative modification and estimation of carboxyl groups in proteins under mild conditions⁴⁴. In a similar reaction Wilchek et al.⁴⁹ used a carbodiimide to condense the carboxyl groups of ribonuclease with glycine and alanylglycine. Lewis and Shafer⁵⁰ have used this method to convert Glu and Asp residues in proteins to Gln and Asn by using ammonia instead of glycine methylester. Fersht and Sperling⁵¹ have shown that semicarbazide is more efficient than glycine derivatives in the carbodiimide mediated condensation with carboxyl groups in proteins. Presumably the increased effectiveness of semicarbazide ($pK_a \sim 3.9$) arises because a greater fraction of it is in the unprotonated form at pH 4.75, the pH optimum for the carbodiimide mediated condensation.

Hoare et al.⁵² used a carbodiimide to effect a Lossen rearrangement on a hydroxamic acid (see Fig. 3), and thereby converted the hydroxamic acid group to an amine. This reaction could be used to convert carboxyl groups to amines in proteins. Since it would convert the negatively charged carboxyl group to a positively charged amino group, this reaction should be valuable in studying the nature of electrostatic forces in maintaining protein conformation.

Hydroxyl groups add to carbodiimides to form O-alkyl- or O-arylisoureyene derivatives. Formation of O-alkylisoureyene derivatives is generally considered to require high temperatures or the presence of a strong base such as alkoxide ion^{43, 53}. Carraway and Koshland⁵⁴ showed that tyrosyl residues reacted under the relatively mild conditions which they used for modifying the carboxyl groups of a protein. They also showed that 1-ethyl-3-dimethylamino-propyl carbodiimide (EDC) reacted with N-acetyltyrosine ethyl ester to form the O-arylisoureyene derivative. Free sulfhydryl groups might be expected to react in a similar manner. Carraway and Triplett⁵⁵ showed that free sulfhydryl groups in proteins are indeed modified by EDC.

Carbodiimides can also react with amino groups to form guanidine derivatives⁴³. Riehm and Scheraga⁴⁵ reported that when they treated ribonuclease with CMC at pH 9.5, acid hydrolysis of the treated ribonuclease followed by amino acid analysis showed lysyl residues had been modified.

Water soluble carbodiimides have proved to be versatile reagents. They have been used to modify nucleotides and nucleic acids as well as proteins. In 1962, P. T. Gilham⁵⁶ reported an addition reaction of CMC, specific for uridine and guanosine nucleotides. He proposed that the product of the reaction between CMC and uridine is the derivative shown below.



Naylor, Ho, and Gilham⁵⁷ modified uridine and pseudouridine in polynucleotides with CMC and studied the effect that the modification had on the specificity of ribonuclease and phosphodiesterases toward the polynucleotides. Other groups^{58, 59} have done similar studies.

One of the most general and potentially useful applications of carbodiimides to protein chemistry is in the modification of carboxyl groups, as described by Hoare and Koshland⁴⁴. The present characterization of the specific inactivation reaction between CMC and α -chymotrypsin should shed light on the limitations and possibilities of carboxyl modification, as well as provide further information on the nature of α -chymotrypsin's active center.

II. EXPERIMENTAL

A. MATERIALS

1. α -Chymotrypsin (EC 3.4.4.5, 3x recrystallized, lot 6121-2) was obtained from Worthington Biochemical Corp. For experiments in which ^{14}C -CMC was reacted with α -chymotrypsin, α -chymotrypsin was purified by an adaptation of the method of Yapel *et al.*⁶⁰. Ten ml of a 3% solution of α -chymotrypsin in 0.001 M HCl was placed on a 4.4 x 46 cm column of Sephadex G-25 (fine), which had been equilibrated at 4° with 0.001 M HCl, and eluted at 4° with 0.001 M HCl at a flow rate of 20-30 ml/hr. Five ml-fractions were collected, and the protein was located by its absorbance at 280 mu.
2. Other Proteins. Chymotrypsinogen-A, (CG, lot 8 CA), diisopropylphosphoryl chymotrypsin (DIP-chymotrypsin, lot 8 BA), and lyophilized, twice-crystallized trypsin (EC 3.4.4.4 lot 6254) were obtained from Worthington Biochemical Corp. Ovalbumin was obtained from Mann Research Laboratories.
3. 2,4-Dinitrofluorobenzene, A grade, was obtained from Calbiochem.
4. 2,4-Dinitrophenyl Amino Acids, A grade, were obtained from Calbiochem.
5. Methyl- ^{14}C Iodide (lot 63-239-16) with a specific activity of approximately 1 mcurie/mmole was obtained from Tracerlab.
6. Sephadex (G-25 and G-75) was obtained from Pharmacia Fine Chemicals, Inc.

7. Tris-(hydroxymethyl)-aminomethane (A grade) was obtained from Calbiochem.

8. Acetonitrile, Benzene, n-Hexane, and Petroleum Ether (used in syntheses) were reagent grade chemicals, which were dried over Linde Type 4A molecular sieve prior to use.

9. 1-Cyclohexyl-3-(2-morpholinyl-(4)-ethyl) Carbodiimide Metho-p-toluenesulfonate (CMC), obtained from Aldrich Chemical Co., was recrystallized three times from benzene, mp 115-116^o, lit.¹ 113-115^o, and stored under reduced pressure over P₂O₅.

CMC was also synthesized by reacting 0.598 g (2.52 mmoles) of 1-cyclohexyl-3-(2-morpholinyl-(4)-ethyl) carbodiimide in a tared tube with 0.28 g (1.98 mmoles) methyl iodide. The tube was stoppered, covered with foil to protect it from light, and shaken intermittently until the mixture solidified (about 1.5 hours). After standing overnight, the white solid was washed by grinding it with 2.00 ml petroleum ether (30-60^o). When all the solid had been ground to a fine powder, the suspension was centrifuged and the supernatant layer discarded. After three more washings with 4.0, 2.0, and 2.0 ml petroleum ether, the solid was dried under reduced pressure over P₂O₅. The dry solid, 1-cyclohexyl-3-(2-morpholinyl-(4)-ethyl) carbodiimide methiodide, weighed 0.614 g. (1.62 mmoles, 82% based on methyl iodide), mp 115-117^o.

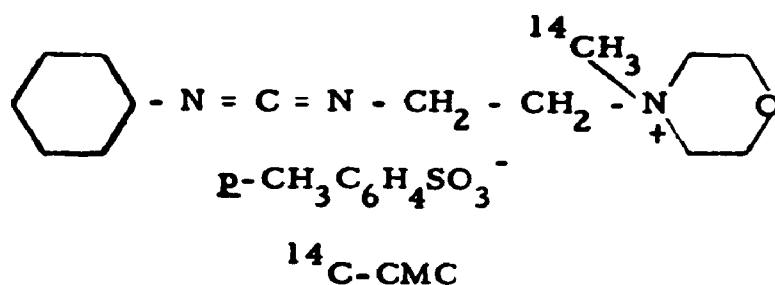
To make CMC, 0.474 g (1.70 mmoles) silver tosylate was almost completely dissolved in 2.0 ml acetonitrile and added all at

once with stirring to 0.614 g (1.62 mmoles) of the carbodiimide methiodide in 2.0 ml of acetonitrile. A yellow precipitate formed immediately. The beaker which had contained the silver tosylate was rinsed twice with a total of 1.0 ml of acetonitrile and the washings added to the reaction mixture. The reaction mixture was stirred for 4 hours in the dark, and then filtered by suction through sintered glass to remove the silver iodide precipitate. The filtrate was dried over 1 g of Linde type 4A molecular sieve overnight, and then the molecular sieve was removed by filtration through Whatman #1 paper. The acetonitrile was evaporated from the filtrate under reduced pressure. The residue, crude CMC, was dried under reduced pressure over P_2O_5 overnight. It was dissolved in 5.5 ml benzene by heating in a $90-93^\circ$ bath, and the solution was transferred to a centrifuge tube using 0.5 ml hot benzene to rinse the original container. The benzene solution was reheated and centrifuged for one minute to remove a small amount of insoluble material. The supernatant solution was transferred to a tared centrifuge tube, allowed to stand until the solid crystallized and then stored at 4° for 0.5 hour. The crystals of CMC were collected by centrifugation and were washed once with 1.5 ml cold benzene. The CMC was recrystallized from 11 ml benzene and dried over P_2O_5 and paraffin shavings under reduced pressure, mp $115-116^\circ$, yield 0.469 g (1.11 mmoles, 56% based on methyl iodide). Further recrystallization resulted in no change in mp. The infrared spectrum showed a band at 4.72μ , which is characteristic of

the $-N=C=N-$ bond⁴³. The ultraviolet spectrum showed a characteristic benzenoid absorption due to the toluenesulfonic acid moiety, with a molar absorptivity (ϵ) of $378 \text{ M}^{-1} \text{ cm}^{-1}$ at 262 m μ .

10. 1-Cyclohexyl-3-(2-morpholinyl-(4)-ethyl) Carbodiimide

Metho-¹⁴C-p-toluenesulfonate (¹⁴C-CMC). The procedure used for synthesizing CMC was also employed to prepare ¹⁴C-CMC, using methyl-¹⁴C iodide with a specific activity of approximately 1.0



mcurie/mmole. The procedure for mixing the carbodiimide and the methyl iodide was altered as follows: In the hood, a sealed flask containing 0.284 g (2.0 mmoles) methyl-¹⁴C iodide was cooled in a dry ice-acetone bath. The seal was broken and 400 μ l (0.39 g, 1.7 mmoles) 1-cyclohexyl-3-(2-morpholinyl-(4)-ethyl) carbodiimide (CDI) was added to the methyl iodide. The mixture was transferred to a tared tube which had been cooled in a dry ice-acetone bath. An additional 200 μ l CDI (0.195 g, 0.085 mmole) was used to rinse any remaining methyl-¹⁴C iodide into the reaction tube. The reaction tube was then sealed and allowed to warm to room temperature.

The ¹⁴C-CMC was recrystallized four times from benzene to constant specific activity. First recrystallization, 8.28×10^5

cpm/ μ mole; second recrystallization, 8.56×10^5 cpm/ μ mole; third recrystallization, 8.52×10^5 cpm/ μ mole; fourth recrystallization, 8.60×10^5 cpm/ μ mole. The ultraviolet spectrum of ^{14}C -CMC (240-280 m μ) was identical to that of the unlabeled CMC.

11. 1-Cyclohexyl-3-(2-morpholinyl-(4)-ethyl) Carbodiimide (CDI)

was prepared using a modification of the method of Sheehan and Hlavka¹.

A mixture of 31.6 g (0.12 mole) of 1-cyclohexyl-3-(2-morpholinyl-(4)-ethyl) thiourea (Aldrich Chemical Co.) and 48.0 g (0.22 mole) mercuric oxide (yellow powder) in 400 ml acetone was refluxed with stirring for 6 hours. The reaction mixture was filtered to remove the mercuric sulfide formed. An additional 24.0 g (0.11 mole) mercuric oxide was added, and the suspension was stirred under reflux for 3 hr. This procedure was repeated once. After the second three hour period, the reaction mixture was filtered through Whatman #42 paper to remove all of the mercuric sulfide. The acetone was evaporated from the filtrate under reduced pressure, and the residual oil was dissolved in 100 ml of benzene and dried overnight at 4^o over 20 g Linde molecular sieve type 4A. A white precipitate which had formed (primarily unchanged starting material) was removed by filtration. The filtrate was concentrated under reduced pressure, and the residual oil was stored in a freezer. To obtain pure product, the oil was filtered (to remove any precipitate) and distilled under reduced pressure, bp 150^o/0.9 mm, yield 9.0 g (28%).

The infrared spectrum of CDI showed a strong band at 4.72 μ , characteristic of the $-N=C=N-$ group.

12. Silver Tosylate was prepared by a modification of the method described by Vogel⁶¹ to make silver maleate. To 47.5 g (0.25 mole) of *p*-toluenesulfonic acid, 84 ml (0.25 mole) of 3.09 M aqueous ammonia solution was added, and then 160 ml of water needed to dissolve the ammonium tosylate which had formed. This mixture was filtered, and a solution of silver nitrate (51 g, 0.30 mole, dissolved in 50 ml of water) was added with stirring. Silver tosylate precipitated immediately. It was collected, washed with about 30 ml of water, and pressed well with the bottom of a beaker. Considerable loss of silver tosylate occurred because of its solubility in water, and therefore ice water was used for three subsequent washings. The salt was protected from light and dried to constant weight in a vacuum oven at 60°, yield 21.3 g (30%). (Presumably the yield could be improved if the suspension of silver tosylate were cooled before it was filtered and if all washings were done with ice water.) Over a period of time, the silver tosylate became discolored. Before using silver tosylate to prepare CMC, it was taken up in acetonitrile and the solution filtered. The acetonitrile was removed under reduced pressure, and the white solid obtained was stored over P_2O_5 in an evacuated dessicator in the dark.

