CHEMICAL MODIFICATION OF CATALYTICALLY ESSENTIAL FUNCTIONAL GROUPS IN THE ACTIVE SITE OF PAPAIN

by

Randolph B. Perfetti

Dissertation submitted to the Graduate Faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Chemistry

APPROVED:

P. L. Hall, Chairman

J. R. Vercellotti

L. T. Taylor

D. G. I. Kingston

M. A. Ogliaruso

April, 1975

Blacksburg, Virginia

To my parents and my wife

ACKNOWLEDGEMENTS

The author would like to sincerely thank Dr. Philip L. Hall for his financial and moral support and guidance during the performance of this work and preparation of this manuscript. I would also like to thank the members of my committee, Dr. D.G.I. Kingston, Dr. M.A. Ogliaruso, Dr. L.T. Taylor and Dr. J.R. Vercellotti for their contributions to this work, and the Chemistry Department of Virginia Polytechnic Institute and State University. My thanks also, to Dr. B.M. Anderson and the Biochemistry and Nutrition Department for allowing me to use their scintillation counter, and to Mrs. B. Hall for running amino acid analyses.

Finally, very special thanks to Mrs. C.D. Anderson for typing the first draft of this dissertation and for her moral support and invaluable technical advice, without which the author would probably have taken much longer to complete this work.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

Abbreviation

EEDQ	- N-Ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline
NEPIS	- N-Ethyl-5-phenylisoxazolium-3'-sulfonate
NEPIT	- N-Ethyl-5-phenylisoxazolium Tetrafluoroborate
NEMIT	- N-Ethyl-5-methylisoxazolium Tetrafluoroborate
EDC	- 1-Ethyl-3-(3-dimethylaminopropyl) Carbodiimide
BzArgOEt	- α-N-Benzoyl-L-arginine Ethyl Ester
GlyOEt	- Glycine Ethyl Ester
$A^{1\%}_{278nm,\ 1cm}$	- Absorbance at 278 nm of a 10 mg/ml solution of protein in a 1 cm cell

INTRODUCTION

Papain (EC 3.4.4.10)[1] is the best characterized and most extensively studied member of a group of enzymes known as the sulfhydryl proteinases, so-called because the sulfhydryl group of an active-site cysteine residue is involved as a nucleophile in the peptide bond hydrolysis which these enzymes catalyze, forming a covalent enzyme-substrate intermediate. In addition to this essential sulfhydryl group of papain, a second group having a pK_a of 4.0(1) appears also to be necessary for catalysis. The nature of this group in the active site of papain has been postulated to be either a free carboxyl group (Asp-158), or an imidazole moiety of a histidine residue (His-159). Kinetic studies(2,3) have implicated either one of these groups as catalytically essential depending upon the evaluation of the data. However, the exact identity of the second catalytically essential group in the active site of the enzyme remains an open question.(4)

Chemical modification of carboxyl groups in papain has not been reported in the literature to date. Therefore, a survey of the reaction of five carboxyl group "specific" reagents with papain was undertaken and the results of this study will be discussed in relation to the identity of the catalytically essential group of pK_a 4 in the active site of the enzyme.

In addition, a new technique developed to reduce carboxyl groups to carbinols in aqueous media, under mild conditions, will be described.

---

[1] Systematic classification and nomenclature of the Commission on Enzymes of the International Union of Biochemistry.

HISTORICAL

Modification of Carboxyl Groups in Proteins and Enzymes - Modification of carboxyl groups of enzymes has, to date, been limited to formation of carboxylate derivatives such as esters or amides. Six types of reagents have been used to perform these modifications. They are methanolic-hydrochloric acid, diazo compounds, N-alkylisoxazolium salts, triethyloxonium tetrafluoroborate, N-ethoxycarbonyl-2-ethoxy-1, 2-dihydroquinoline (EEDQ),[2] and water-soluble carbodiimides.

Methanolic-hydrochloric Acid - The procedure for esterification of carboxyl groups with methanolic-HCl was first described in 1945 by Fraenkel-Conrat and Olcott[5] and has been used by most investigators to date. The general reaction pathway is shown in Equation 1.[6]

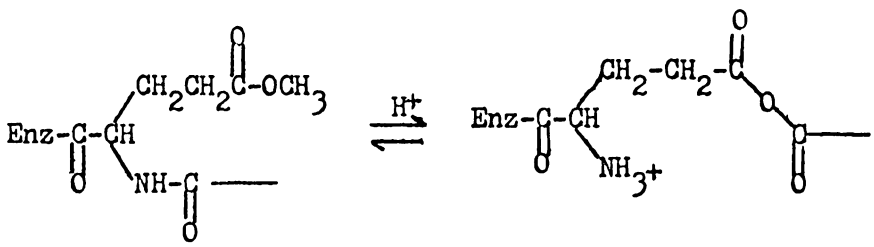
$$\text{Enz-}\overset{\overset{O}{\|}}{C}\text{OH} + CH_3OH \xrightarrow[HCl]{0.02\text{-}0.1M} \text{Enz-}\overset{\overset{O}{\|}}{C}\text{-O-}CH_3 + H_2O \qquad (1)$$

This method, however, is limited in that it requires a dry sample of protein and the conditions for esterification are rather vigorous,[7] causing considerable change in conformation of the protein[6] which almost invariably destroys enzymic activity. Thus, activity measurements during modification of the enzyme cannot be performed. Only two minor side reactions, methanolysis of amide groups, and the so-called N $\rightarrow$ O acyl shift can be found under the conditions described above.

---

[2] The abbreviations used in this text are defined in the List of Abbreviations, page vii .

The N → O acyl shift is shown in Equation 2.[6]

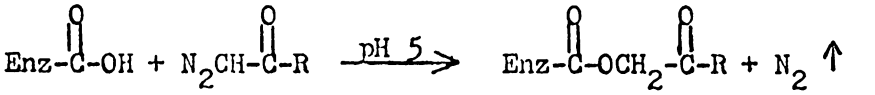


(2)

Methanolic-HCl has been used to modify the carboxyl groups of such enzymes and proteins as egg white lysozyme,[5,8] α-chymotrypsinogen,[9] pancreatic ribonuclease,[10] and bovine serum albumin.[11]

The remaining five reagents, on the other hand, may be used in aqueous solution, allowing, in most cases, assay of enzyme activity during the course of reaction. In addition, the conditions required for carboxyl group modification using these reagents are, for the most part, less vigorous, and protein denaturation can be held to a minimum.

Diazo Compounds - Diazo compounds have been shown to modify carboxyl groups rapidly and under mild conditions.[6] The reaction occurs optimally near pH 5[6] and the unionized carboxyl groups appear to be the reactive species.[9] The proposed reaction pathway is shown in Equation 3.[6]



(3)

Diazoacetates react with water and with many simple inorganic anions.[6] Their rapid reaction with water in aqueous protein solutions makes the use of excess reagent necessary, in addition to limiting the modification to the more accessible and reactive carboxyl groups of the protein.[6]

One other important side reaction which must be considered is the alkylation of sulfhydryl groups.[6] A wide variety of diazo compounds have been used to esterify carboxyl groups in pancreatic ribonuclease,[12] α-chymotrypsinogen,[9] pepsin,[13,14] β-lactoglobulin,[15] and human serum albumin.[16]

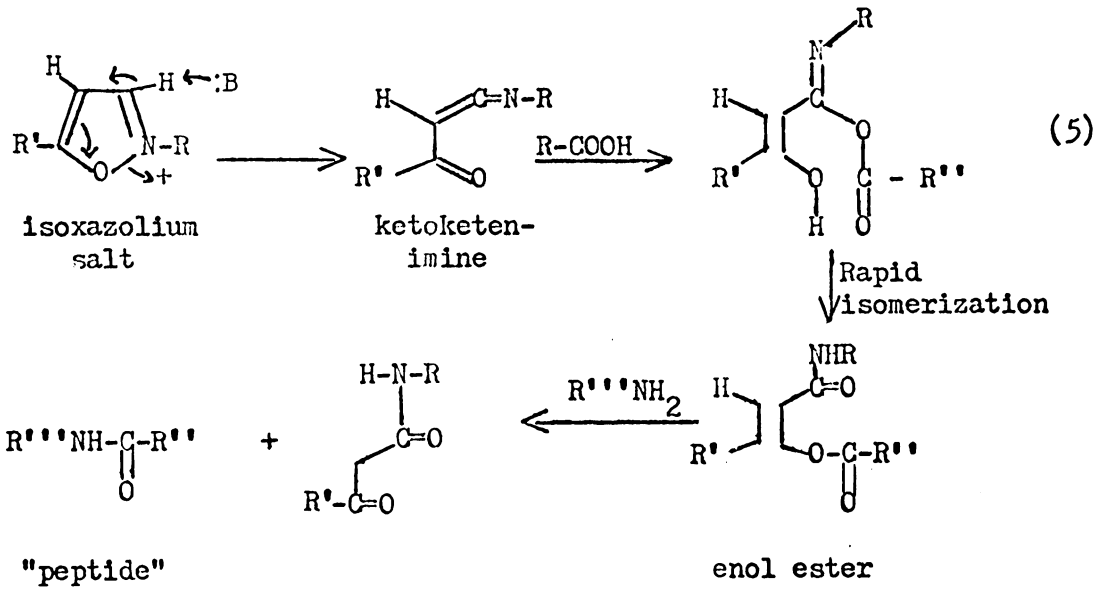
Triethyloxonium Tetrafluoroborate - Triethyloxonium tetrafluoroborate was first synthesized by Meerwein.[17] He and coworkers reported that when a concentrated solution of sodium benzoate was treated with triethyloxonium tetrafluoroborate, ethyl benzoate was produced.[18] In non-aqueous media triethyloxonium tetrafluoroborate will ethylate such compounds as ethers, sulfides, ketones, esters, and amides on oxygen, nitrogen, or sulfur to give the appropriate onium fluoroborate.[19] In aqueous medium, hydrolysis occurs rapidly, the reagent having a half-life of about 10 minutes at room temperature and pH values near six. In this respect triethyloxonium tetrafluoroborate resembles diazo compounds in that excesses of reagent are required and only the most accessible and reactive carboxyl groups will be esterified.[19] The proposed reaction pathway is shown in Equation 4.[19]

$$\text{Enz-}\overset{\overset{O}{\|}}{C}\text{-}O^{-}\,Na^{+} + CH_3CH_2\text{-}O(CH_2CH_3)_2\,BF_4^{-} \longrightarrow \text{Enz-}\overset{\overset{O}{\|}}{C}\text{-}OCH_2CH_3 + NaBF_4 + (CH_3CH_2)_2O \qquad (4)$$

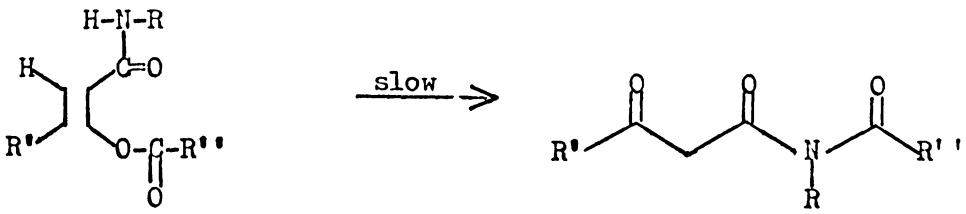
Unlike the diazo compounds, triethyloxonium tetrafluoroborate has an apparent preference for ionized carboxyl groups over nonionized groups.[19] In a study of the selectivity of the reagent towards amino acid side chains in model peptides, Yonemitsu et al.[20] found that, in

aqueous sodium bicarbonate, at room temperature, only the sulfur atom of Met, the nitrogen atom of the imidazole ring of His, and the free carboxyl group of Asp or Glu reacted with triethyloxonium tetrafluoroborate, and in fact, the Met and His reacted only after the carboxyl group had been esterified.[20] Alcoholic and phenolic hydroxyl groups and amino acid guanidino groups were found to be completely unreactive.[20] Triethyloxonium tetrafluoroborate has been used to esterify carboxyl groups in lysozyme,[21] trypsin,[22] pepsin,[23] and human carbonic anhydraseB.[24]