13. N-Benzoyl-L-tyrosine p-Nitroanilide (BTpNA) was synthesized by the method of Bundy⁶². *p*-Nitroaniline hydrochloride (0.314 g,

1.8 mmoles) and N-benzoyl-L-tyrosine amide (0.461 g, 1.8 mmoles) were ground together and then heated to 155-165° in an oil bath until the vigorous bubbling subsided. The resulting hard mass was ground with water using a glass rod. As much water as possible was removed from the solid, and the solid was recrystallized from ethanol-water. The crystals were collected and dissolved in ethanol. The ethanolic solution was boiled with Darco activated carbon, filtered, and cooled. Water was added to produce cloudiness and the mixture was stored overnight at 4°. The yellow crystals were collected and washed with water, 20% alcohol, 0.01 M HCl, and water. The washings were colorless. The BTpNA was recrystallized from ethanol-water and dried, mp 235-236° cor., lit.⁶² 227-228°.

14. N-trans-Cinnamoyl Imidazole (CI) was obtained from Mr. Stephen Su, who synthesized it according to the method of Schonbaum et al.⁶³, and recrystallized it from n-hexane, mp 132-133° cor., lit.⁶³ 133-135°.

15. 1-Cyclohexyl-3-(2-morpholinyl-(4)-ethyl)-urea Metho-p-toluene-sulfonate was synthesized by stirring 5.0 g (0.0118 mole) of CMC from Aldrich Chemical Co. with 3.5 g (0.059 mole) glacial acetic acid and 200 ml water in a round-bottomed flask overnight. The water and acetic acid were evaporated under reduced pressure. The white residue was recrystallized three times from 2-propanol and dried under reduced pressure at 56° over P₂O₅, mp 208° cor. Anal.

calculated for $C_{21}H_{35}N_3O_5S$: C, 57.11; H, 7.98; N, 9.51. Found: C, 56.91; H, 7.81; N, 9.42.

16. Bray Solution⁶⁴ for liquid scintillation counting was prepared by dissolving 8.00 g scintillation grade 2,5-diphenyloxazole obtained from Packard Instrument Company, Inc., 0.400 g p-bis-[2-(5-phenyl-oxazolyl)]-1-benzene obtained from New England Nuclear Corp., 40 ml ethylene glycol, 120 g naphthalene obtained from Matheson, Coleman, and Bell in 200 ml anhydrous methanol and enough p-dioxane obtained from Matheson, Coleman, and Bell to yield a final volume of 2 liters.

17. Other Chemicals unless otherwise specified, were Mallinckrodt, Baker-Adamson, Calbiochem, or Fisher analytical reagents.

18. Distilled Water supplied to the laboratory was run through a demineralizer and redistilled in an all-glass still.

B. METHODS

1. Melting Points were determined on a Mel-Temp apparatus using a thermometer calibrated from 81-237° with a set of melting point standards from Arthur H. Thomas Co.

2. Centrifugation was done at the top speed of a Clinical centrifuge model CL from International Equipment Co.

3. Infrared Spectra were recorded on a Perkin Elmer Model 237 spectrophotometer. Solid samples were in potassium bromide pellets, whereas liquid samples were neat.

4. Ultraviolet Spectra were obtained on a Cary Model 15 recording spectrophotometer.

5. Elemental Analyses were done by Galbraith Laboratories, Inc.

6. Measurements of pH were made using a Radiometer Model 4b pH meter (or a Radiometer Model TTT1C titrator) which was standardized with a 1:1 phosphate, NBS primary standard solution⁶⁵. The response of the glass electrode was checked with another NBS primary standard solution (either borax or phthalate)⁶⁵. Any non-linearity in the glass electrode response was corrected with the temperature compensator. This correction never corresponded to more than 1° per pH unit difference between the primary standards.

7. Specific Activity of ¹⁴C-CMC. After each recrystallization, approximately one mg of ¹⁴C-CMC was dissolved in 3.00 ml of water. An ultraviolet spectrum of this solution was obtained, and the concentration of ¹⁴C-CMC in the solution was calculated from the absorbance at 262 mμ ($\epsilon = 378 \text{ M}^{-1} \text{ cm}^{-1}$). An aliquot of the same solution was put into a counting vial, and water added to make a total volume of 0.600 ml. Then ml of Bray solution was added, and the solution mixed and immediately placed in a Packard Automatic Tri Carb Model 314 liquid scintillation spectrometer for counting at 4°. The high voltage tap was set at 6 (950 V), which gave the maximum cpm for a sample of ¹⁴C-labeled material dissolved in a mixture of 10.0 ml Bray solution and 0.6 ml water. The radioactivity of each sample was counted

for two minute periods. Between 44,000 and 100,000 counts were obtained for each sample.

8. Radioactivity of Protein Samples was determined by adding 0.600 ml of water containing less than 0.12 mg of protein to 10 ml of Bray solution and immediately measuring the radioactivity in the scintillation spectrometer. The radioactivity of a standard sample of ^{14}C -benzoic acid in toluene (Packard) was measured with each set of samples to verify that the spectrometer was always operating at the same efficiency.

9. Concentrations of α -Chymotrypsin in aqueous solution were determined from the A_{280} by using an optical factor of $0.465 \text{ mg ml}^{-1} (\text{absorbance unit})^{-1}$ ⁶⁶. The same optical factor was used to calculate the (approximate) concentration of solutions of DIP-CT and CTgen. An optical factor of $0.64 \text{ mg ml}^{-1} (\text{absorbance unit})^{-1}$ at 280 m μ was used for trypsin solutions⁶⁷.

10. Rate Assays for Enzymic Activity of α -Chymotrypsin. Enzymic activities of α -chymotrypsin solutions were measured by a modification of the method of Bundy⁶² using N-benzoyl-L-tyrosine p-nitroanilide (BTpNA) as substrate. The hydrolysis of BTpNA was followed by the increase in absorbance at 385 m μ associated with the liberation of p-nitroaniline. A BTpNA assay solution was prepared by diluting 7.00 ml acetone containing 5.35×10^{-3} mmoles BTpNA to 50 ml with pH 8.0 Tris buffer (0.040 F Tris, 0.024 F HCl, 0.005 M CaCl_2). For routine assays, 3.00 ml of BTpNA assay solution was allowed to come

to $25^{\circ} \pm 0.2$ in a thermostated cell compartment of the spectrophotometer, and 0.100 ml of an α -chymotrypsin solution was added. After mixing the solution, the linear dependence of the absorbance at 385 m μ on the time was recorded for 16 min or until the absorbance reached a value of 0.22. Under these conditions, the rate of liberation of *p*-nitroaniline was directly proportional to the α -chymotrypsin concentration for chymotrypsin concentrations (in the cuvette) up to 7 μ g/ml (Fig. 4). For assays of the activity of α -chymotrypsin or its derivatives during treatment with hydroxylamine, a different BTpNA solution was used to allow for larger volumes of enzyme solution in the assay procedure.

11. Rate Assays for the Enzymic Activity of Trypsin. The assay of Hummel⁶⁸ with *p*-toluenesulfonyl-L-arginine methyl ester (TAME) was used to measure the activity of trypsin solutions. The rate of increase of the absorbance accompanying the hydrolysis of TAME was followed at 247 m μ . The assays were done by incubating 0.200 ml of 0.165 M TAME and 3.00 ml Tris buffer (0.05 F Tris, 0.026 F HCl, 0.0115 M CaCl₂, pH 8.15) in a cuvette at 25.0° and then adding with mixing, 0.100 ml of trypsin solution (~ 20 μ g/ml).

12. All-Or-None Assays, Using N-trans-Cinnamoyl Imidazole. The concentration of active sites in samples of native, purified (method of Yapel et al.⁶⁰), and CMC-treated α -chymotrypsin was determined by a modification of the method of Schonbaum et al.⁶³. This method is a spectrophotometric titration based on the fact that N-trans-cinnamoyl

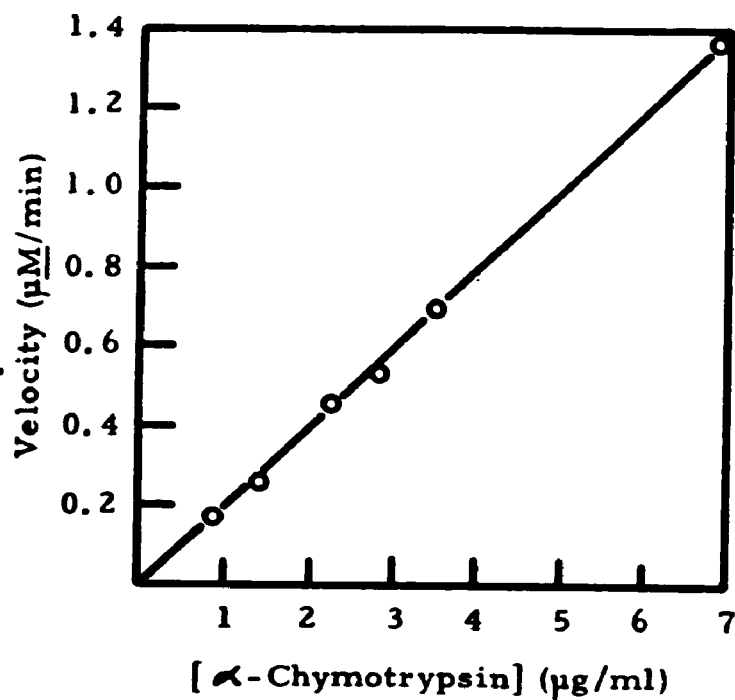


Figure 4. Dependence of velocity of substrate hydrolysis on the concentration of α -chymotrypsin for the N-benzoyl-L-tyrosine p-nitroanilide assay.

imidazole (CI) rapidly acylates α -chymotrypsin to form relatively stable trans-cinnamoyl chymotrypsin (CCT).

The concentration of active sites in an enzyme solution was determined by mixing 0.200 ml of acetate buffer (0.48 M acetic acid, 1.12 M sodium acetate) with 3.0 ml of the enzyme solution in order to adjust the pH of the enzyme solution to 5.05. The absorbance at 310 m μ of this solution (A_i) was measured, and 0.100 ml of CI stock solution (1.5-3.0 moles of CI per mole of active sites), was added with mixing to the 3.2 ml. The absorbance of the solution at 310 m μ was monitored. A rapid drop in absorbance occurred followed by a slow decrease in absorbance. The slow decrease in absorbance which is associated with the hydrolysis of CI and CCT was extrapolated to the time of mixing (A_{mix}).

The concentration of active sites in the 3.3 ml of enzyme solution (N_e) containing CI is given by equation 7.

$$N_e = \frac{A_{CI} - (A_{mix} - \frac{3.2}{3.3} A_i)}{\epsilon_{CI} - (\epsilon_{CCT} - \epsilon_e)} \quad (7)$$

A_{CI} is the resulting absorbance (at the time of mixing) when 0.1 ml of CI is added to 3.0 ml of water and 0.2 ml of the acetate buffer. ϵ_{CI} , ϵ_{CCT} , and ϵ_e are the molar absorptivities (at 310 m μ) of CI, CCT, and active chymotrypsin, respectively.

Equation 7 is obtained from the following relationships:

$$A_{CI} = N_{CI} \epsilon_{CI} + B. \quad (8)$$

where N_{CI} is the concentration of CI prior to its reaction with enzyme and B is the background absorbance associated with the solution CI.

$$A_i = \frac{3.3}{3.2} N_e \epsilon_e + Bi \quad (9)$$

where Bi is the background absorbance of the enzyme solution.

$$A_{mix} = (N_{CI} - N_e) \epsilon_{CI} + N_e \epsilon_{CCT} + \frac{3.2}{3.3} Bi + B \quad (10)$$

Thus,

$$A_{CI} - (A_{mix} - \frac{3.2}{3.3} Ai) = N_e (\epsilon_{CI} - \epsilon_{CCT} + \epsilon_e). \quad (11)$$

Solving (11) for N_e yields equation 7.

The quantity, $\epsilon_{CI} - (\epsilon_{CCT} - \epsilon_e)$, was determined from equation (12), using the procedure employed for determining N_e , except that the concentration of active sites was in excess over the concentration of CI.

$$\epsilon_{CI} - (\epsilon_{CCT} - \epsilon_e) = \frac{A_{CI} - (A_{mix} - \frac{3.2}{3.3} Ai)}{N_{CI}} \quad (12)$$

Equation 12 is obtained from the fact that when $N_e > N_{CI}$

$$A_{mix} = N_{CI} \epsilon_{CCT} + (N_e - N_{CI}) \epsilon_e + \frac{3.2}{3.3} Bi + B, \quad (13)$$

$$Ai = \frac{3.3}{3.2} N_e \epsilon_e + Bi, \text{ and} \quad (14)$$

$$A_{CI} = N_{CI} \epsilon_{CI} + B. \quad (15)$$

$$\text{Thus } A_{CI} - (A_{mix} - \frac{3.2}{3.3} Ai) = N_{CI} (\epsilon_{CI} - \epsilon_{CCT} + \epsilon_e) \quad (16)$$

and rearrangement of equation 16 yields equation 12.

Since the determination of $\epsilon_{CI} - (\epsilon_{CCT} - \epsilon_e)$ requires an accurate value for the concentration of CI in the assay mixture (N_{CI}), prior to its reaction with enzyme, a freshly synthesized sample of CI was used to determine $\epsilon_{CI} - (\epsilon_{CCT} - \epsilon_e)$. A value of 10,200 vs. a reported value of 10,900⁶³ was obtained for $\epsilon_{CI} - (\epsilon_{CCT} - \epsilon_e)$.

13. Reaction of α -Chymotrypsin with CMC. A known weight of α -chymotrypsin was dissolved in 0.100 M KCl. If necessary, the solution was centrifuged to remove any insoluble material and the supernatant layer adjusted to the desired pH with sodium hydroxide. A known weight of CMC was dissolved in a suitable volume of 0.10 M KCl in a 25.0° thermostated beaker, and the pH adjusted to that of the chymotrypsin solution. An aliquot of the chymotrypsin solution was then mixed with the CMC solution. The pH was kept constant with a Radiometer Model TTT1C titrator. The remaining α -chymotrypsin solution was diluted to a suitable concentration, its A_{280} read, and an aliquot of the solution was assayed for its activity with BTpNA. The change in the activity with time of α -chymotrypsin in the reaction mixture was followed by withdrawing 0.1 ml aliquots from the reaction mixture at suitable time intervals, and assaying these samples with 3 ml of BTpNA assay solution. If necessary, the aliquots from the reaction mixture were diluted to less than 200 $\mu\text{g/ml}$ of active α -chymotrypsin with water before they were assayed with BTpNA.

14. Gel Filtration on Sephadex G-25 Columns (2.5 x 30 cm for 10-30 ml samples and 0.9 x 50 cm for 1 ml samples) was employed to

separate α -chymotrypsin from smaller molecules such as CMC. After equilibration of the Sephadex G-25 (fine) columns with 0.010 M NaCl, the samples were applied and eluted with 0.010 M NaCl. The fractions containing α -chymotrypsin were located by their A_{280} and were combined and lyophilized.

Gel filtration of the reaction mixtures containing α -chymotrypsin and ^{14}C -CMC was done at 4° on a 4.4 x 46 cm column equilibrated with 0.010 M NaCl. The column was eluted with 0.010 M NaCl (flow rate 20 ml/hr) and 10-ml fractions were collected. The protein was located by its A_{280} . The radioactivity of aliquots of the fractions at the beginning and end of the peak was counted. If the fractions at the beginning and end of the protein peak did not have the same specific radioactivity, the fractions containing proteins were combined and re-chromatographed at 4° using 0.01 M NaCl on the same 4.4 x 46 cm column at a low rate of 85 ml/hr.

15. The Maximum Molecular Weight of CMC-Inactivated (at pH 7)

α -Chymotrypsin was estimated by gel filtration on a 1 x 40 cm column of Sephadex G-75 (fine). The column was calibrated with ovalbumin (MW 45,000) and native α -chymotrypsin (MW 25,000). A 2 ml sample containing ovalbumin (1.1 mg) and α -chymotrypsin (0.5 mg) was resolved by gel filtration on this column. A solution of CMC-inactivated α -chymotrypsin (0.9 mg in 2 ml) was subjected to gel filtration under conditions used for calibration of the column.

16. Hydroxylamine Treatment.

a. Solutions.

(1) 2.0 M hydroxylamine at pH 8.0 was prepared by adding (~50 ml) 2.0 M NaOH to 100 mmoles of hydroxylamine hydrochloride until the pH of the solution reached 8.0.

(2) 2.0 M hydroxylamine, 0.005 CaCl_2 at pH 8.0 was prepared by adding 0.25 mmole of $\text{CaCl}_2 \cdot 6 \text{H}_2\text{O}$ to the 50 ml of 2.0 M hydroxylamine at pH 8.0.

(3) Tris buffer (pH 8.0) was 0.040 F Tris, 0.024 F HCl, and 0.005 M CaCl_2 .

b. ^{14}C -CMC-Treated Chymotrypsin was prepared by reacting α -chymotrypsin (4-5 mg/ml) with 0.002 M ^{14}C -CMC at pH 8.0 for four hours, and the protein was separated from the ^{14}C -CMC by gel filtration on Sephadex G-25.

c. Treatment of CMC-Treated α -Chymotrypsin with 0.95 M Hydroxylamine and pH 8.0 Tris Buffer. One volume of 0.103 mg/ml α -chymotrypsin in 0.010 M CMC, pH 7.0, for 18 hours was mixed with ten volumes of 2.0 M hydroxylamine at pH 8.0, and ten volumes of pH 8.0 tris buffer. A control without NH_2OH present was prepared by mixing 20 volumes of pH 8.0 tris buffer with one volume of the same α -chymotrypsin-CMC reaction mixture. The catalytic activity of these solutions toward BTpNA was measured at various times.

d. Treatment of ^{14}C -CMC-Treated α -Chymotrypsin and Untreated α -Chymotrypsin with 1.9 M Hydroxylamine. One volume

of a solution of 0.22 mg/ml ^{14}C -CMC-treated chymotrypsin was mixed with 20 volumes of 2.0 M hydroxylamine, 0.005 M CaCl_2 at pH 8.0. A control, containing 20 $\mu\text{g}/\text{ml}$ of untreated α -chymotrypsin, was prepared by mixing one volume of a solution of 0.22 mg/ml α -chymotrypsin with 20 volumes of the same hydroxylamine- CaCl_2 solution. At various times, samples of the test solution containing ^{14}C -CMC treated chymotrypsin and the control were assayed for their activity toward BTpNA.

A second test solution containing 20 $\mu\text{g}/\text{ml}$ of ^{14}C -CMC-treated chymotrypsin was prepared by mixing one volume of 0.22 mg/ml of the ^{14}C -CMC-treated chymotrypsin with twenty volumes of the 2.0 M hydroxylamine, 0.005 M CaCl_2 at pH 8.0. The activity of the chymotrypsin in this solution was assayed after 6 minutes and 18 hours of hydroxylamine treatment. After eighteen hours of hydroxylamine treatment, this test solution was chromatographed on Sephadex G-25. The two fractions which contained chymotryptic activity toward BTpNA were combined and lyophilized. The lyophilized chymotrypsin was dissolved in 1.3 ml of 0.001 N HCl , and its specific catalytic activity toward BTpNA as well as its specific radioactivity determined.

17. Reaction of ^{14}C -CMC with Chymotrypsinogen-A. Chymotrypsinogen-A (4.82 mg/ml) was treated with 0.002 M ^{14}C -CMC at pH 8 using the same procedure employed for the reaction of CMC with α -chymotrypsin and after 4 hours it was subjected to gel filtration on Sephadex G-25. The fractions containing protein were combined and

subjected again to gel filtration to remove any traces of ^{14}C -CMC, and the specific radioactivity of the ^{14}C -CMC-treated chymotrypsinogen-A was determined.

18. Reaction of ^{14}C -CMC with DIP-Chymotrypsin. DIP-chymotrypsin (5.35 mg/ml) was treated with 0.002 M ^{14}C -CMC at pH 8 using the same procedure employed for the reaction of ^{14}C -CMC with chymotrypsinogen-A.

19. Treatment of Trypsin with CMC. Trypsin was treated with CMC at pH 8.0 (0.02 mg/ml trypsin, 5.4 mM CMC and 4.8 mg/ml trypsin, 10 mM CMC) using the same procedure employed for the reaction of α -chymotrypsin with CMC. The activity of trypsin toward TAME was assayed at various time intervals. The change of activity toward BTPNA with time was also followed in the run containing 4.8 mg/ml trypsin.

20. Determination of Amino Groups in Native and CMC-Treated α -Chymotrypsin using 2,4-Dinitrofluorobenzene (DNFB).

a. Dinitrophenylation of Native and CMC-Treated α -Chymotrypsin.

(1) Method 1: An aqueous solution of 0.2 mg/ml enzyme (9 volumes) was stirred with one volume of 2 mg/ml NaHCO_3 solution and 0.4 volume DNFB in a thermostated beaker at 40° for 60-70 minutes.

(2) Method 2 (method of Levy⁶⁹): DNFB (0.200 ml) was added to 6.0 ml of water containing 3.1 mg α -chymotrypsin at pH 8.0

in a beaker thermostated at 40° . The mixture was stirred and the pH was maintained at pH 8.0 by the addition of 0.05 M NaOH from an American Instrument Co. burette controlled by a Radiometer TTT1C automatic titrator. After 90 minutes, the reaction mixture was extracted with ether twice and acidified with hydrochloric acid.

(3) Method 3 (modified method of Sanger⁷⁰): An aqueous solution containing a sample of chymotrypsin and 5 mg NaHCO_3 was mixed with twice the volume of a 5% solution of DNFB in 95% ethanol. The reaction mixture was stirred for two hours at room temperature.

b. Extraction with Ether to Remove Excess DNFB and By-Products. The reaction mixture was acidified (pH 2) with concentrated hydrochloric acid and then extracted with ether until the ether layer showed no yellow color when it was shaken with 1 M NaOH.

c. Absorbancies at 350 m μ of Solutions of 2,4-Dinitro-phenyl- (DNP-) Proteins in Acetic Acid. For this determination, samples of α -chymotrypsin and CMC-treated chymotrypsin were dinitrophenylated using method 1. After ether extraction the water layer containing the precipitated DNP-protein was dissolved by adding glacial acetic acid to a known volume. The solution was centrifuged to remove a small amount of lint-like material and the A_{350} of the solution was determined.

d. Hydrolyses of DNP-Proteins. DNP-proteins were hydrolyzed at 105° in 6 N HCl (less than 1 mg/ml DNP-protein) in a flask equipped with a reflux condenser. After either 12 or 22 hours, the

hydrolysate was diluted to 1 M HCl and extracted four or five times with ether.

e. Amino Acid Analyses of the Water Layer. After ether extraction, the water layer of the hydrolysate was evaporated to dryness under reduced pressure. Water was added to the residue and the solution evaporated again. This procedure was repeated four times. The residue was dissolved in 1.2 ml of 0.100 M HCl, and five-sixths of the solution was chromatographed on a Phoenix amino acid analyzer for determination of basic amino acids.

f. Thin Layer Chromatography of the Ether Soluble DNP-Amino Acids. After extraction of the hydrolysate with ether the combined ether layer was washed with 1.0 M HCl and evaporated under reduced pressure. The residue was taken up in acetone and spotted on Eastman silica gel chromatogram paper (K301R2) which had been dried at 110° for 30 minutes. A solution of DNP-isoleucine in acetone was spotted on one side of the residue and a solution of equal amounts of DNP-isoleucine and DNP-alanine on the other. The chromatogram was developed in the Eastman Chromatogram Chamber in the dark using a solvent system (developed by Green and Kay⁷¹ for separating DNP-amino acids on silicic acid-celite columns) containing 8.0 ml glacial acetic acid and 4.0 ml acetone diluted to 100 ml with 30-60° petroleum ether.

21. Amino Acid Analysis of CMC-Treated Chymotrypsin and Native α -Chymotrypsin. After hydrolysis of the protein in 6 N HCl (less

than 1 mg/ml protein) for 22 hours at 108° in an evacuated sealed tube, the hydrolysate was brought to dryness on a rotary evaporator under reduced pressure, and taken up in an appropriate volume of 0.1 N HCl. Amino acids were determined by the method of Spackman et al.⁷², except after development of the color, the absorbance was measured continuously at 570 mu on a Gilford Model 2000 multiple sample absorbance recorder using 0.5 and 1 cm flow-through cells. Areas under the peaks were measured with a compensating polar planimeter sold by the Gelman Instrument Co.

III. RESULTS

A. REACTION OF CMC WITH α -CHYMOTRYPSIN

1. General Description of the Inactivation Reaction. Figures 5 and 6 illustrate the effect on catalytic efficiency of incubating CMC with α -chymotrypsin at pH 7 and pH 8. The dependence of catalytic activity on time of incubation with CMC is illustrated in Figure 7. This figure indicates that reaction with CMC produces an essentially inactive enzyme at pH 7 and pH 8, whereas at pH 5 a partially active derivative of α -chymotrypsin is produced.

2. Residual Activity After Long Reaction Times. After CMC is allowed to react with α -chymotrypsin for times equivalent to more than ten half-lives at pH 7 and pH 8, a small residual activity remains which corresponds to between 2% and 4% of the initial enzymic activity. This activity may be associated with a form of α -chymotrypsin which is unaffected by CMC or it may be due to proteolytic enzyme impurities in the chymotrypsin which are unreactive toward CMC. An alternative possibility, that the products of reaction between α -chymotrypsin and CMC at pH 7 and pH 8 have low catalytic efficiencies, will be considered later. The possibility that any of the reactions between CMC and α -chymotrypsin failed to go to completion because the CMC decomposed before it inactivated all the α -chymotrypsin was excluded by the observation that extra additions of CMC after several half-lives resulted in no further loss of chymotryptic activity.