N-Alkylisoxazolium Salts - N-Alkylisoxazolium salts were first shown to react with carboxyl groups by Mumm.[25] In a reinvestigation of these salts Woodward and coworkers showed that a number of 5-substituted isoxazolium salts react with carboxylic acids in the presence of a base to yield enol esters which are powerful acylating agents, especially useful in peptide synthesis.[26-28] The principal features of this chemistry are shown in Equations 5 and 6.[29]



(5)



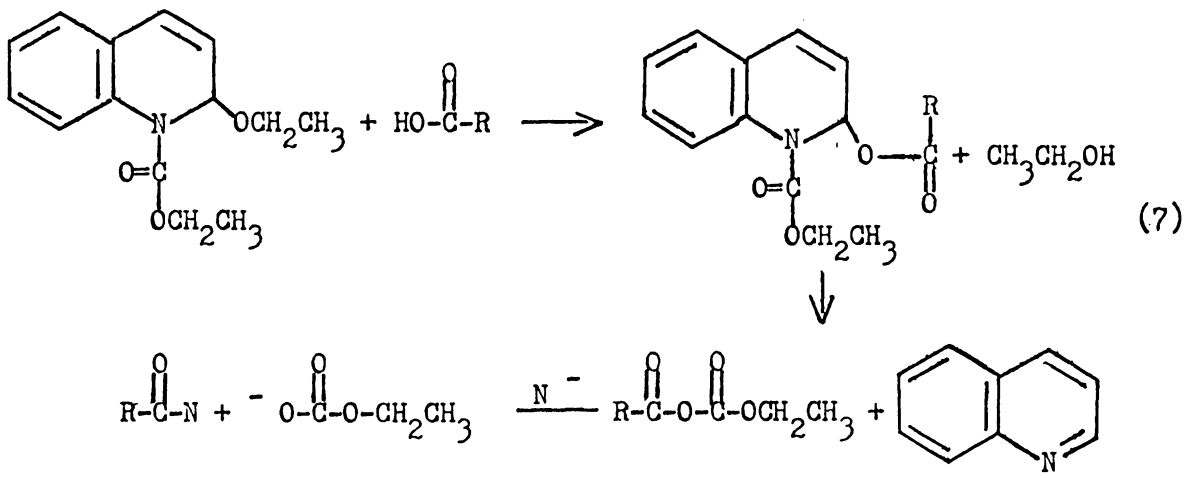
(6)

enol ester

These isoxazolium salts have been shown to react under mild conditions with carboxyl groups in proteins at pH values below 4.75.[6] The enol esters formed initially are sufficiently stable to allow isolation and subsequent reaction with appropriate nucleophiles.[26] A review of isoxazolium salts and their reactions has been prepared by King.[30] N-Ethyl-5-phenylisoxazolium-3'-sulfonate (or "Woodward's Reagent K") has been used to modify carboxyl groups in bovine carboxypeptidase A,[31,32] trypsin,[33,34] and yeast phosphoglycerate kinase.[35] Other isoxazolium salts which have been used to modify carboxyl groups in trypsin are, N-methyl-5-phenylisoxazolium tetrafluoroborate, N-ethyl-5-phenylisoxazolium tetrafluoroborate, and N-methylbenzisoxazolium tetrafluoroborate.[33,34]

N-Ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) - N-Ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) was first synthesized by Belleau *et al.*[36] They found that this compound was a potent central nervous system depressant.[36] In addition, they reported that EEDQ has the ability to activate carboxyl groups toward attack by appropriate nucleophiles. Belleau *et al.* employed EEDQ in the synthesis of several peptides and found it to be very satisfactory in non-aqueous solvents.[37] The presumed chemistry of the reaction of EEDQ with carboxyl groups is

shown in Equation 7.[37]

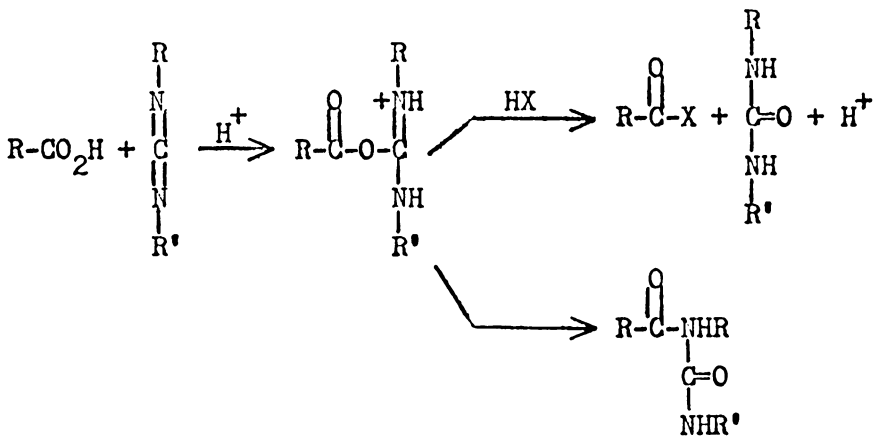


(7)

In aqueous medium, at acid pH values EEDQ is rapidly hydrolyzed to quinoline, carbon dioxide, and ethanol. Thus the use of excess reagent is required in modification of carboxyl groups in enzymes at acid pH. At pH values near 7 the reagent is more stable and extensive hydrolysis does not occur.[38] Side reactions with amines do not appear to occur under conditions of ready peptide synthesis.[37] EEDQ has been used as an activating agent for carboxyl group derivatization on the serine hydrolases,[38] in peptide synthesis,[39] in the preparation of steroid amides,[40] and to antagonize cholinergic receptors in the rat jejunum.[41]

Water-soluble Carbodiimides - Water-soluble carbodiimides were first used for the modification of carboxyl groups in proteins in a mild procedure reported by Sheehan and Hlavka[42] for the synthesis of small peptides, and the cross-linking of gelatin.[43] Hoare and Koshland later reported a procedure for the irreversible modification of carboxyl groups in proteins and enzymes using a water-soluble carbodiimide and an

added nucleophile.[44] The reaction pathway as proposed by Hoare and Koshland is shown in Equation 8.[45]



(8)

Carbodiimides react with a number of organic functional groups.[46] In aqueous solution at acidic pH values the major protein groups which react with carbodiimides are carboxyls,[45] sulfhydryls,[47] some amines at basic pH,[48,49] and tyrosines.[50] The rates of reaction of model sulfhydryl and carboxyl compounds with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) are approximately equal, while tyrosines react more slowly.[46] Tyrosine modification, however, can be reversed by treatment with hydroxylamine at pH 7.0 and 25°.[50] In addition, the number of modified tyrosines can be approximated by amino acid analysis, since the product of the reaction of carbodiimide with tyrosine is relatively stable to acid hydrolysis conditions.[50] Attempts to regenerate sulfhydryl groups by nucleophilic displacement, however, have not been successful.[47] Thus protection of the thiol groups by prior reactions with SH reagents of the type R-S-S-R where R= $-CH_2CH_2COOH$ or $-CH_2CH_2NH_3^+$

allows regeneration of the thiol group after carboxyl modification, and is one way of avoiding sulfhydryl modification.[46]

Due to the possibility of reaction of carbodiimides with a wide variety of nucleophiles, one must be careful in attributing enzyme activity changes resulting from carbodiimide treatment to carboxyl group modification until possible side reactions can be ruled out.[46] This is often possible by observation of the effects of carbodiimide in the absence of a nucleophile, since most of the side reactions are dependent only on carbodiimide.[46]

Water-soluble carbodiimides have been used in the modification of carboxyl groups in such enzymes and proteins as lysozyme,[44,45,51-54] ribonuclease,[45,51,55] bacitracin,[53] bovine insulin,[45,53,56] α-chymotrypsin,[44,51,57] α-chymotrypsinogen,[57] trypsin,[44,51,57-59] trypsinogen,[59] acetylcholinesterase,[60] α-lactalbumin,[61] β-lactoglobulin,[47,62] L-glutamate dehydrogenase,[63] carboxypeptidase A,[64] glucoamylase,[65] sucrose phosphorylase[66] and glutamyl peptides and proteins.[67]

Papain - Papain is the principal protein-cleaving enzyme found in the latex of the branches, leaves and green fruit of Carica papaya, the familiar softwood tropical shrub. As early as 1879 Wrutz and Bouchut performed controlled experiments on the active material in papaya latex.[68] Between 1900 and 1940 many papers were published using only purified extracts of papaya latex. A review of the literature of this period was prepared by Hwang and Ivy.[69]

The interest in papain came about chiefly because it was the first proteinase found to be capable of hydrolyzing synthetic substrates.[70]

It was not, however, until 1954 that Kimmel and Smith,[71,72] using a modification of the method of Balls and Lineweaver,[73] prepared crystalline papain from dried papaya latex. Today papain can be prepared relatively easily and economically from dried latex and in a state of relative purity. Papain, as prepared by the method of Kimmel and Smith[71,72] contains a free sulfhydryl titre of between 0.1 and 0.5 moles of -SH per mole of protein. Elicitation of maximal hydrolytic activity of the enzyme requires activation of papain.[74] Papain can be activated by a variety of thiol reagents such as cysteine, 2-mercaptoethanol or glutathione, or by certain reducing agents such as sodium borohydride.[74] In addition to these thiol reagents a chelator is required for maximal activation, presumably to remove any traces of metal ions which might otherwise bind to the essential thiol group of the enzyme.[74] These chelators can be incorporated into the thiol reagent as in the case of 2,3-dimercaptopropanol,[74] or dithiothreitol,[75a] or chelators such as EDTA may be used.[74]

The details of activation of papain have been studied by a number of investigators.[76-78] The best documented form of inactive papain is that prepared by the method of Kimmel and Smith,[71,72] where the essential thiol group of Cys-25 is involved in a mixed disulfide bond with cysteine,[77,78] and it is this mixed disulfide bond which is reduced by addition of excess thiol reagent.[74]

A more recent hypothesis on the activation of papain has been proposed by Brocklehurst and Kierstan.[75b] According to these authors, papain exists in dried latex as a zymogen (propapain) in which the active SH group of cysteine-25 is involved in a disulfide bond with a

neighboring cysteine residue, but upon treatment with a suitable activating agent, the zymogen undergoes an intramolecular thiol-disulfide interchange to produce active papain.[75c]