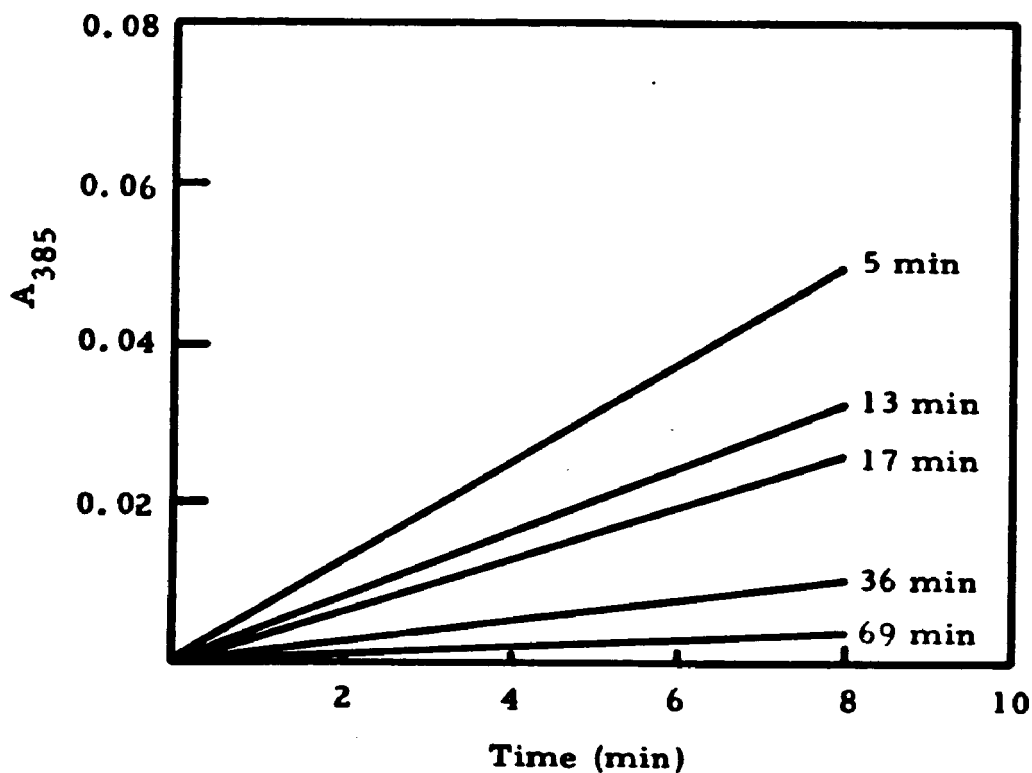


Figure 5. Recorder traces of α -chymotrypsin's catalytic activity after incubation with 0.01 M CMC, as measured by the BTpNA assay. The times shown indicate the time of incubation of α -chymotrypsin with CMC (pH 7.0, 0.10 mg/ml α -chymotrypsin) before the sample was diluted into the BtpNA assay mixture.

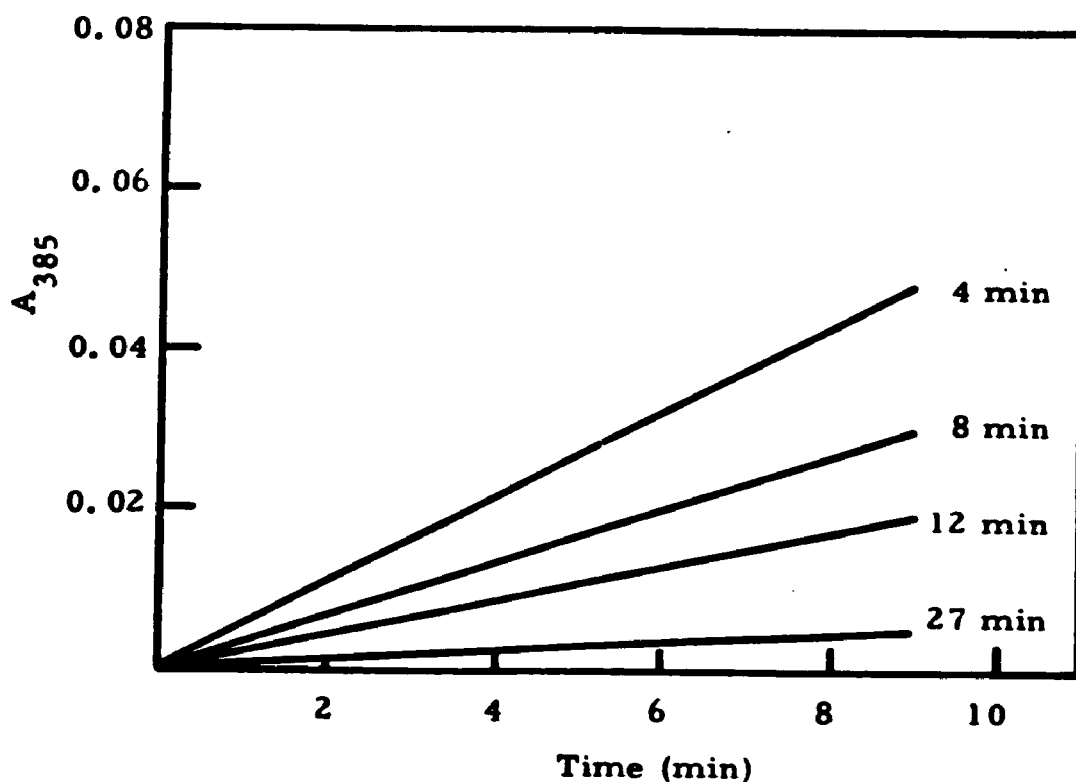


Figure 6. Recorder traces of α -chymotrypsin's catalytic activity after incubation with 0.01 M CMC, as measured by the BTpNA assay. The times shown indicate the time of incubation of α -chymotrypsin with CMC (pH 8.0, 0.10 mg/ml α -chymotrypsin) before the sample was diluted into the BTpNA assay mixture.

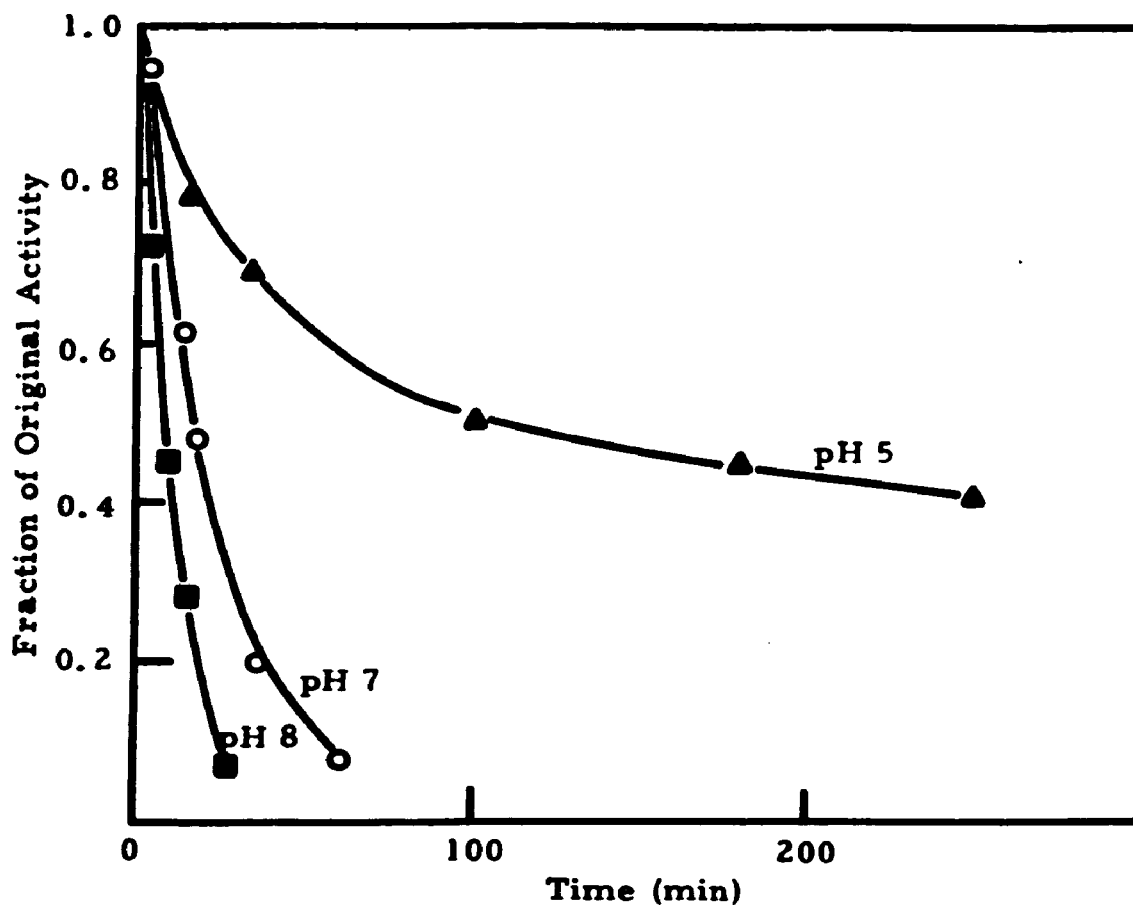
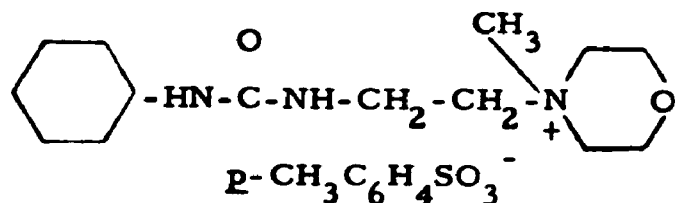


Figure 7. The dependence of catalytic activity on time of incubation with CMC. Conditions for pH 5.0 are 0.01 M CMC, 0.6 mg/ml at 25°, 0.1 M KCl. Conditions for pH 7.0 are 0.01 mg/ml α -chymotrypsin; 0.01 M CMC, 25°. Conditions for pH 8.0 are 0.01 M CMC, 0.10 mg/ml α -chymotrypsin, 25°.

3. Stability of α -Chymotrypsin Under the Conditions of Its Reaction with CMC. The stability of native α -chymotrypsin under the conditions of inactivation was checked in order to verify that reaction with CMC and not autolysis of the enzyme was responsible for the observed inactivation. At pH 8.0 and 0.1 M KCl α -chymotrypsin (5 mg/ml) was found to lose only 5% of its activity in 165 min. The effect of 1-cyclohexyl-3-(2-morpholinyl-(4)-ethyl) ureylene metho-*p*-toluene-sulfonate (CMU), the expected reaction product from hydrolysis of



CMU

CMC, on native α -chymotrypsin was checked. Incubation of α -chymotrypsin for 2 hr at pH 7 in 0.01 M CMC resulted in only a 9% loss in α -chymotrypsin's catalytic activity. This result clearly indicates that CMU is not responsible for the rapid loss in α -chymotryptic activity on incubating the enzyme with CMC.

4. Kinetics of Inactivation of α -Chymotrypsin by CMC. Since the concentration of active enzyme, $[e]$, is proportional to the catalytic activity, v , (see Fig. 4)

$$v = k'[e] \quad (17)$$

If the rate of inactivation is first order with respect to active enzyme,

$$\frac{-d[e]}{dt} = k_{\text{obs}}[e] . \quad (18)$$

Integration of equation 18 yields equation 19.

$$- \ln[e] = k_{\text{obs}} t - \ln[e^0] \quad (19)$$

Substituting equation 17 into equation 19 yields equation 20.

$$- \ln \frac{v}{v_0} = k_{\text{obs}} t , \quad (20)$$

where k_{obs} is the pseudo-first order rate constant for the loss of active enzyme. This rate constant can be obtained from the slope of $\ln \frac{v}{v_0}$ vs. time. Figure 8 illustrates that inactivation of α -chymotrypsin at pH 7 and pH 8 is indeed first order with respect to the active chymotrypsin concentration as indicated by the linear dependence of $\ln v$ on time. However, a marked deviation from the pseudo-first order rate law is seen at pH 5.

Table I shows that the apparent pseudo-first order rate constant is essentially independent of the initial concentration of α -chymotrypsin, as predicted by equations 19 and 20.