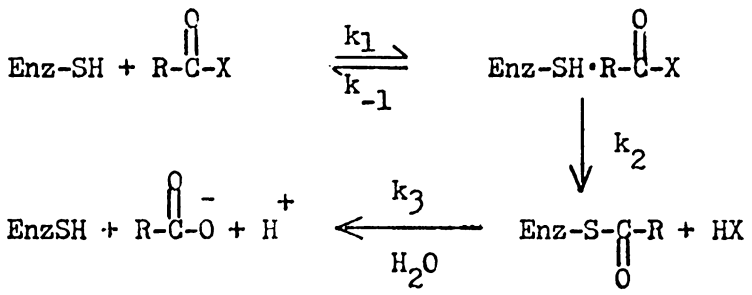
In addition, a purification technique reported by Blumberg et al.,[79] using affinity chromatography enabled these workers to obtain a pure homogeneous completely activatable fraction of the enzyme. Before the Blumberg report, all crystalline papain preparations consisted of at least two components, one of which could not be activated, presumably because the essential thiol group was in an oxidation state which could not be reduced by thiol group reducing agents such as 2-mercaptoethanol or cysteine, and a second component which could be activated by thiol reagents.[80]

Finally, if papain is to be stored for any length of time, or if protection of the essential thiol group is required, conversion of active papain to its soluble mercury derivative can be achieved by addition of one mole of mercuric chloride per mole of protein.[79] This mercury derivative is inactive but can be instantaneously reactivated by addition of 5 mM cysteine and 1 mM EDTA during assay, as indicated in Equation 9.[81]

$$\underset{\text{inactive}}{\text{Enz -S- HgCl}} + \text{R - SH} \longrightarrow \underset{\text{active}}{\text{Enz - SH}} + \text{R - S - HgCl} \qquad (9)$$

Since the 1950's a vast amount of work has been done on papain, so that today the amino acid sequence,[82] as well as the 3-dimensional structure from X-ray crystallography of the papain molecule is known.[83]

Much evidence has also been obtained concerning the mechanism of action of papain, and several different mechanistic pathways for catalysis have been proposed.[84-86] These mechanisms differ in detail, but all incorporate the essential feature implicating a free thiol group of a cysteine residue as the major functional group in catalysis. A brief outline of the proposed reaction pathway is given in Equation 10.[74]



(10)

Extensive recent reviews on papain are available in which the physical, structural, and catalytic properties of the enzyme as well as the progress which has been made toward understanding its mechanism of action are discussed.[75c,83,85,74,87-89]

Structure and Properties - Some of the more important physical properties of the enzyme papain are given in Table I. Table II gives the amino acid composition of papain.[74]

The primary structure of papain was published in 1970 by Mitchel et al.[82] The secondary and tertiary structural features of crystalline papain were determined from the X-ray data of Drenth et al.[83] The papain molecule has three disulfide bridges. These occur between residues 22 and 63, 56 and 95, and residues 153 and 200.[82] The 3-dimensional structure of the enzyme is that of a globular protein

TABLE I

PHYSICAL PROPERTIES OF PAPAIN

		Reference
Isoelectric Point, pI	8.75	74
Absorbancy, $A^{1\%}_{1cm}$ at 278 nm	25.0	90
Molecular Weight	23430	91

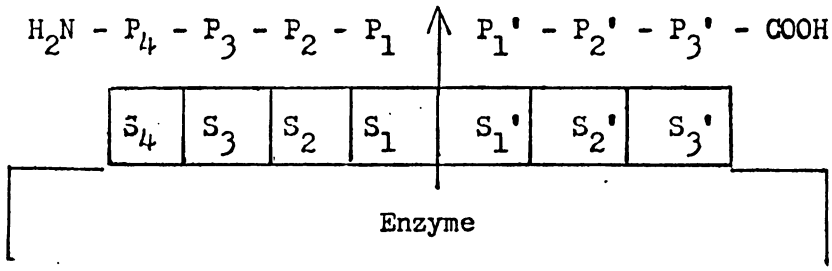
TABLE II

AMINO ACID COMPOSITION OF PAPAIN

Amino Acid	mole/mole of Enzyme
Lysine	10
Histidine	2
Arginine	12
Aspartic acid	6
Asparagine	13
Glutamic acid	8
Glutamine	12
Threonine	8
Serine	13
Proline	10
Glycine	28
Alanine	14
Valine	18
Isoleucine	12
Leucine	11
Tyrosine	19
Phenylalanine	4
Tryptophan	5
Cysteine	1
Cystine (half)	6
total	212

with two wing-like hydrophobic cores which contain approximately equal numbers of residues. The two wings of the molecule are divided by a deep cleft which is the location of the active site. In addition to the essential thiol of Cys-25, the active site contains several other residues of possible catalytic importance, including His-159 and Asp-158 which are in proximity to the Cys-25 sulfhydryl group.(74) The active site groove is further lined by side chains of Tyr-61, Tyr-67, and Trp-69.(74) The molecule measures approximately 5.0 x 3.7 x 3.7 nm.(83)

Berger and Schecter have mapped the extended active site of papain using a series of alanine peptides.(89) The results of this study showed that the active site of the enzyme is approximately 25 Å long which corresponds to 7 amino acid residues. Berger and Schecter labeled the binding sites for the seven amino acid residues S_1 - S_4 and S_1' - S_3' as shown below:



The essential thiol group of the enzyme is found in the position between the S_1 and S_1' subsites. Also this is the position of cleavage of the peptide bond. It was further found that one of the subsites, S_2, specifically interacts with a phenylalanine residue.(92) In addition, subsite S_1 shows some preference for lysine over alanine. Finally it has been shown recently that subsite S_1' has a predilection for hydrophobic residues, in particular, L-tryptophan and L-leucine.(93a)

A recent study by Lowbridge and Fruton[93b] has, however, set forth the hypothesis that the minimum size of the active site of an enzyme cannot conclusively be estimated as described by Berger and Schecter.[89] They argue that while such formulations of the results of kinetic studies on a series of oligopeptides lend themselves to a graphical representation, the increasing evidence for the conformational flexibility at the active sites of proteolytic enzymes suggest that such mapping may be an oversimplification. These authors contend that the active site may undergo changes in its conformation in response to interaction with the substrate, thus making the delination of the complete binding area of an enzyme active site in terms of specific subsites a task of doubtful validity.[93b]

Some catalytic properties of papain with respect to synthetic N-acylamino esters and amides are given in Table III.[74]

Assay - Probably the most widely used assay for papain is the pH-stat titration of the rate of hydrolysis of α-N-benzoyl-L-arginine ethyl ester (BzArgOEt) as described by Smith and Parker[94] and later modified by Hall et al.[81] A second often used esterase assay technique using N-benzyloxycarbonylglycine p-nitrophenyl ester and following the appearance of the p-nitrophenylate ion spectrophotometrically at 400 nm has been reported by Kirsch and Igelström.[2] An assay for amidase activity using α-N-benzoyl-L-argininamide was described by Kimmel and Smith[72] and involved a titrimetric technique similar to that used in the BzArgOEt assay. A spectrophotometric assay using BzArgOEt under conditions and kinetic parameters which relate this assay to absolute papain concentra-

TABLE III

KINETIC PARAMETERS FOR PAPAIN-CATALYZED HYDROLYSIS OF SOME N-ACYLAMINO ESTER AND AMIDE SUBSTRATES

Substrate	$K_{m(app)}$ (M)	$k_{cat(lim)}$ (sec^{-1})	Temperature (degrees)	Reference
α-N-Benzoyl-L-arginine ethyl ester	0.023	9.0	25	94
	0.018	12.0	37	94
	0.014	16.1	25	95
α-N-Benzoyl-L-argininamide	0.040	11.0	38	84
	0.032	8.7	25	95
N-Benzoylglycine ethyl ester	0.021	3.1	40	97
N-Benzoyl glycinamide	0.16	0.6[a]	38	98
N-Benzyloxycarbonylglycine p-nitrophenyl ester[b]	8×10^{-6}	5.2	25	99

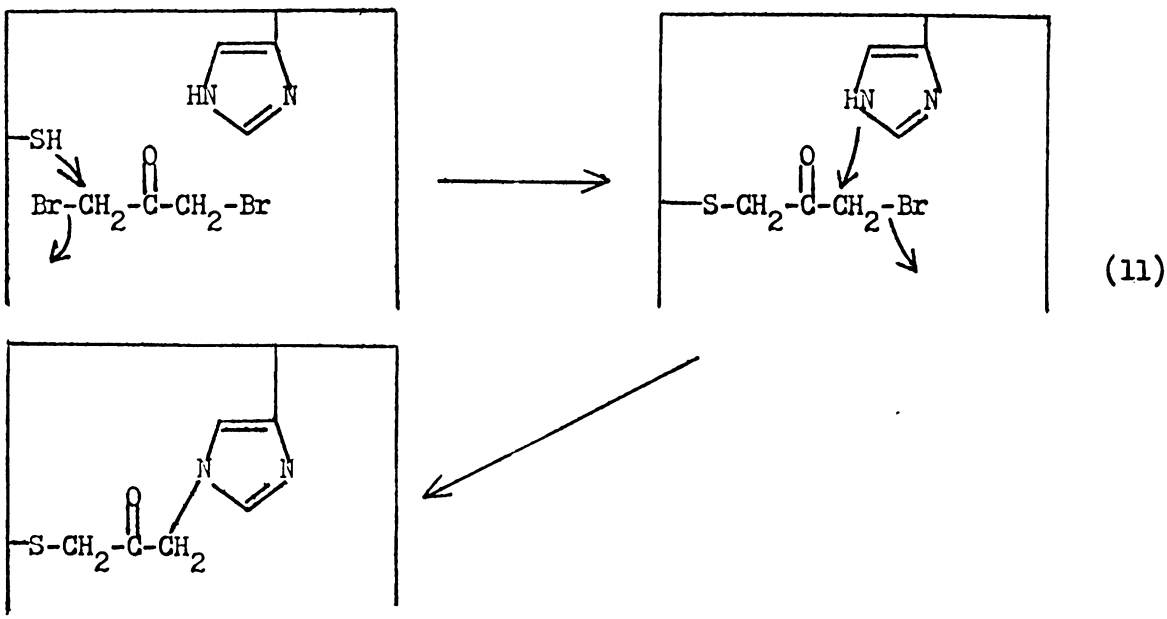
[a] At pH 5.2, and is not maximal

[b] In the presence of 1.6% acetonitrile, by volume

tions determined by direct active-site titration was reported by Whitaker and Bender.[95]

Finally a technique for the titrimetric determination of the active thiol content of papain using 2,2'-dipyridyldisulfide was described by Brocklehurst and Little.[75]

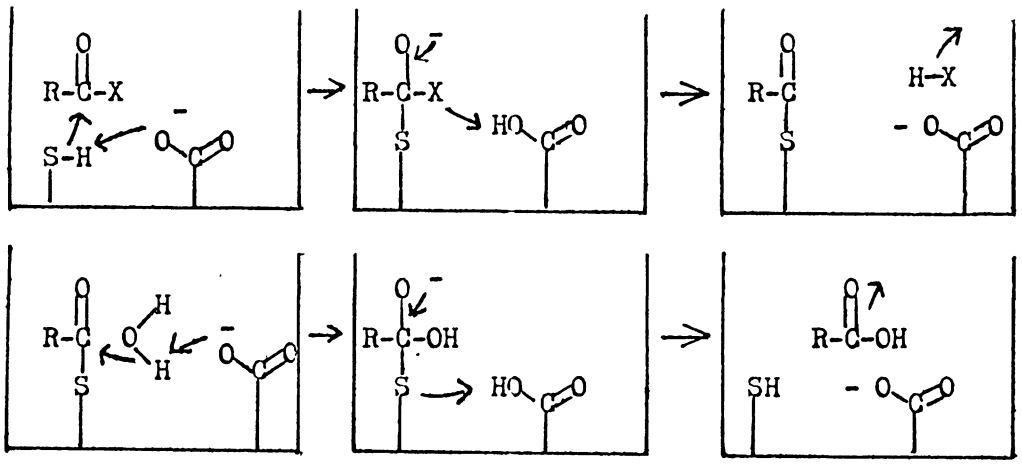
The Active Site - Williams has shown that the reaction pathway for papain-catalyzed hydrolysis of α-N-acyl-L-amino acid derivatives involves formation of an acyl enzyme intermediate via reaction with a group having an apparent pK_a of 8.2.[96] The assignment of this apparent pK_a to the active site thiol group of Cys-25 is consistent with a large number of chemical modification studies. These studies have involved alkylating agents which invariably react specifically with the essential thiol group of the enzyme and result in inactivation. Papain has thus been inactivated via alkylation of its essential thiol group by α-halo carboxylic acid derivatives,[100-108] N-alkylmaleimides,[109] chloromethylketones derived from N-tosyl-L-lysine (TLCK),[110-112] and N-tosyl-L-phenylalanine (TPCK),[110-113] phenylmethanesulfonylfluoride,[110] and N-tosyl-glycine.[114] In addition the inactivation of papain with the bifunctional alkylating agent 1,3-dibromoacetone[115-117] involved cross linking of the essential thiol group of papain to the imidazole ring of His-159 in the active site as shown in Equation 11:



(11)

In addition to this thiol group, Lowe and Yuthavong found that the bell-shaped pH dependence of acylation also indicated that a second group with an apparent pK_a of 4.2 was involved in catalysis and probably functioned as a general base.(1) Wallenfels and Eisele proposed that this second group was a carboxyl group because the apparent pK_a of 4 and the low heat of ionization of the group was consistent with those of a normal carboxyl group.(108)

Kirsch and Igelström(2) have studied the kinetics of papain-catalyzed hydrolysis of esters of benzyloxycarbonylglycine and from their kinetic data have proposed a minimal mechanism of action of papain as shown in Equation 12:



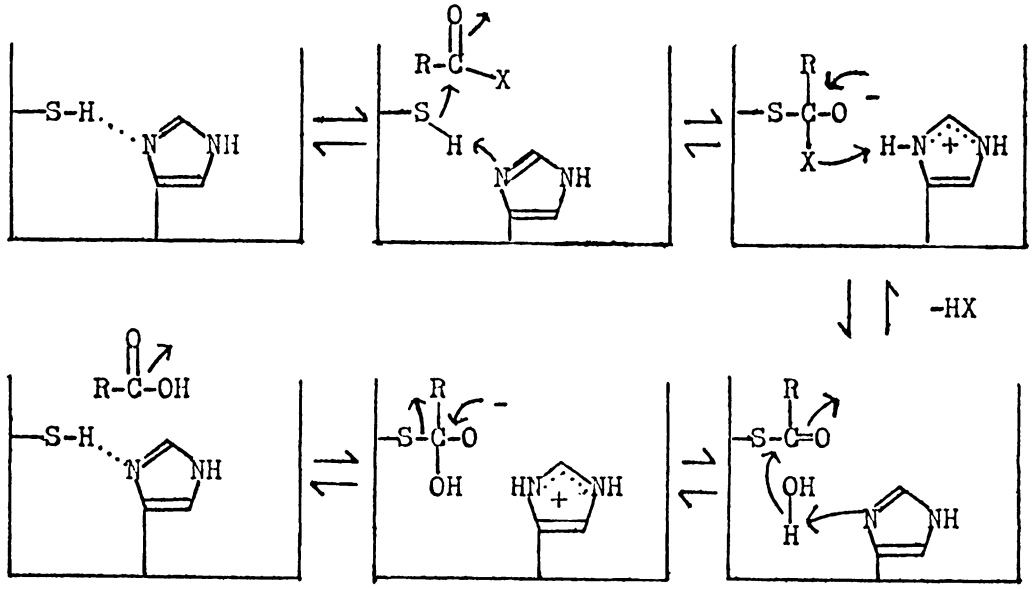
(12)

The function of the carboxyl group is one of proton transfer, initially by acting as a general base to increase the nucleophilicity of the unionized mercaptan, thus aiding the formation of a tetrahedral addition intermediate, and then, through general acid catalysis, facilitating the expulsion of the leaving group to form the thiol ester.

Lowe and Williams,[118] however, suggested that the group involved was not a carboxyl group but an imidazole moiety of a histidine residue because they found that the rate of intramolecular catalysis of S-hippuryl thioglycolate and S-ethylmonosuccinate hydrolysis was 10^5 to 10^6 times slower than the rates of deacylation of hippuryl-papain and α-N-benzoyl-L-arginine-papain, while the rate of imidazole intramolecular catalysis of the hydrolysis of n-propyl-α-4-imidazoyl thiobutyrate was only one order of magnitude slower than the deacylation step of reasonably good substrates.

In addition, Lucas and Williams[3] agree with the assignment of a catalytic role to His-159 on the basis of data obtained on the pH dependencies of individual rate constants in certain papain-catalyzed reactions of synthetic hippuryl and glycine esters. Their proposed

mechanism assigns to His-159 the role of acting as a general base attacking a thiol proton in acylation, and a water proton in deacylation. Also, they postulate that the apparent pK_a of the imidazole is lowered to 4 by the donation of a hydrogen bond from the amide nitrogen of the adjacent Asp-174 to the tertiary nitrogen of the imidazole ring. The pathway for this reaction involving His-159 is shown in Equation 13:[3,85]



(13)

Also, the cross-linking of the essential thiol of papain to His-159 by 1,3-dibromoacetone, as mentioned above,[115-117] further supports claims that this imidazole ring may participate as an essential group in papain catalysis.[3,86] In addition, the later work of Drenth et al.[119] showed that indeed there was an imidazole moiety of His-159 in the active site of papain in proximity to the essential thiol of Cys-25 as had been proposed by Husain and Lowe.[115]

Amino groups[120,121] and tyrosine hydroxyl groups[122-124] in papain may be chemically modified without loss of enzymatic activity. No investigation directed toward the chemical modifications of carboxyl

groups in papain have, as yet, been reported in the literature. Thus, because of the lack of direct chemical evidence, the exact identity of the second catalytically essential group in the active site of papain remains an open question.[4]

In addition, recent evidence has been published[125] which indicates that His-159 has a "normal" pK_a of ~ 6.7 rather than a perturbed pK_a near 4. So while it does seem likely that the imidazole group may indeed participate in papain catalysis, the evidence associating this group with the acidic limb of the pH-rate profile for acylation is not compelling, and the possibility remains that the apparent pK_a of ~ 4 might be ascribed to a carboxyl group such as that of Asp-158.

Finally, very few competitive inhibitors for papain are known. One of the best competitive inhibitors for papain to date is benzamidoacetonitrile. Benzamidoacetonitrile was first synthesized by Klages and Haak[126] and later shown to have a pH-independent K_i of 0.4 mM.[3] A second very excellent competitive inhibitor of papain is the tetrapeptide, Gly-Gly-Tyr(Bzl)-Arg, which has a K_i of 10 μM at pH 4.3 and a K_i of 150 μM at pH 6.0.[89,92] This tetrapeptide was used by Blumberg *et al.* to purify papain *via* affinity chromatography.[79]

EXPERIMENTAL PROCEDURE

Materials

N-Ethyl-5-phenylisoxazolium-3'-sulfonate, 4-methoxy-3-butene-2-one, 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-p-toluene sulfonate, L-histidine, t-butoxycarbonylazide, N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (Gold Label) (recrystallized before use, m.p. 65-66°, Lit. m.p. 63.5-65°[127]) and monoethyladipate were obtained from Aldrich Chemical Company, Milwaukee, Wisconsin.

Sodium borohydride, 1-butanol, acetonitrile, benzoic acid, benzyl alcohol, 1-propanol and thioglycolic acid were purchased from Fisher Scientific Company, Fairlawn, New Jersey.

N-Benzyloxycarbonylglycine-p-nitrophenyl ester, L-glutamic acid, glycine methyl ester hydrochloride, glycine ethyl ester hydrochloride (sublimed before use), α-N-benzoyl-L-arginine ethyl ester hydrochloride, L-cysteine (free base), EDTA (disodium salt), dithiothreitol, 2,2'-dipyridyldisulfide (2,2'-dithiodipyridine), and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (m.p. 114-115°, Lit. m.p. 113.5-114.5°[128]) were obtained from Sigma Chemical Company, St. Louis, Missouri.

Sephadex G-25 (medium) was purchased from Pharmacia Fine Chemicals, Piscataway, New Jersey.

Triethylamine, 13181 Silica Gel thin layer chromatography plates, 1-butanoic acid, acrylic acid, 2-mercaptoethanol (distilled before use), and diphenyl acetic acid were purchased from Eastman Organic Chemicals, Rochester, New York.

[$^{14}C_1$] -Glycine ethyl ester hydrochloride (Lot #600-281) was obtained from New England Nuclear, Boston, Massachusetts.

Papain (2x recrystallized, Lot #PAP33K857 and #PAP34D677) was purchased from Worthington Biochemical Corporation, Freehold, New Jersey.

All other chemicals (solvents, buffer salts, and common chemicals) employed were the highest grade obtainable. Oxygen-free, nitrogen-purged, glass distilled water was used throughout.

Benzamidoacetonitrile was prepared by the method of Klages and Haak,[126] m.p. 140° (Lit. m.p. 139-141° [3]).

Triethylamine hydrochloride was prepared by lyophilizing a solution of predistilled triethylamine in constant boiling 6 N HCl. The white powdery salt melted at 255-256° (Lit. m.p. 253-254° [129]).

L-Histidine methyl ester dihydrochloride was prepared by the method described by Greenstein and Winitz,[130] m.p. 212-214° (Lit. m.p. 200-201°[131]). Analysis: Calculated for $C_7H_{13}Cl_2N_3O_2$: C, 34.71; H, 5.37; N, 17.32. Found: C, 34.56; H, 5.32; N. 17.32.

N-t-Butoxycarbonyl-L-histidine methyl ester was prepared by the method of Schröder and Gibian,[132] m.p. 126-128° (Lit. m.p. 127-130° [133]).

Although available commercially (vide supra), N-ethyl-5-phenyl-isoxazolium-3'-sulfonate was also synthesized by the procedure of Woodward et al.,[28] m.p. 206-208° d (Lit. m.p. 206-208° d [28]).

N-Ethyl-5-phenylisoxazolium tetrafluoroborate was prepared by the method of Woodward and Olofson,[26] m.p. 99.5-100.5° (Lit. m.p. 100-100.5° [26]).

N-Ethyl-5-methylisoxazolium tetrafluoroborate was prepared from 4-methoxy-3-butene-2-one using the method of Wilson and Burness,[134] m.p. 39-40° d (Lit. m.p. 39-40° d [134]).

Triethyloxonium tetrafluoroborate was prepared by the method of Meerwein,[17] m.p. 91-92° d (Lit. m.p. 91-92° [17]).

Diazoacetamide was synthesized by the procedures described by Wilcox,[7] m.p. 119-121° d (Lit. m.p. 120° d[7]).

2,2'-Diphenylethanol was prepared from diphenylacetic acid by the method of Yoon et al.,[135] m.p. 58-59° (Lit. m.p. 58-59°[136]).