If the reaction of chymotrypsin with CMC was a simple second order process the rate of loss of active enzyme should be given by

$$\frac{d[e]}{dt} = k[\text{CMC}][e] . \quad (21)$$

When $[\text{CMC}] \gg [e]$, $[\text{CMC}]$ can be assumed constant during the reaction, so that the product $k[\text{CMC}]$ can be set equal to k_{obs} . The value of k_{obs} as determined by analyzing the time course of inactivation in terms of equation 21 should therefore be proportional to the

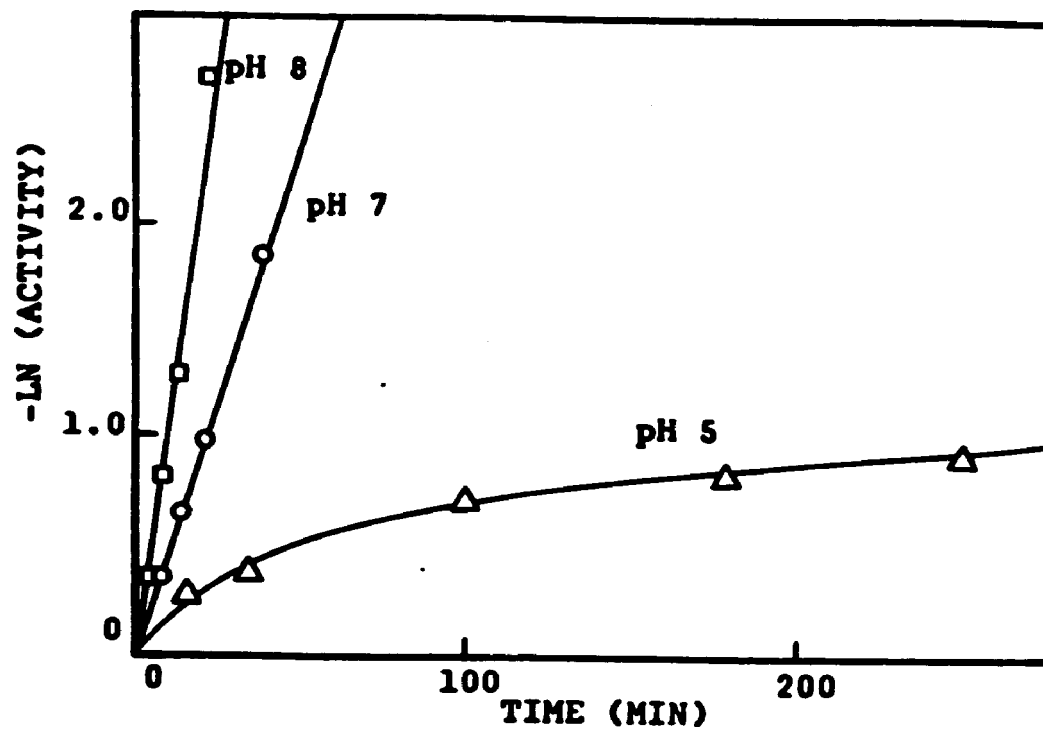


Figure 8. Effect of pH on the inactivation of α -chymotrypsin (0.1 mg/ml) by 0.01 M CMC at 25° in 0.1 M KCl. Activity is defined as v/v^0 .

Table I
Inactivation of α -Chymotrypsin with CMC^a

[CMC] (M)	[α -Chymotrypsin] (mg/ml) ^b	$10^2 k_{obs}^c$ (min ⁻¹)
0.01	9.6	3.9
0.01	0.96	4.3
0.01	0.41	4.9
0.01	0.2	4.5
0.01	0.1	4.6

^a At 25°, 0.1 M KCl, pH 7.0.

^b From absorbance at 280 m μ and an optical factor of 0.465 mg ml⁻¹ (absorbance units)⁻¹.

^c From the slope of the plot of $-\ln$ (activity) against time (Fig. 8).

concentration of CMC. A plot (Fig. 9) of the data in Table II plainly indicates that the inactivation of α -chymotrypsin is not first order with respect to the concentration of CMC, and the inactivation of α -chymotrypsin by CMC is not a simple second order process.

5. Effect of Indole on the Rate of Inactivation of α -Chymotrypsin

by CMC. Indole, a competitive inhibitor in α -chymotrypsin-catalyzed reactions, occupies part of the substrate binding site on the enzyme. At pH 7, 10 mM CMC, 0.1 mg/ml α -chymotrypsin, 2.6 mM indole causes the pseudo-first order rate constant for inactivation (k_{obs}) to decrease from 0.046 min^{-1} to 0.016 min^{-1} .

6. All-Or-None Assays on CMC-Chymotrypsin. To verify that CMC reacts with α -chymotrypsin to produce a derivative completely devoid of chymotryptic activity, all-or-none assays using N-trans-cinnamoyl imidazole (CI)^{63,19} were done.

In a rate assay, the rate of conversion of a substrate to product, e. g. . BTPNA to p-nitroaniline and benzoyl-L-tyrosine is measured, in order to obtain the average catalytic efficiency of the enzyme. However, a rate assay does not allow one to distinguish between a solution in which all the enzyme molecules are partially active and a solution in which some of the enzyme molecules are fully active and the rest of the enzyme molecules completely inactive.

An all-or-none assay, under ideal circumstances, measures the concentration of enzyme molecules that have any activity at all and by difference the concentration of enzyme molecules with no

Table II

Dependence of the Pseudo-First Order Rate Constant (k_{obs}) on
the Concentration of CMC^a

[CMC] (mM)	$10^2 k_{\text{obs}}$ (min ⁻¹)
1	0.72
2	1.32
5	2.7
7	3.8
15	5.6
20	5.9
30	7.1

^a At 25°, 0.1 M KCl, pH 7, 0.10 mg/ml α -chymotrypsin.

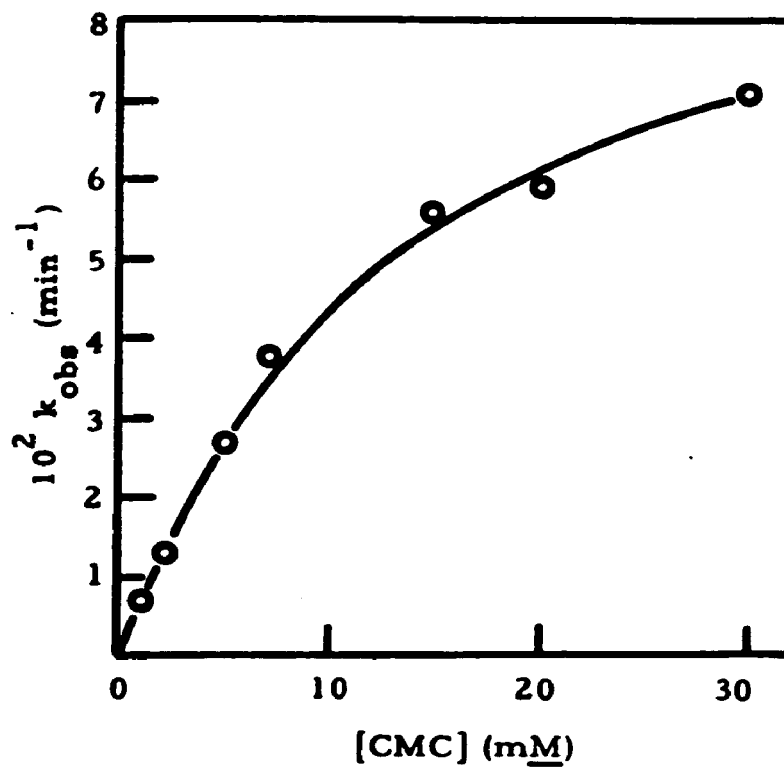
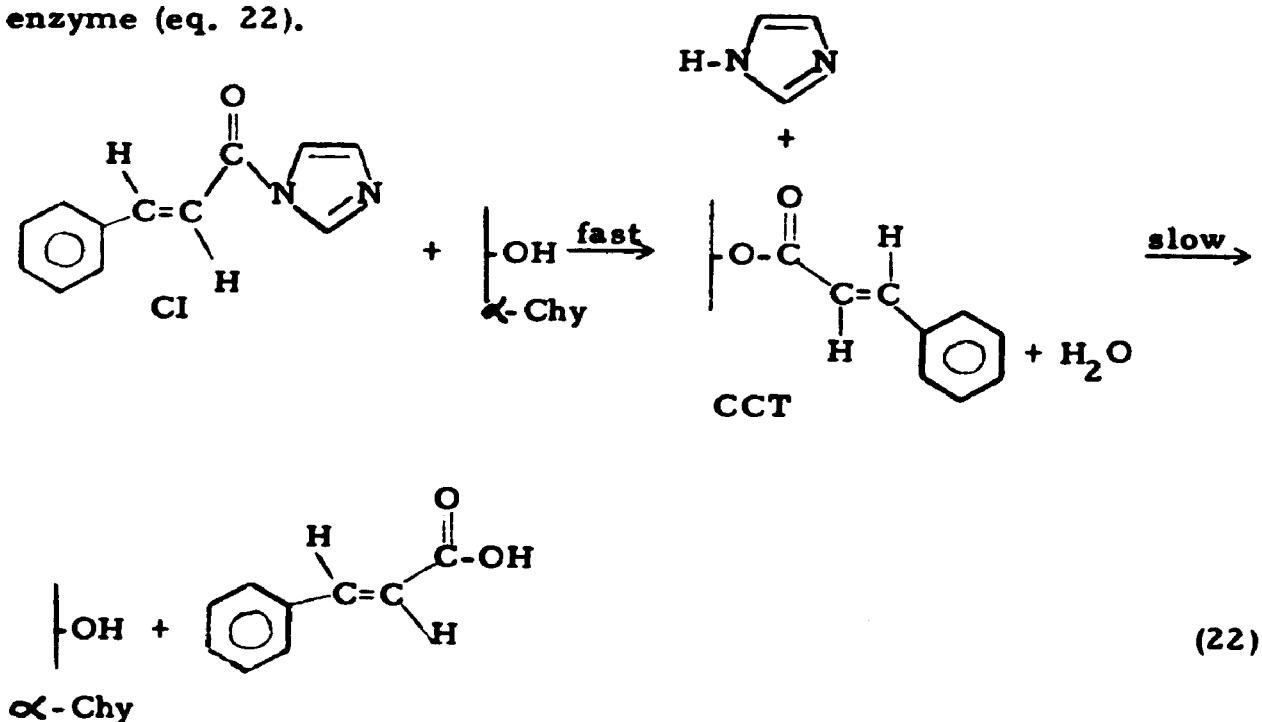


Figure 9. Dependence of rate of inactivation of α -chymotrypsin on the concentration of carbodiimide.

activity whatsoever. It does not depend on time as does the rate assay, so that enzyme molecules with reduced efficiency react in the same manner as those that are fully active.

This type of assay is convenient in modification studies because it allows one to determine whether the modification results in completely inactive enzyme molecules or whether there is in fact some low residual activity.

The all-or-none assay used in this work uses CI as substrate and is based on the fact that CI acylates α -chymotrypsin very much faster than the cinnamoyl group is hydrolyzed from the cinnamoylated enzyme (eq. 22).



The spectral change accompanying the formation of cinnamoyl- α -chymotrypsin (CCT) allows one to determine the amount of CCT formed, and thus one can measure the amount of enzyme capable of reacting

with Cl. Koshland et al.¹⁹ determined that this assay will detect more than 90% of enzyme which has a catalytic efficiency of 1%. Thus two enzyme solutions at the same concentration, one containing native enzyme and the other containing enzyme having a catalytic efficiency only 1% of that of the native enzyme will yield the same result for the concentration of active sites in the all-or-none assay. Other more sensitive all-or-none assays have been recently employed by Henderson²⁶ to measure the amount of enzyme having catalytic efficiencies of 1/10,000 that of the native enzyme.

For an all-or-none assay on CMC-chymotrypsin, a sample of α -chymotrypsin was inactivated by CMC (0.1 mg/ml α -chymotrypsin, 0.01 M CMC, pH 7.0) to 13% of its initial activity toward BTpNA. After it was separated from the CMC, the sample which had lost 87% of its activity toward BTpNA was found to contain $85 \pm 5\%$ inactive enzyme molecules by the all-or-none assay with N-trans-cinnamoyl imidazole. If the reaction with CMC were producing partially active enzyme, the all-or-none assay would have indicated that none of the enzyme molecules were completely inactive.

A result showing partially active enzyme was obtained for α -chymotrypsin inactivated at pH 5.0. α -Chymotrypsin inactivated at pH 5.0 had a catalytic efficiency of approximately 40%. However, the all-or-none assay indicated that none of the enzyme molecules was completely inactive. That is, this sample of CMC-treated chymotrypsin gave the same all-or-none assay expected for fully

active α -chymotrypsin. These results indicate that CMC treatment at pH 5 produces a derivative having a catalytic efficiency roughly 40% that of native α -chymotrypsin.

However, an enzyme with a catalytic efficiency of 2% which was produced by incubating α -chymotrypsin with CMC at pH 8 gave an all-or-none assay which was only 1-2% that of native α -chymotrypsin. Thus, it is clear that at pH 7.0 and pH 8.0 CMC reacts with α -chymotrypsin to produce a completely inactive derivative.

The close correspondence between the fraction of catalytic activity lost (p) and the fraction of totally inactive enzyme present (f) during the inactivation of α -chymotrypsin with ^{14}C -CMC is illustrated in Table III.

7. Reaction of α -Chymotrypsin with ^{14}C -CMC. Table III also shows a one to one relationship between the loss of chymotryptic activity and the uptake of covalently bonded ^{14}C -CMC. This data suggests that the inactivation of α -chymotrypsin is caused by the formation of a single covalent bond between enzyme and CMC.

If the enzyme is reacted with ^{14}C -CMC for many half-lives, more than one mole of ^{14}C -CMC per mole of inactivated enzyme is incorporated. For example, the treatment of 5 mg/ml α -chymotrypsin with 0.010 M ^{14}C -CMC at pH 7.0 for about 8 half-lives yielded an enzyme sample that was 4% active (toward BTpNA) and contained 1.65 moles of bound ^{14}C -CMC per mole of inactivated enzyme. At pH 8.0 the reaction between 5 mg/ml α -chymotrypsin and 0.002 M ^{14}C -CMC

Table III

Inactivation of α -Chymotrypsin with 2 mM CMC^a

Time (min)	Fractional loss in catalytic efficiency p^b	Fraction totally inactivated enzyme f^c	$\frac{\text{Mole } ^{14}\text{C-CMC bound}^d}{\text{Mole protein}}$ i
20	0.48	0.47	0.47
35	0.60	0.62	0.61
60	0.78	0.75	0.80

^a At 25°, 0.1 M KCl, pH 8, 4.4 mg/ml α -chymotrypsin. After the states times, protein was separated from the reaction mixture by gel filtration, and the values of p , f , and i determined.

^b $p = 1 - \frac{\text{specific activity toward BTpNA}}{\text{initial specific activity toward BtpNA}}$

^c $f = 1 - \frac{\text{concentration of active sites/mg protein}}{\text{initial concentration of active sites/mg protein}}$

^d Determined from the specific radioactivity of the protein, using an optical factor $0.465 \text{ mg ml}^{-1} (\text{absorbance unit})^{-1}$ at 280 m μ and a molecular weight of 24,800 for α -chymotrypsin.

for about 8 half-lives yielded enzyme that was 4% active toward BTpNA and contained 1.22 moles of bound ^{14}C -CMC per mole of inactivated enzyme.

B. REACTION OF CMC WITH RELATED PROTEINS

When ^{14}C -CMC was allowed to react with chymotrypsinogen-A (CTgen) (0.002 M ^{14}C -CMC, 4.8 mg/ml CTgen, pH 8.0) for 4 hours by the procedure used to prepare ^{14}C -CMC chymotrypsin, only 0.13 mole of ^{14}C -CMC per mole of CTgen became covalently bonded to protein. Similarly ^{14}C -CMC was reacted with DIP-chymotrypsin (0.002 M ^{14}C -CMC, 5.4 mg/ml DIP-chymotrypsin, pH 8.0) and only 0.38 mole of ^{14}C -CMC per mole of DIP-chymotrypsin became covalently bound to protein. It was also determined that CMC does not affect the activity of trypsin toward p-toluene-L-arginine methyl ester under conditions that result in the inactivation of α -chymotrypsin. Interestingly, CMC effectively eliminates the apparent catalytic activity of trypsin toward BTpNA. The specific catalytic activity of untreated trypsin toward BTpNA is $\sim 0.3\%$ that of native α -chymotrypsin. When α -chymotrypsin was added to a solution of trypsin, which had been incubated with CMC until the catalytic activity toward BTpNA was eliminated, the pseudo-first order rate constant for the loss of chymotryptic activity toward BTpNA was identical with the pseudo-first order rate constant for the loss of activity toward BTpNA in the original solution of trypsin. These results are consistent with the presence of α -chymotrypsin in the sample of trypsin.

C. PROPERTIES OF CMC-CHYMOTRYPSIN

1. Amino Acid Composition After Acid Hydrolysis. Table IV illustrates that reaction with CMC produces no significant differences in the composition of amino acids of chymotrypsin which are stable to the conditions of acid hydrolysis.

2. Number of Primary Amino Groups in Dinitrophenylated CMC-Chymotrypsin. The absorbance at 350 mμ of a known amount of dinitrophenylated chymotrypsin dissolved in glacial acetic acid was related to the number of dinitrophenylated primary amino groups (N_a), using the relationship of Levy and Lang⁶⁹.

$$N_a = \frac{A_{350} - 0.15 \times 6 \times N_p \times \epsilon_{350}}{N_p \times \epsilon_{350}}$$

where N_p is the molar concentration of protein in the sample and

ϵ_{350} is the molar absorptivity of a dinitrophenylated amino residue ($14,500 \text{ cm}^{-1} \text{ M}^{-1}$)⁶⁹. The term $0.15 \times 6 \times N_p \times \epsilon_{350}$ represents the contribution of the 4 tyrosyl and 2 histidyl residues to the A_{350} , assuming that these groups are dinitrophenylated.

As judged from the A_{350} , 16.4 amino groups were dinitrophenylated in CMC-chymotrypsin and 15.7 amino groups were dinitrophenylated in untreated α -chymotrypsin.

3. Number of Lysyl- ϵ -amino Groups. The number of lysyl residues unavailable for reaction with 2,4-dinitrofluorobenzene (as determined by the yield of free lysine after the acid hydrolysis of dinitrophenylated protein) was compared for native and CMC-chymotrypsin.

Table IV
Amino Acid Analysis of CMC-Chymotrypsin

	Found ^a (residues per molecule)	Expected ^b
Lys	16.0	14
His	2.2	2
Arg	(3.0)	3
Asp + Asn	21.0	22
Thr	22.3	22
Ser	23.4	27
Glu + Gln	16.3	15
Gly	23.2	23
Ala	22.6	22
1/2 Cys	8.5	10
Val	19.2	23
Met	(2.0)	2
Ile	8.9	10
Leu	18.9	19
Tyr	4.2	4
Phe	6.5	6

^a The basic amino acids were normalized to 3.0 Arg and the rest of the amino acids were normalized to 2.0 Met.

^b From the primary sequence reference³³ and the corrections appearing in references 27, 73, and 74.

Native α -chymotrypsin was dinitrophenylated and hydrolyzed.

CMC-chymotrypsin, which retained less than 4% of its initial activity toward BTpNA, was dinitrophenylated and hydrolyzed. Native

α -chymotrypsin showed 2.48 ± 1.0 moles of lysine per mole of protein. CMC-chymotrypsin showed 1.26 ± 0.5 moles of lysine per mole of protein. If the ϵ -amino groups of lysine were modified on reaction with CMC to form an acid labile derivative, they would be unavailable for reaction with DNFB and these amino groups so modified would be recovered as free lysine on acid hydrolysis. However, no extra lysine was observed on hydrolysis of dinitrophenylated CMC-chymotrypsin.

4. Presence of Amino-Terminal Isoleucyl and Alanil Residues. The presence of amino-terminal isoleucyl and alanyl residues was determined by thin layer chromatography of acid hydrolyzed dinitrophenylated CMC-chymotrypsin and untreated dinitrophenylated α -chymotrypsin. Equal recoveries of both DNP-Ile and DNP-Ala were obtained from dinitrophenylated CMC-chymotrypsin and α -chymotrypsin. No attempt was made to compare the content of N-terminal cystine in CMC-chymotrypsin and untreated α -chymotrypsin.

5. Maximum Molecular Weight. The elution volumes of CMC-chymotrypsin and untreated native chymotrypsin were compared on a column of Sephadex G-75 which gave different elution volumes for α -chymotrypsin (mol. wt. 25,000) and ovalbumin (mol. wt. 45,000). Identical elution volumes were observed for CMC-chymotrypsin and untreated

α -chymotrypsin indicating that reaction with CMC did not cause the protein to dimerize.

D. REACTIVATION WITH HYDROXYLAMINE

It was found that treatment of the inactive CMC-chymotrypsin with hydroxylamine resulted in reactivation of the chymotryptic activity. A sample of CMC-chymotrypsin which retained only 4% of its initial activity was treated with 0.95 M NH_2OH , pH 8.0, and found to have recovered to 88% of its initial activity after 36 hr. A control sample of the same CMC-chymotrypsin was treated with 0.038 N Tris buffer at pH 8.0 and found to reach only 17% of its initial activity over the same time period. As an additional control, native α -chymotrypsin was treated with 0.95 M NH_2OH at pH 8 and found to lose only 8% of its initial activity over a similar time period. For treatments with hydroxylamine, the concentration of α -chymotrypsin was kept low (0.01 mg/ml) to prevent autolysis.

Treatment of ^{14}C -CMC-chymotrypsin with 1.9 M hydroxylamine (11 $\mu\text{g}/\text{ml}$ protein) resulted in the regeneration of chymotryptic activity equivalent to 0.80 mole of fully active enzyme and the liberation of radiochemical label equivalent to 1.0 mole of CMC (Table V). If reactivation of the enzyme is due to reaction at a single residue, the rate of reactivation should be first order with respect to the concentration of CMC-chymotrypsin remaining. Since controls with native α -chymotrypsin showed that the enzyme does not lose activity

Table V

Treatment of ^{14}C -CMC-Chymotrypsin with 1.9 M NH_2OH ^a

Time with NH_2OH (hr)	Specific catalytic activity ^b	Mole ^{14}C -CMC bound ^c Mole protein
0	0.10	1.098
18	0.90 (0.30 ^{d, e})	0.108 ^d

^a At pH 8, 25°.

^b The specific activity of native α -chymotrypsin was defined as 1.00.

^c Determined using an optical factor of 0.465 mg ml^{-1} (absorbance unit)⁻¹ and a molecular weight of 24,800 for α -chymotrypsin.

^d Determined after gel filtration of the reaction mixture followed by lyophilization of the fractions containing protein.

^e This decrease in specific activity (0.90 to 0.30) was caused by lyophilization. Lyophilization of dilute solutions of α -chymotrypsin was shown to cause similar losses in specific activity.

in hydroxylamine, the fraction of CMC-chymotrypsin remaining (f_{CMC}) is given by equation 24.

$$f_{\text{CMC}} = \frac{v_f - v_t}{v_f} , \quad (24)$$

where v_t and v_f represent the catalytic activities in the hydroxylamine solution at time t and after several (8) half-lives, respectively. If reactivation of CMC-chymotrypsin is first order with respect to the concentration of CMC-chymotrypsin, the time dependence of f_{CMC} should be given by equation 25.

$$\ln(f_{\text{CMC}}) = k_r t + \ln f_{\text{CMC}}^0 , \quad (25)$$

where k_r represents the first order rate constant for reactivation and $\ln f_{\text{CMC}}^0$ represents the fraction of the chymotrypsin which is CMC-chymotrypsin at $t = 0$. Figure 10 shows that the time dependence of f_{CMC} is given by equation 25 (with $k_r = 3.4 \times 10^{-3} \text{ min}^{-1}$). This result indicates that reactivation of CMC-chymotrypsin is first order with respect to the concentration of CMC-chymotrypsin.

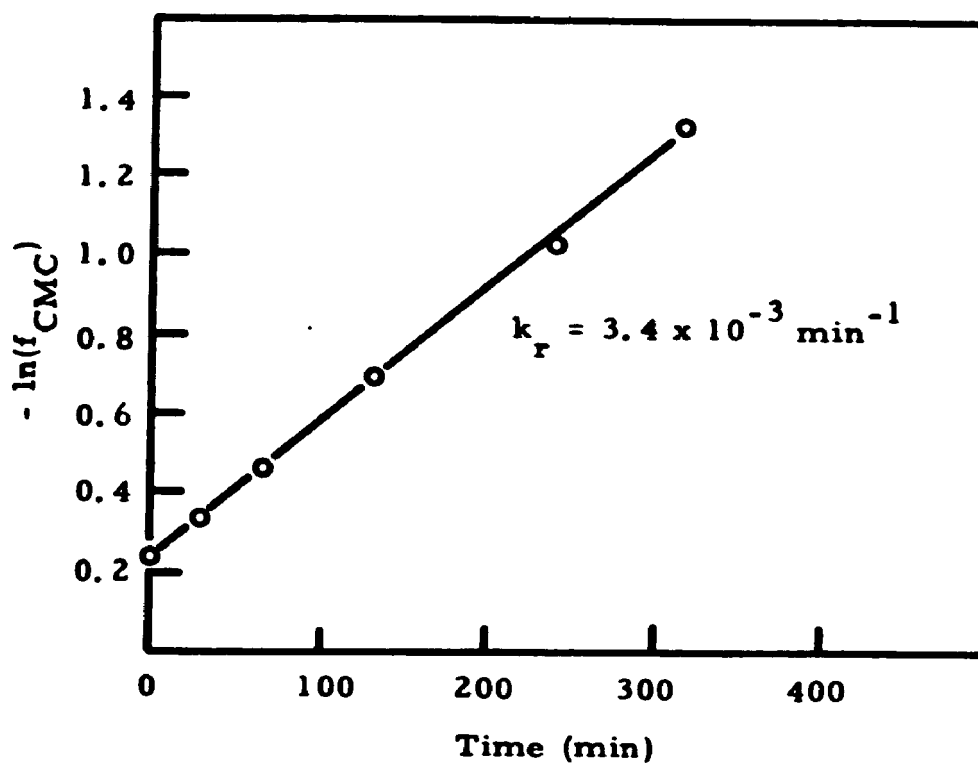


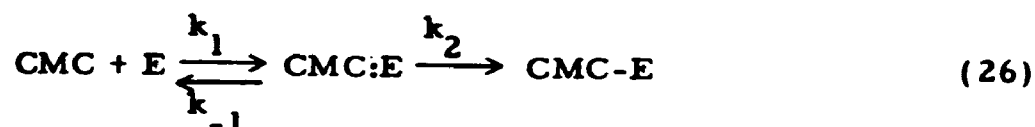
Figure 10. Time dependence of fraction of CMC-chymotrypsin remaining, f_{CMC} , during incubation of $20 \mu\text{g/ml}$ ^{14}C -CMC-chymotrypsin in 1.82 M hydroxylamine, pH 8.0, 0.005 M CaCl_2 .