Methods

Infrared (ir) spectra were taken on a Beckman Model IR 20A spectrophotometer either as thin films, or KBr pellets. Nuclear magnetic resonance (nmr) spectra were obtained on a JEOL-PS-100 NMR spectrometer, using tetramethylsilane (TMS) as an internal standard. Chemical shift values (δ) are given in ppm relative to TMS. Ultraviolet-visible spectra and spectrophotometric rate assays were acquired using a Cary Model 14 recording spectrophotometer equipped with a thermostatted cell compartment and cuvette holder for standard 1 cm quartz cuvettes.

Gas chromatography was performed using an Aerograph HY-FI Model 600-C gas chromatograph, equipped with a hydrogen flame ionization detector. A 6 ft x 0.25 in stainless steel column packed with 10% Carbowax on 60/80 Chromosorb W was used throughout.

Melting points were taken in open capillary tubes in a Thomas-Hoover melting point apparatus and are uncorrected.

Measurements of pH and pH-stat titrimetric rate assays were carried out with a Radiometer PHM-26 pH meter equipped with a Radiometer TTT-11 titrator, SBR-2 recorder, and SBU-1 syringe buret. The electrode system was a Radiometer Model GK 2321 C. The electrode was calibrated using Fisher standard buffers at pH 6.86 and 4.01 at 25° as described in the Radiometer Instruction Manual for pH Meter Type PHM-26. Titrants were prepared from standard solutions of NaOH or $HClO_4$.

Radioactivity was determined using a Beckman Model LS-133 liquid scintillation counter.

When appropriate, kinetic data were subjected to analysis by computer (DEC PDP 81) using a linear least squares program which provided slopes and intercepts along with their standard error estimates, from which rate constants could be derived.

Elemental analyses were performed in the Chemistry Department Analytical Services section at Virginia Polytechnic Institute and State University by Thomas E. Glass. Analyses were performed on a Perkin Elmer Model 240 Elemental Analyzer.

Papain Preparation - Papain (EC 3.4.4.10) (2x recrystallized) was further purified by the method of Blumberg et al.[79] using a glycyl-glycyl - O-benzoyl-L-tyrosyl-L-arginine Sepharose affinity column. This column and the peptide bound to it were both prepared by Mrs. Constance D. Anderson. The purified, fully active enzyme was converted to its more stable but catalytically inactive mercury derivative by addition of a stoichiometric amount of mercuric chloride for storage at 4° as a solution (approximately 3.5 mg/ml). Fully active papain is readily regnerated from mercuripapain as described below.

In experiments requiring mercuripapain, an aliquot of the stock solution was first dialyzed against 3 x 500 ml portions of 10 mM KCl to remove excess mercuric ions. Active, activator-free papain was prepared by adding dithiothreitol (to a final concentration of 2.9 mM) to a 2.5 ml aliquot of mercuripapain stock solution and stirring at room temperature for 30 minutes. The activator was then removed by gel filtration on a Sephadex G-25 (medium) column (1 x 37 cm). The eluent solvent was oxygen-free, nitrogen-purged 10 mM KCl. The activator-free enzyme usually appeared between 15 and 18 ml of eluate volume and was used immediately.

Papain Assays - Total papain concentration was determined spectrophotometrically using the published parameters $A^{1\%}_{278\ nm,\ 1\ cm} = 25.0$[74] and M.W. = 23430.[117] The concentration of "activatable papain"[80] in a purified (see above) mercuripapain stock solution was determined by spectrophotometric rate assay using α-N-benzoyl-L-arginine ethyl ester as described by Whitaker and Bender[95] under conditions and using kinetic parameters which relate this assay to absolute papain concentrations determined by direct active site titration. Comparison of the rate assay data with concentrations determined from protein absorbance at 278 nm established that the stock solutions contained no "inactivatable"[80] enzyme.

Determinations of relative papain activity during the course of inactivation reactions were carried out by periodic titrimetric or spectrophotometric rate assays. In the case of the titrimetric assays, each assay was initiated by adding an appropriate small aliquot (typically 35 μl) of the reaction mixture to a titration vessel thermo-

statted at 25° and containing 1 mM EDTA, 50 mM α-N-benzoyl-L-arginine ethyl ester, 0.23 M KCl and water to a final volume of 2.5 ml. For those assays performed on mercuripapain, the 2.5 ml assay mixture was made 5 mM in cysteine. All assays were carried out at pH 6.6, delivering freshly standardized 0.05 N NaOH titrant from a 0.5 ml syringe buret. The calculated final protein concentration was 0.85, 1.44 or 1.49 μM depending on the nature of the inactivation reaction. Initial rates of enzyme catalyzed ester hydrolysis, recorded as percent of syringe capacity per minute, were normalized to an averaged control rate using fully active papain, and are reported as percent active enzyme.

Determinations of the free oxidizable thiol content of papain either before, during, or after inactivation reactions were routinely performed according to the method of Brocklehurst and Little[75] using 2,2'-dipyridyldisulfide in a spectrophotometric titration at 343 nm. However, in conjunction with inactivations of active, activator-free papain with N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline, determinations of free oxidizable thiol during inactivation required modification of the published procedure. This modification was needed because N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline had a significant absorption at 343 nm. The reference cuvette contained 30 mM formate buffer, pH 3.8, 2.5% acetonitrile, 0.75 mM KCl, 1.22 mM EEDQ and water to a final volume of 2.9 ml. The sample cuvette contained 30 mM formate buffer, pH 3.8, 0.75 mM KCl, an aliquot of the reaction mixture containing acetonitrile, papain, and EEDQ, plus water to a volume of 2.9 ml. Using the 0.0 - 0.2 slidewire of the Cary 14 spectrophotometer, an initial

baseline was recorded. When 0.1 ml of 1.78 mM 2,2'-dipyridyldisulfide was quickly added first to the reference and then to the sample cuvette, the instantaneous absorbance change was recorded at 343 nm. The concentration of 2-thiopyridone released was calculated using $\varepsilon_{343\ nm} = 7.0 \times 10^3\ M^{-1}\ cm^{-1}$.(75)

Spectrophotometric papain assays using N-benzyloxycarbonylglycine-p-nitrophenyl ester were performed as described by Kirsch and Igelström,(2) with enzyme activity calculated from the change in optical density at 400 nm per minute. In all enzyme modifications parallel control reactions identical in every respect to the inactivation reaction mixtures without the modifying reagent were assayed periodically during all reactions. In no case was any activity loss or reduction in thiol titre observed in these controls.

Reduction of Carboxylic Acids Using N-Ethyl-5-phenylisoxazolium-3'-sulfonate and Sodium Borohydride. Method A - Into a 50 ml Erlenmeyer flask equipped with a magnetic stirrer were placed 1 m mole of the acid to be reduced, 1 m mole of N-ethyl-5-phenylisoxazolium-3'-sulfonate, and 5 ml of acetonitrile at room temperature. Triethylamine (1 m mole) in 1 ml of acetonitrile was added dropwise with stirring. After 90 minutes (by which time all solids had dissolved) the reaction mixture was evaporated to dryness *in vacuo* at room temperature, and the residue, which was usually an oil was either recrystallized from acetonitrile to give the expected enol ester derivative in high yield (characterized by ir and nmr spectra and by elemental analysis) as was reported by Woodward *et al.*,(26) or dissolved immediately in 5 ml of water, followed quickly by addition of 10 m moles of sodium borohydride. After 90

minutes, the mixture was cooled in an ice bath and acidified with 6 N HCl to destroy excess sodium borohydride. The carbinol products were then either subjected to analysis by direct injection of the acidified reaction mixture into the gas chromatograph or extracted with ether and isolated and purified by conventional means.

Reduction of Carboxylic Acids Using N-Ethyl-5-phenylisoxazolium-3'-sulfonate and Sodium Borohydride. Method B - Into a 500 ml round bottom flask equipped with a stirrer and dropping funnel were placed 3 gm of N-ethyl-5-phenylisoxazolium-3'-sulfonate (12 m moles) and 12 m moles of the acid to be reduced along with 75 ml of acetonitrile. Triethylamine (12 m moles) dissolved in 25 ml of acetonitrile was added dropwise with stirring. In the case of some of the acids which were reduced by this method the corresponding N-ethyl-5-phenylisoxazolium-3'-sulfonate enol esters formed fluffy white crystals which were not completely soluble in acetonitrile. Thus, disappearance of undissolved materials could not serve to indicate complete reaction. Nonetheless, 90 minutes always proved sufficient to attain complete derivatization of all the acids tested in this investigation. Therefore, after 90 minutes the reaction mixture was evaporated to dryness in vacuo at room temperature and to the residue was added 100 ml of water followed by 120 m moles of sodium borohydride. The reaction mixture was magnetically stirred at room temperature for 90 minutes and then cooled in an isopropanol-dry ice bath. Next, 50-65 ml of ethanolic HCl were added carefully with stirring to destroy excess sodium borohydride after which the pH of the reaction mixture was adjusted to 11 with 4 N NaOH. This alkaline aqueous solution was subjected to continuous ether extraction for 24-72

hours. The ether extract was concentrated *in vacuo*, washed with water to remove any salts and dried over anhydrous $MgSO_4$. The ether was then carefully distilled off and the products purified and identified by conventional means.

O-Benzoyl-N-ethylcinnamamide-3'-sulfonate was prepared from benzoic acid in acetonitrile using Method A in a 94% yield, m.p. 158-159° d. Analysis: Calculated for $C_{24}H_{33}N_2O_6S$: C, 60.30; H, 6.92; N, 5.87. Found: C, 60.21; H, 6.94; N, 6.00.

O-Butyryl-N-ethylcinnamamide-3'-sulfonate was prepared from 1-butanoic acid in acetonitrile using Method A in 92% yield, m.p. 126-129°, nmr ($CDCl_3$) δ 9.21 (1,s), 8.00 (1,s), 7.79 (1,d), 7.39 (3,q), 6.55 (1,s), 3.31 (2,t), 3.02 (2,q), 2.65 (2,q), 1.75 (2,m), 1.18 (15,t). Analysis: Calculated for $C_{21}H_{34}N_2O_6S$: C, 57.01; H, 7.69; N, 6.33. Found: C, 56.84; H, 7.30; N, 6.24.

O-Cinnamoyl-N-ethylcinnamamide-3'-sulfonate was prepared from cinnamic acid using Method B in 92% yield, m.p. 133.5-134° d, nmr ($CDCl_3$) δ 1.19 (12,t), 3.01 (8,q), 6.75 (1,s), 7.35 (3,m), 7.91 (1,d), 8.30 (4,s), 9.55 (1,s). Analysis: Calculated for $C_{26}H_{34}N_2O_6S$: C, 62.15; H, 6.77; N, 5.58. Found: C, 62.43; H, 6.42; N, 5.92.

O-(p-Nitrobenzoyl)-N-ethylcinnamamide-3'-sulfonate was prepared from p-nitrobenzoic acid using Method B in 63% yield, m.p. 143-143.5° d (orange), nmr ($CDCl_3$) δ 1.05 (12,t), 2.90 (6,q), 3.18 (2,q), 6.62 (1,d), 7.30 (5,s), 7.79 (1,d), 8.09 (1,s), 7.51 (4,m), 9.15 (1,s). Analysis: Calculated for $C_{24}H_{31}N_3O_8S$: C, 58.41; H, 6.29; N, 8.52. Found: C, 58.42; H, 6.23; N, 8.58.