IV. DISCUSSION OF THE RESULTS

A. INACTIVATION REACTION

1. Dependence of the Rate of Inactivation on the Concentration of CMC

The saturation effect depicted in Figure 9 for the dependence of the reaction of α -chymotrypsin with CMC on the concentration of CMC suggests formation of a complex between CMC and α -chymotrypsin prior to the reaction (eq. 26).



Assuming that $k_{-1} \gg k_2$, the dependence of the concentration of CMC:E on $[e]$, the total concentration of enzyme not yet covalently linked to CMC, is obtained from the following relationships when $[\text{CMC}] \gg [e]$:

$$[e] = [\text{CMC:E}] + [E], \quad (27)$$

$$[e] = [\text{CMC:E}] \left(1 + \frac{K_D}{[\text{CMC}]} \right), \quad (28)$$

$$[\text{CMC:E}] = \frac{[e]}{1 + \frac{K_D}{[\text{CMC}]}} \quad (29)$$

where $K_D = \frac{k_{-1}}{k_1} = \frac{[\text{CMC}][E]}{[\text{CMC:E}]}$. (30)

On diluting the reaction mixture into an assay solution the complex, CMC:E, dissociates and one obtains a measure of $[e]$ from eq. 17.

$$v = k'[e] \quad (17)$$

Since

$$-\frac{d[e]}{dt} = k_2[\text{CMC:E}] , \quad (32)$$

$$-\frac{d[e]}{dt} = k_2 \frac{[e]}{1 + \frac{K_D}{[\text{CMC}]}} . \quad (33)$$

Integrating equation 33 yields

$$-\ln[e] = \frac{k_2 t}{1 + \frac{K_D}{[\text{CMC}]}} - \ln[e^0] . \quad (34)$$

Substituting equation 17 into equation 34 yields equation 35,

$$-\ln\left(\frac{v}{v^0}\right) = \frac{k_2}{1 + \frac{K_D}{[\text{CMC}]}} t = k_{\text{obs}} t . \quad (35)$$

Since $k_2/1+(K_D/[\text{CMC}])$ is essentially constant during any one inactivation reaction, we can set $k_2/1 + (K_D/[\text{CMC}])$ equal to k_{obs} . Values of k_{obs} were obtained from the slopes of the linear plots of $-\ln(v/v^0)$ versus time. The relationship between $[\text{CMC}]$ and k_{obs} leads to the linear double reciprocal relationship of equation 36,

$$\frac{1}{k_{\text{obs}}} = \frac{K_D}{k_2} \cdot \frac{1}{[\text{CMC}]} + \frac{1}{k_2} \quad (36)$$

Figure 11 shows the fit of data in Table II to equation 36.

Values of 0.10 min^{-1} for k_2 and 0.013 M for $K_D(k_{-1}/k_1)$ were calculated from the intercept and slope of the plot in Figure 11.

2. Effect of Indole on the Rate of Inactivation. The decrease in the rate constant for inactivation when indole (I), a competitive inhibitor in α -chymotrypsin-catalyzed reactions, is present suggests that

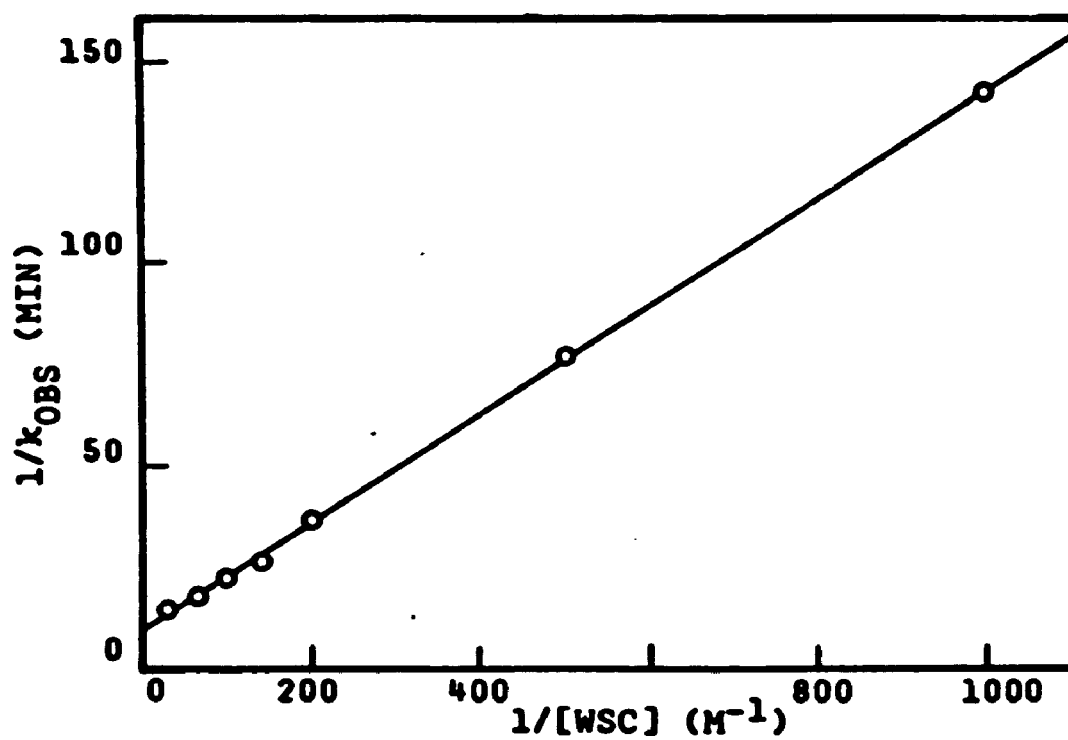


Figure 11. Determination of the dissociation constant (K_D) for the CMC-chymotrypsin adsorption complex.

CMC also interacts with the substrate binding site of α -chymotrypsin. If CMC cannot complex with enzyme which is complexed with indole, the concentration of CMC:E is obtained from the following relationships;

$$[e] = [CMC:E] + [E] + [EI], \quad (37)$$

$$[e] = [CMC:E] \left(1 + \frac{K_D}{[CMC]} + \frac{K_D}{[CMC]} \cdot \frac{[I]}{K_I} \right), \quad (38)$$

$$[CMC:E] = \frac{[e]}{1 + \frac{K_D}{[CMC]} + \frac{K_D}{[CMC]} \cdot \frac{[I]}{K_I}}, \quad (39)$$

where $[EI]$ represents the concentration of the enzyme-indole complex and K_I , the dissociation constant for this complex, is $[E][I]/[EI]$. If CMC can not react with EI to produce inactive enzyme,

then
$$-\frac{d[e]}{dt} = k_2[CMC:E]. \quad (40)$$

Substituting equation 39 into equation 40 yields equation 41;

$$-\frac{d[e]}{dt} = \frac{k_2[e]}{1 + \frac{K_D}{[CMC]} \left(1 + \frac{[I]}{K_I} \right)}. \quad (41)$$

Integrating equation 41 yields equation 42;

$$-\ln[e] = \frac{k_2 t}{1 + \frac{K_D}{[CMC]} \left(1 + \frac{[I]}{K_I} \right)} - \ln[e^0]. \quad (42)$$

On dilution into the assay mixture both the CMC:E and the EI complexes undergo essentially complete dissociation, so that a measure of $[e]$ is obtained from the BTpNA assay and equation 17 may

be substituted into equation 42;

$$-\ln \frac{v}{v_o} = \frac{k_2 t}{1 + \frac{K_D}{[CMC]} \left(1 + \frac{[I]}{K_I}\right)} = k_{obs}^I t \quad (43)$$

The definitions of k_{obs} and k_{obs}^I set forth in equation 35 and equation 43 leads to equation 44 for the ratio k_{obs}^I/k_{obs} ;

$$\frac{k_{obs}^I}{k_{obs}} = \frac{1}{1 + \frac{K_D}{[CMC] + K_D} \cdot \frac{[I]}{K_I}} \quad (44)$$

Using the value of $8 \times 10^{-4} \text{ M}$ for the inhibitor constant for indole (K_I) reported by Wallace, Kurtz, and Niemann⁷⁵ along with a value of 0.013 M for K_D , equation 44 predicts a value of 0.35 for k_{obs}^I/k_{obs} when $[I] = 2.6 \times 10^{-3} \text{ M}$ and $[CMC] = 0.01 \text{ M}$. The observed value of k_{obs}^I/k_{obs} under these conditions is also 0.35 ($0.016 \text{ min}^{-1}/0.046 \text{ min}^{-1}$). The close correspondence between the expected and observed values of k_{obs}^I/k_{obs} is strong evidence that CMC first forms a non-covalent complex with α -chymotrypsin before inactivating the enzyme and that the non-covalent complex (CMC:E) cannot form when indole is bound to the enzyme. Because indole is a small molecule relative to the enzyme, the indole protection against inactivation by CMC suggests that inactivation is caused by the reaction of one or two residues at or near the active site of the enzyme. The failure of CMC to inactivate trypsin or to react to a significant extent with chymotrypsinogen-A and diisopropylphosphoryl

chymotrypsin is another indication that CMC reacts at the active site of α -chymotrypsin.

3. Stoichiometry of Inactivation. The results of the all-or-none assays show two things. First, the assays show that the inactivation produces completely inactive enzyme. Since 2-4% of the original chymotryptic activity remains even after long reaction times with CMC, it is possible that the reaction, when completed, produces chymotrypsin which has greatly reduced efficiency but is still catalytically active. However, if this were so the cinnamoyl imidazole assay would show that all the chymotrypsin present was active. Secondly, the results of the all-or-none assays together with those of the rate assays suggest that it is the reaction of a single residue with CMC which causes inactivation of α -chymotrypsin.

One might imagine that reactions at several singly unimportant residues collectively cause the enzyme to become inactivated. These groups might somehow have been shielded by an indole-induced conformational change of the enzyme. When the rate assay indicates that the reaction is at a certain degree of completion, p , the fraction of totally inactive enzyme present, f , depends on the number of groups, n , which must be modified to produce totally inactive enzyme. If all these groups are equivalent in reactivity and in the amount by which their modification decreases the catalytic efficiency of the enzyme, then $f = p^n$. Consequently, the enzyme solution which was inactivated to 13% ($p = 0.87$) of its initial activity should show 87%

totally inactive enzyme as measured by the all-or-none assay if $n = 1$, and 76% or 66% totally inactive enzyme if $n = 2$, or 3. The observed value of 0.85 for f when $p = 0.87$ and the close correspondence between f and p shown in Table III are most consistent with inactivation by CMC through reaction at a single residue.

It is clear from Table III that the incorporation of ^{14}C -CMC closely parallels the loss of enzymic activity. Thus, a single residue of the enzyme, at the active site, reacts to form a covalent bond between enzyme and CMC and this reaction causes the enzyme to become inactive. This conclusion is confirmed by the data in Table V which indicates that 0.80 mole of fully active enzyme is produced for each mole of radiochemical label lost during treatment of the inactivated enzyme with hydroxylamine.

B. REACTIONS EXCLUDED AS A CAUSE OF INACTIVATION

Since carbodiimides can undergo several reactions with the functional groups of proteins, some of these reactions were excluded as a cause of the inactivation of α -chymotrypsin by CMC.

1. Reactions with Carboxyl Groups. The reactions of carboxyl groups shown in Figure 2 are unlikely causes of inactivation of α -chymotrypsin by CMC. Hoare and Koshland⁴⁴ determined that the pH optimum for these reactions is between 4 and 5. The rate of inactivation by CMC increases when pH is increased from 7 to 8 (Fig. 8).

Amide bond formation to any large extent was shown not to occur. The results indicate little difference between the number of free amino groups in CMC-chymotrypsin and those in native α -chymotrypsin. For example, end group analysis using DNFB showed no difference between native and CMC-chymotrypsin in yield of DNP-alanine and DNP-isoleucine. Also, if many of the ϵ -amino groups of lysine had reacted to form amide bonds with carboxyl groups, one would expect that dinitrophenylated and then hydrolyzed CMC-chymotrypsin would have a higher yield of free lysine than native chymotrypsin, which was dinitrophenylated and then hydrolyzed. Within experimental error, the yield of free lysine from dinitrophenylated and hydrolyzed CMC-chymotrypsin and native ϵ -chymotrypsin was the same.