O-Butyryl-N-ethylcinnamamide was prepared from 1-butanoic acid and 5-phenylisoxazolium tetrafluoroborate in water at pH 7.0, at 4°, using the procedure of Woodward and Olofson[26] for the analogous esterification of acetic acid. The enol ester was obtained in a 69% yield, m.p. 107-107.5°; ir (KBr pellet) 3280, 3070, 2970, 1780, 1670, 1634, 1563, 1285, 1235, 1145, 1121, and 980 cm^{-1}. Analysis: Calculated for $C_{15}H_{19}NO_3$: C, 68.97; H, 7.28; N, 5.36. Found: C, 70.47; H, 7.73; N, 5.18.

O-Methyl-N-benzyloxycarbonyl-α-L-glutamyl glycinate was prepared from N-benzyloxycarbonyl-L-glutamic anhydride and methyl glycinate according to the procedure of Le Quesne and Young.[137] The product mixture of γ- and α-glutamyl dipeptides was fractionally extracted with aqueous sodium carbonate. The last fraction extracted showed only one spot on TLC and the ir, nmr, and mass spectra were consistent with the α-dipeptide, m.p. 99-100°; ir (KBr pellet), 3310, 1745, 1690, 1655, 1540, 1455 (doublet), 1300, 1230, 1055, and 1035 cm^{-1}; nmr ($CDCl_3$) δ 2.10 (2,t), 2.53 (2,d), 3.74 (3,s), 4.01 (2,m), 4.53 (1,t), 5.16 (2,s), 6.12 (1,d), 7.33 (5,s); mass spectrum m/e 352. Analysis: Calculated for $C_{16}H_{20}N_2O_2$: C, 54.54; H, 5.68; N, 7.95. Found: C, 54.25; H, 5.81; N, 7.91.

Reduction of O-Methyl-N-benzyloxycarbonyl-α-L-glutamyl Glycinate Using N-Ethyl-5-phenylisoxazolium-3'-sulfonate and Sodium Borohydride - This peptide was reduced using Method A, forming the diol derivative N-benzyloxycarbonyl-α-amino-δ-hydroxyvaleryl ethanolamide in 50% yield, m.p. 95-96°; ir (KBr pellet) 3330 (doublet), 1740, 1650, 1550, 1280, 1180,

and 1060 cm^{-1}. Analysis: Calculated for $C_{15}H_{22}N_2O_5$: C, 58.00; H, 7.09; N, 9.03. Found: C, 57.67; H, 7.45; N, 9.02.

Reduction of O-Methyl-N-benzyloxycarbonyl-α-L-glutamyl Glycinate With Sodium Borohydride - Into a 50 ml Erlenmeyer flask equipped with a magnetic stirrer were placed 1.42 m moles of the peptide, 14.2 m moles of sodium borohydride and 5 ml of water. The reaction mixture was stirred for 90 minutes, cooled on an ice bath, acidified with 6 N HCl and extracted with ethyl acetate. The ethyl acetate was removed *in vacuo* and the residue was recrystallized from acetone - petroleum ether to give a 73% yield of the alcohol N-α-benzyloxycarbonylglutamyl ethanolamide, m.p. 156-157°, ir (KBr pellet) 3320, 1735, 1650, 1560, 1275, 1180 and 1065 cm^{-1}. Analysis: Calculated for $C_{15}H_{20}N_2O_6$: C, 55.56; H, 6.17; N, 8.64. Found: C, 55.43; H, 6.21; N, 9.00.

Inactivations of Papain With N-Ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline - All inactivations were carried out at 25°, using either mercuripapain or freshly activated, activator-free papain.

Inactivation reaction mixtures were made up in 10 mM KCl to contain 0.1 mM enzyme, 25% acetonitrile, and 12.2 mM N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (122 fold molar excess), in the presence or absence of the desired added reagents. These added reagents, when present, had the following initial concentrations in the reaction mixture: glycine methyl ester, 1.0 M; and $\left[^{14}C_1\right]$-glycine ethyl ester, 0.5 M. The initial pH was adjusted to 4.6, 6.5, or 7.5 and maintained at that value throughout the course of inactivation. Inactivation was initiated by addition of the enzyme solution to an N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline solution to give the final concentrations shown above. Aliquots of

the reaction mixture were removed at timed intervals for assay of enzyme activity and thiol content.

Incorporation of $[^{14}C_1]$-Glycine Ethyl Ester - N-Ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline inactivations carried out in the presence of $[^{14}C_1]$-glycine ethyl ester (Sp. act. 9μc/m mole) resulted in no irreversible incorporation of radioactivity by papain. To determine irreversibly bound radioactive label associated with the enzyme in a given experiment, the final reaction mixture was dialyzed under nitrogen pressure at 25° against oxygen-free, nitrogen-purged 10 mM KCl, on an Amicon Micro Ultrafiltration System Model 8MC using an Amicon PM-10 Diaflo membrane. When the dialysis eluate no longer contained any radioactivity, the dialysate was concentrated to approximately 1 ml and its protein concentration was determined spectrophotometrically at 278 nm. Aliquots of this dialysate were then assayed with α-N-benzoyl-L-arginine ethyl ester for residual enzyme activity and then counted in duplicate in Bray's solution.(138) The stoichiometry of label incorporation was determined from the specific activity of the $[^{14}C_1]$-glycine ethyl ester employed, as determined by counting aliquots of standard solutions of the labeled ester.

Inactivations of Papain With 1-Ethyl-3-(3-dimethylaminopropyl) Carbodiimide - All inactivations were carried out at pH 4.75, 25° using either mercuripapain or freshly activated, activator-free papain. Inactivation reaction mixtures were made up in 10 mM KCl to contain 0.103 mM enzyme in the presence or absence of the desired added reagents. These added reagents, when present, had the following initial concentrations in the reaction mixture after addition of 1-ethyl-3-(3-dimethyl-

aminopropyl)carbodiimide: glycine ethyl ester, 0.5 M; triethylamine, 0.5 M; benzamidoacetonitrile, 18.7 mM (when this competitive inhibitor was present, the reaction mixture was also 1% v:v in acetonitrile); $[^{14}C]$-glycine ethyl ester, 0.5 M (in experiments involving labeled glycine ethyl ester, the enzyme concentration was only 0.061 mM). The initial pH was adjusted to 4.75 and maintained at that value by adding 0.2 N HCl throughout the course of inactivation. Inactivation was initiated by addition of the appropriate amount of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, either as the pure crystals, or as an aqueous solution in 10 mM KCl at pH 4.75. In some experiments a second addition of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide was made after the reaction had been allowed to proceed for 60 minutes. Aliquots of the reaction mixture were removed at timed intervals for assays of enzyme activity. After inactivation, the reaction mixtures were dialyzed for 20 hours against 10 mM KCl before a final assay for enzymatic activity and free thiol content.

For the experiments involving mercuripapain reacting with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide for long times a reaction mixture described above was used. At 60, 90, 120, and 180 minutes, 0.75 ml aliquots of the reaction were removed, quenched with 2 volumes of 1 M acetic acid buffer at pH 4.75 and placed on a G-25 (medium) Sephadex column (1 x 37 cm). The protein was eluted with 10 mM KCl and the protein fraction came out at 15-18 ml. This fraction was concentrated on the Amicon and the resulting protein concentration was read at 278 nm. The protein was then assayed with α-N-benzoyl-L-arginine ethyl

ester as described above to determine the total amount of active protein in the reaction mixture.

Incorporation of [^{14}C]-Glycine Ethyl Ester - 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide inactivations carried out in the presence of labeled glycine ethyl ester (Sp. act. 9 μc/m mole) resulted in the irreversible incorporation of radioactivity by papain. The stoichiometry of incorporation of label into the enzyme in a given experiment was determined as described above for N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline. In experiments involving the competitive inhibitor, benzamidoacetonitrile, the dialysis step was preceeded by a 1:1 dilution of the final inactivation reaction mixture with 1 M acetic acid buffer, pH 4.75, to destroy any remaining 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide.

Assay of 1-Ethyl-3-(3-dimethylaminopropyl) Carbodiimide in Solution - The concentration of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide in aqueous solution can be determined spectrophotometrically, in the absence of interfering chromophoric solution components at 212 nm as described by Ozawa.[56] This method cannot be employed for solutions containing high protein concentrations. Two methods were used for the estimation of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide concentrations in solutions containing mercuripapain. Ultrafiltration of an aliquot of such a solution on the Amicon unit described above gave an aliquot of eluate which could be analyzed for 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide at 212 nm. Alternately, an aliquot of active, activator-free papain could be added and the resulting mixture assayed for papain activity in the absence of cysteine. The decrease of α-N-

benzoyl-L-arginine ethyl ester activity with time under these conditions served to provide a rough estimate of the 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide concentration present in the solution.

Modification and Regeneration of Tyrosine Residues in Papain - Inactivations of papain with concomitant modification of tyrosine residues were carried out as described above with 0.103 mM enzyme, 123.6 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, and 0.5 M glycine ethyl ester, all in 10 mM KCl. In these experiments a second addition of the same amount of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, was made at 60 minutes. A control, identical in every respect to the reaction mixture without 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide was assayed periodically during the inactivation. After 90 minutes both the reaction mixture and the control were dialyzed against 5 x 500 ml portions of oxygen-free, nitrogen-purged, 10 mM KCl for 20 hours at 4°. After dialysis, these two solutions were assayed for enzymatic activity and the protein concentration was determined spectrophotometrically at 278 nm. Then one-half of each the reaction and control solutions were treated with 0.59 ml of a 2.19 M hydroxylamine solution at pH 7.0. The final hydroxylamine concentration was 0.5 M. The reaction and control solutions with hydroxylamine were allowed to stand for 5 hours at 25°. The other half of the above solutions were prepared for amino acid analysis. After 5 hours the reaction and control solutions were again dialyzed as described above, assayed for enzymatic activity, and the protein concentration was determined spectrophotometrically at 278 nm. Each of these solutions was then also prepared for amino acid analysis.

Amino Acid Analysis - The amino acid composition of hydrolyzed samples of native or modified papain were determined using a Beckman Model 121 Automatic Amino Acid Analyzer equipped with a Beckman System AA Computing Integrator, using the method of Spackman, Stein and Moore[139] as outlined in the Model 121 Instruction Manual.[140] Lyophilized samples containing approximately 1 mg of either unmodified or modified papain were hydrolyzed with constant boiling hydrochloric acid (Pierce Chemical Company, Sequanal Grade) in evacuated, sealed tubes for 24, 48, or 72 hours. These samples were then lyophilized to remove HCl and sent to the Biochemistry and Nutrition Department of Virginia Polytechnic Institute and State University for amino acid analysis. Values obtained for threonine or serine were extrapolated to zero hydrolysis time to correct for destruction of these amino acids.

Attempted Reaction of N-t-Butoxycarbonyl-L-histidine Methyl Ester With 1-Ethyl-3-(3-dimethylaminopropyl) Carbodiimide or 1-Cyclohexyl-3-(2-morpholinoethyl) Carbodiimide in Aqueous Solution - Into a 50 ml Erlenmeyer flask equipped with a magnetic stirrer were placed 1.9 m mole of N-t-butoxycarbonyl-L-histidine methyl ester and 10 ml of water. The pH of this solution was 8.5. To this solution was added 1.9 m moles of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride or 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-p-toluene sulfonate. The reaction was maintained at pH 8.5 with 0.2 N NaOH. After 8 hours with stirring at room temperature, the reaction was extracted with chloroform. The chloroform extract was dried with anhydrous sodium sulfate and the chloroform was stripped off *in vacuo* at room temperature, to give a light yellow oil. The oil was again taken up in chloroform and care-

fully precipitated with ethyl acetate. Crystallization occurred overnight in the freezer to give 1.7 m moles (1.8 m moles in the reaction with 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide) of the starting material N-t-butoxycarbonyl-L-histidine methyl ester, or essentially quantitative recovery in both cases.

Attempted Reaction of N-t-Butoxycarbonyl-L-histidine Methyl Ester With 1-Ethyl-3-(3-dimethylaminopropyl) Carbodiimide in Methylene Chloride - Into a 100 ml round bottom flask equipped with a magnetic stirrer, reflux condenser, and drying tube were placed 1.9 m moles of N-t-butoxycarbonyl-L-histidine methyl ester, 1.9 m moles of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride, and 10 ml of anhydrous methylene chloride. The reaction was refluxed for 24 hours and then allowed to stand at room temperature for 14 days. After 2 weeks the methylene chloride was stripped off in vacuo at room temperature and 10 ml of water was added. This aqueous solution was then quickly extracted with chloroform, and recrystallized as described above to give 1.8 m moles of N-t-butoxycarbonyl-L-histidine methyl ester or essentially quantitative recovery of the starting material.

Reaction of Papain With Diazoacetamide - Reaction mixtures, thermostatted at 10° at pH 5.0 were made up in water to contain 0.12 mM mercuripapain, in addition 1 drop of 1-octanol was added to reduce foaming. The initial pH was adjusted to 5.0 and maintained at that value throughout the course of the reaction with 0.05 N $HClO_4$. The reaction was initiated by addition of 23 mg of diazoacetamide (362-fold molar excess). Aliquots of the reaction mixture were removed at timed intervals for assay of enzyme activity. After 24 hours reaction time

the enzymatic activity of the control and reaction mixtures were identical.

Reaction of Papain With Triethyloxonium Tetrafluoroborate - Reaction mixtures, thermostatted at 25° at pH 5.0 were made up in 32 mM KCl to contain 0.1 mM mercuripapain. The initial pH was adjusted to 5.0 and maintained at that value throughout the course of the reaction with 0.1 N NaOH. The reaction was initiated by addition of 20 µl of a 0.5 M solution of triethyloxonium tetrafluoroborate in acetonitrile (133-fold molar excess). The final acetonitrile concentration was 0.8%. Aliquots of the reaction mixture were removed at timed intervals for assay for enzyme activity. After 60 minutes reaction time the enzymatic activity of the reaction and control mixtures were identical.

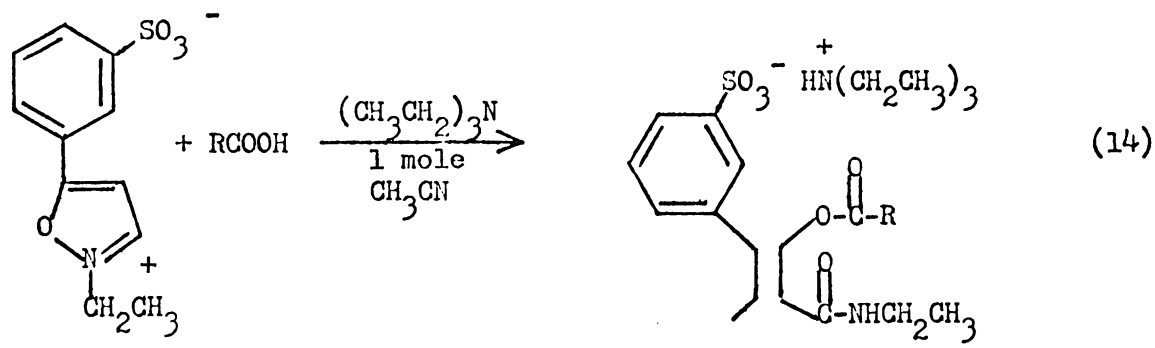
Reaction of Papain With N-Ethyl-5-phenylisoxazolium-3'-sulfonate - Reaction mixtures thermostatted at 25°, at pH 4.5 were made up in water to contain 0.11 mM mercuripapain. The initial pH was adjusted to 4.5 and maintained at that value throughout the course of the reaction with 0.1 N NaOH. The reaction was initiated by addition of 25 µl of a 0.474 M solution of N-ethyl-5-phenylisoxazolium-3'-sulfonate in 1 mM HCl (100-fold molar excess). Aliquots of the reaction mixture were removed at timed intervals for assay of enzyme activity. Reaction times up to 20 minutes showed no loss of enzymatic activity due to N-ethyl-5-phenylisoxazolium-3'-sulfonate. However, after 20 minutes the reaction solution became very milky, due presumably to extensive enzyme denaturation.

RESULTS AND DISCUSSION

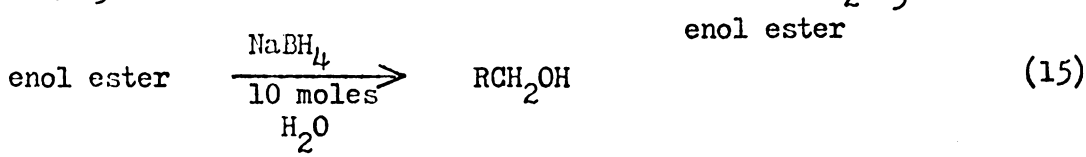
Reduction of Carboxylic Acids to Carbinols

Woodward and co-workers have shown that certain 3-unsubstituted isoxazolium salts react with carboxylic acids to form enol esters in good yields.[26] Woodward *et al.* also found that enol esters were very potent acylating agents. This fact presented the possibility that perhaps the borohydride anion would be a strong enough nucleophile to displace the enol leaving group and allow the reduction of carboxyl groups to carbinols.

The enol ester derivatives of certain representative carboxylic acids were prepared by treating the acids with an equivalent each of NEPIS and triethylamine in acetonitrile at 25°. These enol ester derivatives were then reduced with a ten-fold molar excess of sodium borohydride in water. The overall yields of the two-step reduction procedure shown in Equations 14 and 15 are given in Table IV.



(14)



(15)

TABLE IV

CONVERSION OF CARBOXYLIC ACIDS TO CARBINOLS BY NEPIS ACTIVATION AND SODIUM BOROHYDRIDE REDUCTION

Acid	Carbinol	Method[a]	Overall Yield (%)
CH_3COOH	CH_3CH_2OH	A	90[b]
C_6H_5COOH	$C_6H_5CH_2OH$	A	94[b]
$CH_3CH_2CH_2COOH$	$CH_3CH_2CH_2CH_2OH$	A	100[b]
$CH_3(CH_2)_{15}COOH$	$CH_3(CH_2)_{15}CH_2OH$	B	50[c]
$CH_2=CH-COOH$	$CH_3CH_2CH_2OH$	A	100[b]
$HS-CH_2COOH$	$HS-CH_2CH_2OH$	A	80[b]
$C_2H_5O_2C(CH_2)_4COOH$	$C_2H_5O_2C(CH_2)_5OH$	B	71[d]
$(C_6H_5)_2CHCOOH$	$(C_6H_5)_2CHCH_2OH$[e]	B	40[f]

[a] See Experimental Procedure for a detailed description of the methods employed.

[b] Identification and quantitation by comparison of glpc chromatograms of product mixtures with those of standard solutions of authentic carbinols.

[c] Isolated yield, m.p. 48-49° (Lit. m.p. 49°.[141]). Micelle formation made extraction of this carbinol from the aqueous product mixture extremely inefficient and may account in part for the low isolated yield.

[d] Isolated yield, n_D^{24} 1.4276 (Lit. n_D^{24} 1.4275.[142])

[e] Accompanied by N-ethyl-α,α-diphenylacetamide (35% yield) identified by ir, nmr, mass spectrum (m/e 237) and m.p. 134-135° (Lit. m.p. 134.5-135.5°.[143])

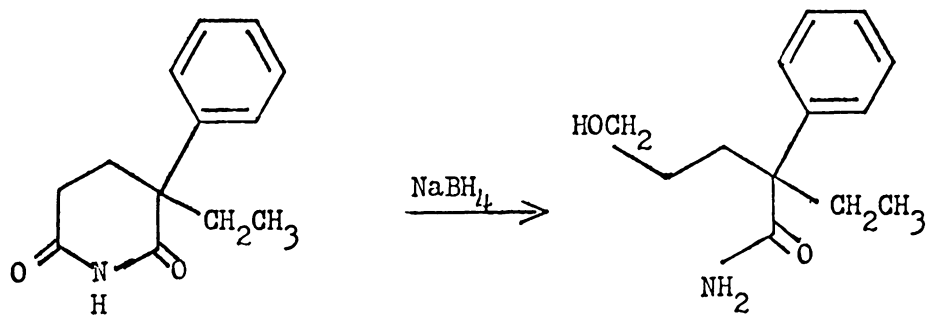
[f] Isolated oil had identical glpc retention time and TLC R_F value as authentic 2,2-diphenylethanol (m.p. 58-59°; Lit. m.p. 59°[136]) prepared from diphenylacetic acid by the method of Yoon et al.[135]

As can be seen in Table IV both aliphatic and aromatic carboxylic acids were reduced by treatment with NEPIS and $NaBH_4$ in overall yields of 40-100% depending on the particular acid used. The ethyl ester function of monoethyl adipate was not reduced or hydrolyzed during the reduction procedure and ethyl-6-hydroxyhexanoate was isolated in a 71% yield. Furthermore, treatment of ethyl heptanoate with a ten-fold molar excess of $NaBH_4$ under the same conditions employed during NEPIS enol ester reductions resulted in a 96% recovery of unreduced ethyl heptanoate, thus confirming that simple carboxylic acid esters are not reduced by sodium borohydride under the conditions employed.

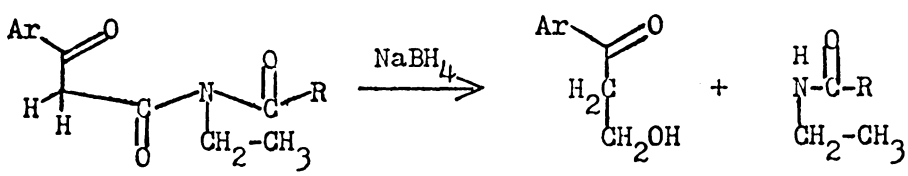
The reduction of 2,2-diphenylacetic acid with NEPIS and $NaBH_4$ resulted in the expected 2,2-diphenylethanol, but in only 40% yield. A comparable quantity of N-ethyl-α, α_1-diphenylacetamide was isolated from the reaction mixture, suggesting that the reduction of some of these enol esters derived from NEPIS may not be as straight-forward as originally envisioned. Indeed, Woodward *et al.*,[26] have shown that these particular enol esters are subject to isomerization *via* an O to N acyl shift, producing the corresponding imide. The isomerization is accelerated by bases,[26] and, under the alkaline conditions which accompany aqueous $NaBH_4$ (pH 9.0-10.0), imide formation may be quite rapid. Indeed this has been shown to be the case by King.[30] Thus isomerization would be expected to compete to some extent with direct reduction of the enol ester by $NaBH_4$, especially when borohydride attack is sterically hindered, as would be the case for the NEPIS enol ester of 2,2-diphenylacetic acid. Also, the imide product of base-catalyzed

isomerization appears to be subject to reduction by aqueous sodium borohydride, as is shown by the isolation of N-ethyl-α,α_1-diphenylacetamide.