2. Reaction with Phenolic Hydroxyl Groups. If the phenolic hydroxyl of a tyrosine residue reacted one would expect CMC-chymotrypsin to have a lower yield of tyrosine after hydrolysis than CMC-chymotrypsin, since Carraway and Koshland⁵⁴ showed that the addition product of carbodiimide and tyrosine is stable to acid hydrolysis. The failure of CMC-chymotrypsin to exhibit a lowered yield of tyrosine (Table IV) eliminates this reaction as the cause of inactivation.

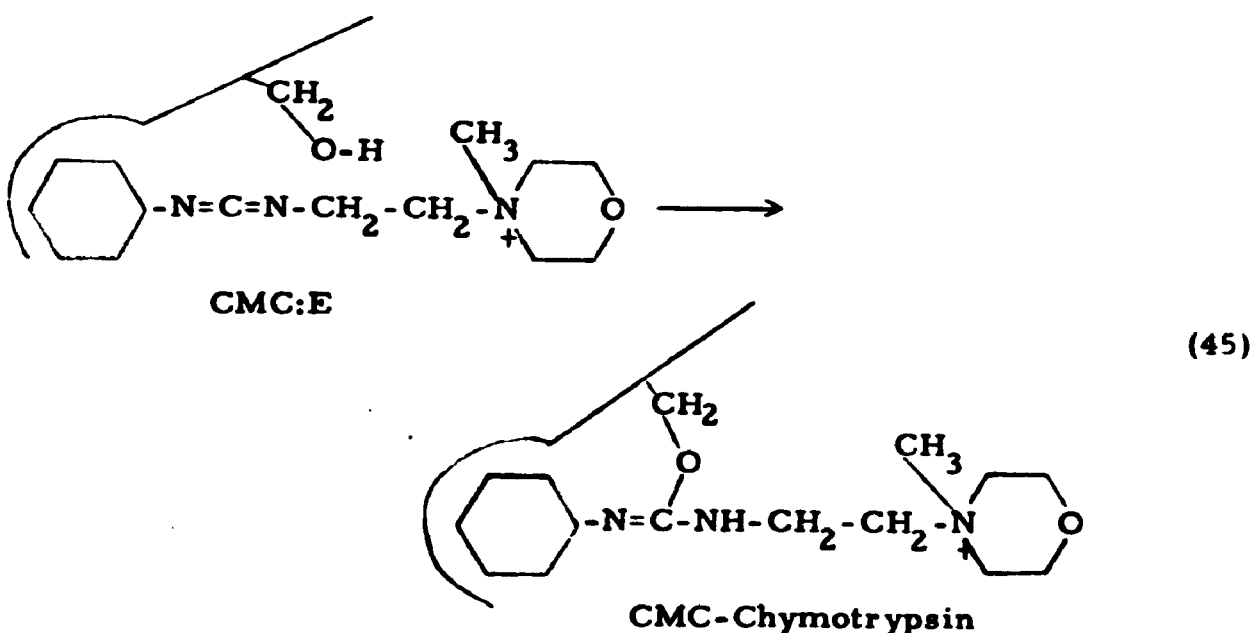
3. Addition of ϵ -Amino Groups to CMC. If the ϵ -amino groups of lysine reacted to form guanidino groups (Fig. 3), one would expect a difference between native and CMC-chymotrypsin in yields of lysine after acid hydrolysis, which was not observed (Table IV). It has

already been noted above that the α -amino groups of alanine and isoleucine appear to be unaffected by the inactivation reaction.

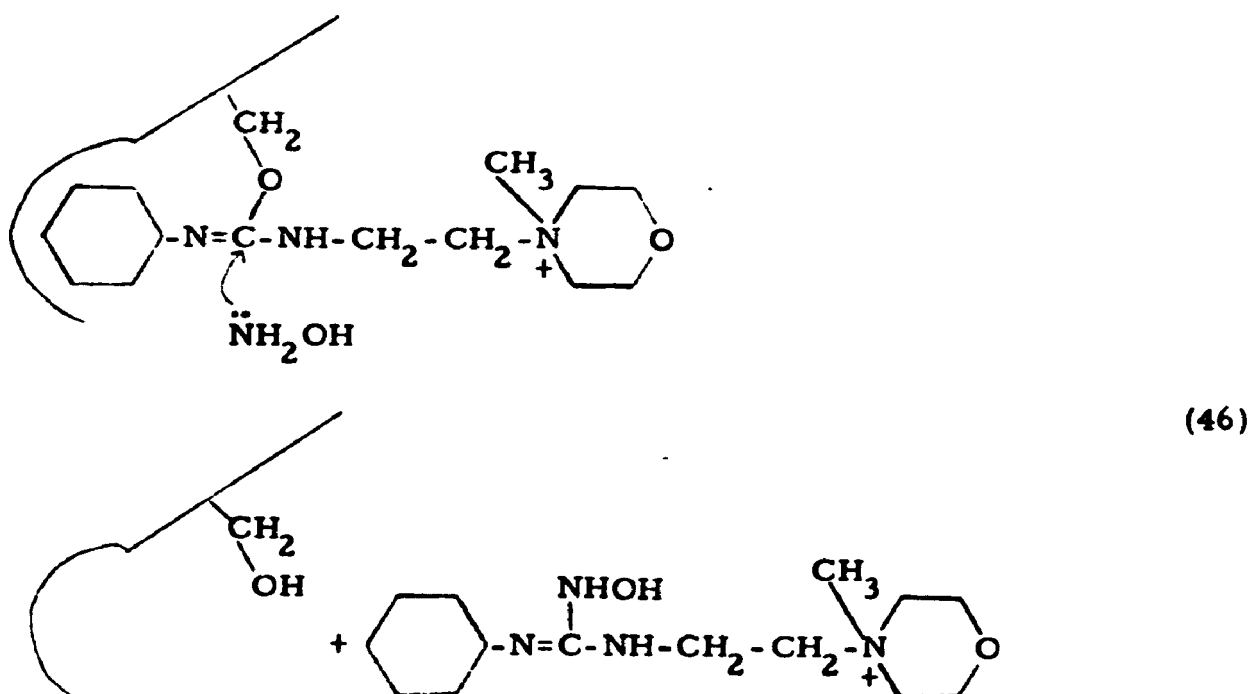
Since the inactivation reaction is first order in the concentration of chymotrypsin, it is clear that the inactivation observed is not caused by polymerization of chymotrypsin (e. g., via interchain amide bond formation), at least not as the rate-limiting step. Chromatography of the product, CMC-chymotrypsin, on Sephadex G-75, indicated that polymers were not formed in observable quantities.

C. ADDITION OF THE "ACTIVE SERINE" TO CMC AS THE CAUSE OF INACTIVATION

By eliminating the reactions described above, by knowing the role of the "active serine" in the activity of α -chymotrypsin, and by knowing that CMC reacts at the active site, it is reasonable to postulate that CMC inactivates α -chymotrypsin by forming an O-isoureyllene derivative with the "active serine" (eq. 45).

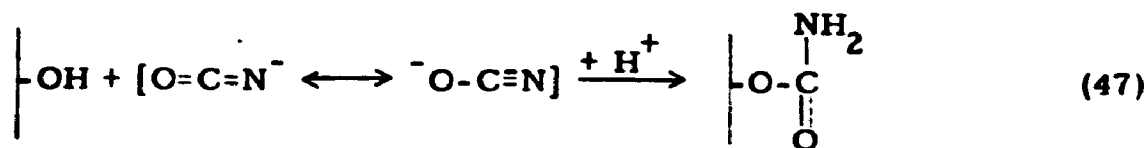


The hydroxylamine-mediated regeneration of the active enzyme and liberation of radiochemical label would be expected to proceed via equation 46;



This reaction is analogous to the well known displacement of methanol from O-methyl isourea by amino groups⁷⁶.

1. Analogies between the Proposed Inactivation Reaction and Other Reactions of α -Chymotrypsin. The proposed inactivation reaction (eq. 45) is formally analogous to the addition of seryl residue 195 to cyanate, reported by Shaw *et al.*⁷⁷ (eq. 47);

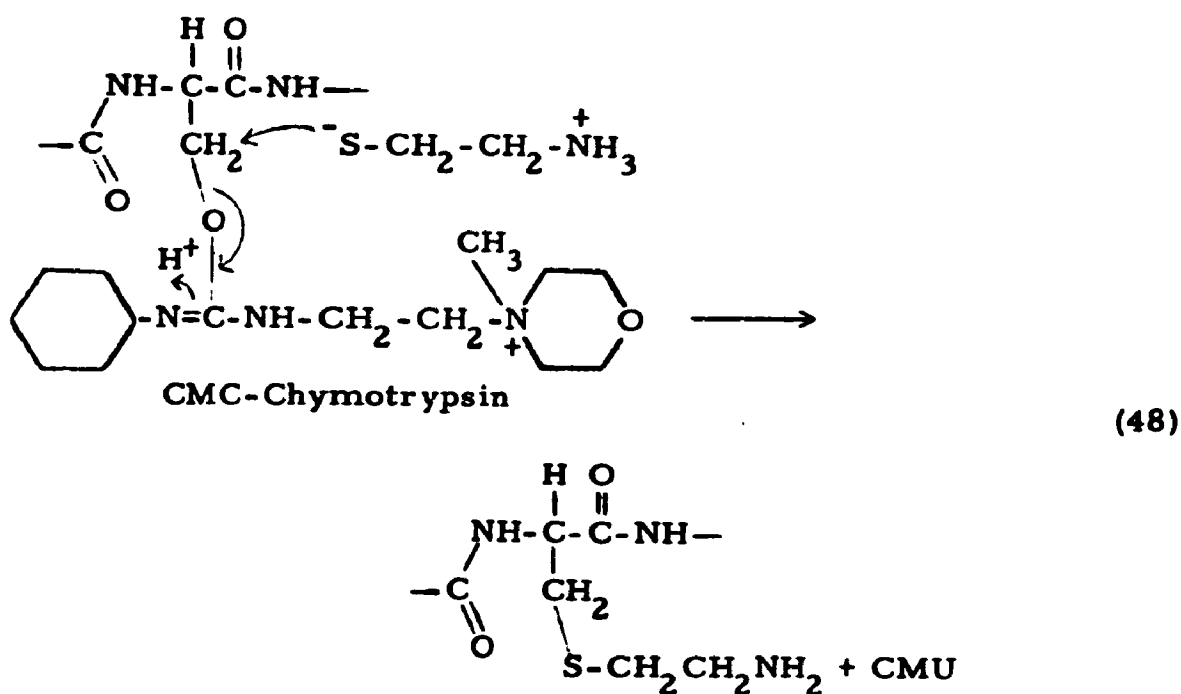


The proposed addition of the "active serine" to the double bond of CMC is also formally analogous to a step in the catalytic pathway in

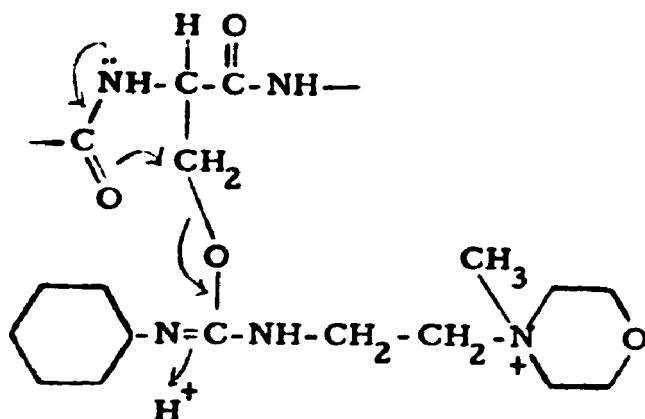
which the "active serine" adds to the C=O double bond of the substrate to form a tetrahedral intermediate which decomposes to acyl enzyme.

2. Further Work⁷⁸ showed that the reaction depicted in equation 45 is indeed the cause of the inactivation of α -chymotrypsin by CMC.

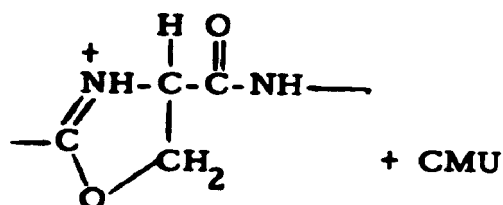
It was shown that: 1) 2-mercaptoethylamine displaces CMC to yield one S-aminoethylcysteinyl residue per inactivated enzyme molecule (eq. 48);



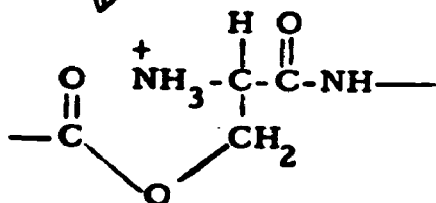
2) On standing in 5% acetic acid, ¹⁴C-CMC-chymotrypsin liberates radiochemical label, and the amino group of a seryl residue is exposed (eq. 49-50);



(49)



+ CMU

+ H₂O

(50)

- 3) ¹⁴C-CMC is bound to an active center peptide obtained by digesting ¹⁴C-CMC-chymotrypsin with pepsin.

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