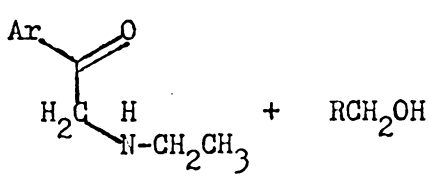
Reductions of cyclic imides by $NaBH_4$ have been reported,[144-147] with borohydride attack directed preferentially to the less hindered carbonyl group, giving rise to an amide and a carbinol as shown in Equation 16 for a cyclic imide[145] and in Equation 17 for the imide derived from a NEPIS enol ester.



(16)

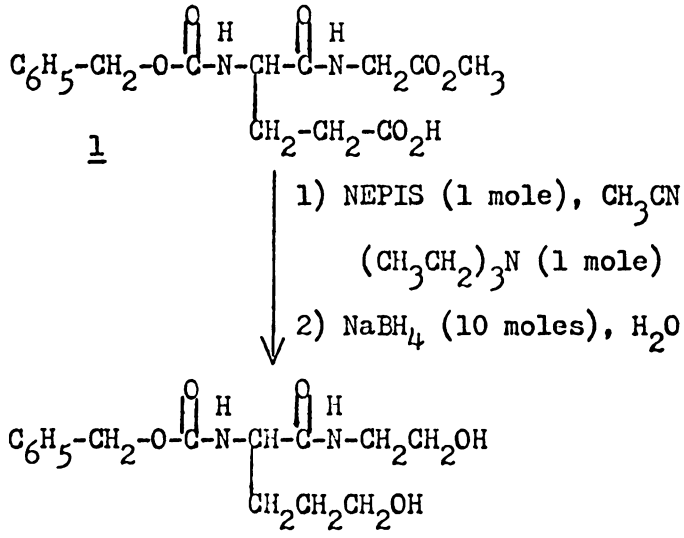


(17)



In order to test the applicability of this reduction procedure to free carboxyl groups in peptides or proteins, the peptide derivative, O-methyl-N-benzyloxycarbonyl-α-L-glutamyl glycinate, 1, was prepared as

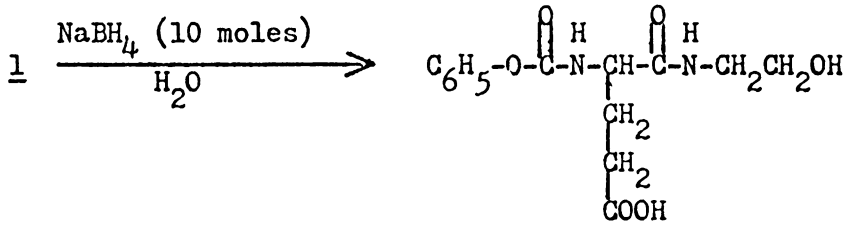
a model substrate, according to the procedure of Le Quesne and Young[137] for the corresponding ethyl ester. The two-step reduction procedure (see Experimental Procedure, Method A) gave the diol derivative N-benzyloxycarbonyl-α-amino-δ-hydroxyvaleryl ethanolamide, 2, in 50% yield.



(18)

The free γ-carboxyl function of the glutamyl residue was reduced as expected. The concomitant reduction of the carboxyl-terminal methyl ester linkage of 1 finds ample precedent in the work of Yonemitsu et al.,[148,149] in which it was determined that carboxylic esters having an α-amino function are susceptible to reduction by a 10-fold molar excess of aqueous sodium borohydride. This fact was confirmed by the finding that a direct reduction of 1 with a 10-fold molar excess of $NaBH_4$ in H_2O, under the conditions employed to effect the second step in the production of 2, afforded a clean selective reduction of the terminal methyl ester group of 1 to yield N-α-benzyloxycarbonyl-

glutamyl ethanolamide, 3, in 75% yield. This treatment was without effect on the free carboxyl group, the peptide linkage or the benzyloxycarbonyl moiety of 1.



(19)

3

In order to determine the ideal reaction conditions for the reduction of the enol esters derived from NEPIS to their corresponding carbinols, a kinetic experiment in which the formation of 1-butanol from O-butyryl-N-ethylcinnamamide-3'-sulfonate was followed with time upon treatment of this enol ester with a ten-fold molar excess of $NaBH_4$ in water. As shown in Figure 1, the reduction of the enol ester was essentially complete after 90 minutes reduction time, and the yield of 1-butanol did not increase appreciably even after 120 minutes. Subsequent data however,[30] showed that the rearrangement of the enol esters derived from NEPIS is quite fast thus indicating that the reaction rate shown in Figure 1 may be that of the reduction of the corresponding imide of the NEPIS enol ester.

To determine if the 10-fold molar excess of $NaBH_4$ was required for high yields of carbinol, O-butyryl-N-ethylcinnamamide-3'-sulfonate was treated with 2- to 14-fold molar excesses of $NaBH_4$ under the usual reaction conditions (see Experimental Procedure), and the yields of 1-butanol were determined in each case. The results of these studies are shown in

FIGURE 1

Reduction of O-Butyryl-N-ethylcinnamamide-3'-sulfonate with Sodium Borohydride in Water

O-Butyryl-N-ethylcinnamide-3'-sulfonate concentration was 1 mM. Sodium borohydride concentration was 10 mM. Formation of 1-butanol was followed by glpc (see Experimental Procedure).

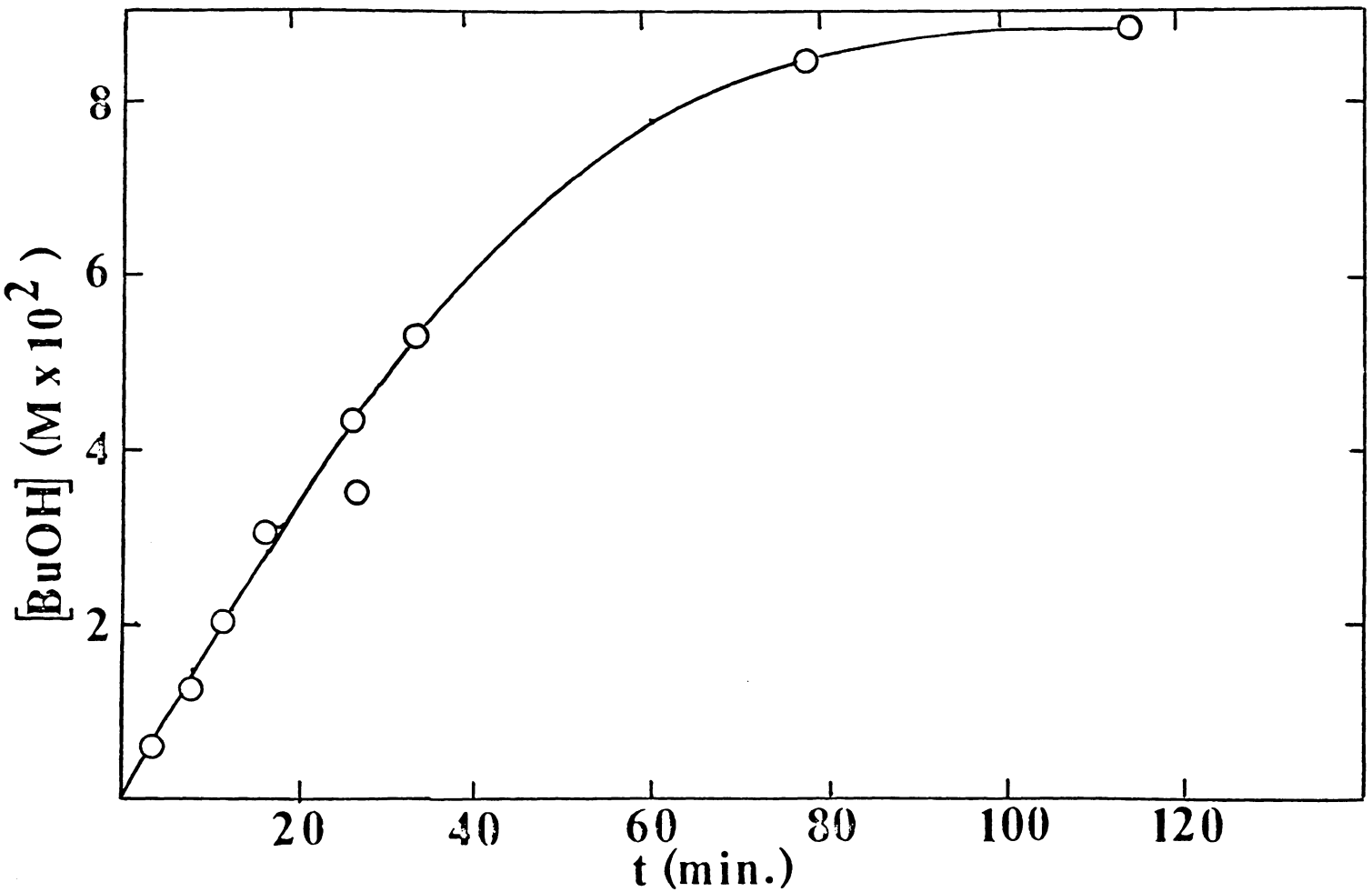

[BuOH] (M x 10^2)
8
6
4
2
20
40
60
80
100
120
t (min.)

Figure 2. The maximum yield of 1-butanol was achieved with a 10-fold molar excess of sodium borohydride, and a 12- or 14-fold molar excess of this reagent did not increase the yield of 1-butanol. The large molar excesses of $NaBH_4$ required for complete reduction may arise because the concentration of $NaBH_4$ must be high enough to compete with water as the nucleophile and thereby effect reduction rather than hydrolysis of the enol ester.

Since this reduction procedure would hopefully be applicable to the reduction of free carboxyl groups in proteins and enzymes in aqueous solution, experiments were performed where both the initial enol ester formation (treatment of 1-butanoic acid with NEPIS) and the subsequent reduction with sodium borohydride were carried out in water, using 1- to 4-fold molar excesses of NEPIS and a 10-fold molar excess of $NaBH_4$.

$$\underset{}{\text{NEPIS}} + \underset{1\ \text{mmole}}{\text{1-butanoic acid}} + \underset{1\ \text{mmole}}{N(CH_2\text{-}CH_3)_3} \xrightarrow[90\ \text{minutes}]{H_2O,\ pH\ 5} \text{Enol Ester} \qquad (20)$$

$$\underset{(\text{not isolated})}{\text{Enol Ester}} \xrightarrow{NaBH_4\ (10\ \text{mmoles})} \text{1-butanol} \qquad (21)$$

Table V shows that even with a 4-fold molar excess of NEPIS over the acid the yield of 1-butanol after reduction was only 55%, suggesting that enol ester formation is not as effective in water as in acetonitrile. However, as the excess of NEPIS is increased, the yield of 1-butanol increases after reduction. This fact is important, since, in the case of proteins and enzymes, molar excesses of 100-fold or greater can be used without requiring large amounts of NEPIS. Therefore, it is not unreasonable to anticipate that with these large molar excesses of

FIGURE 2

Reduction of O-Butyryl-N-ethylcinnamamide-3'-sulfonate with 2- to 14-Fold Molar Excesses of Aqueous Sodium Borohydride

The concentration of O-butyryl-N-ethylcinnamamide-3'-sulfonate was 1 mM. The concentrations of sodium borohydride were 2.0, 4.0, 6.0, 8.0, 10.0, 12.0, and 14.0 mM, respectively. The yield of 1-butanol was determined by glpc (see Experimental Procedure).

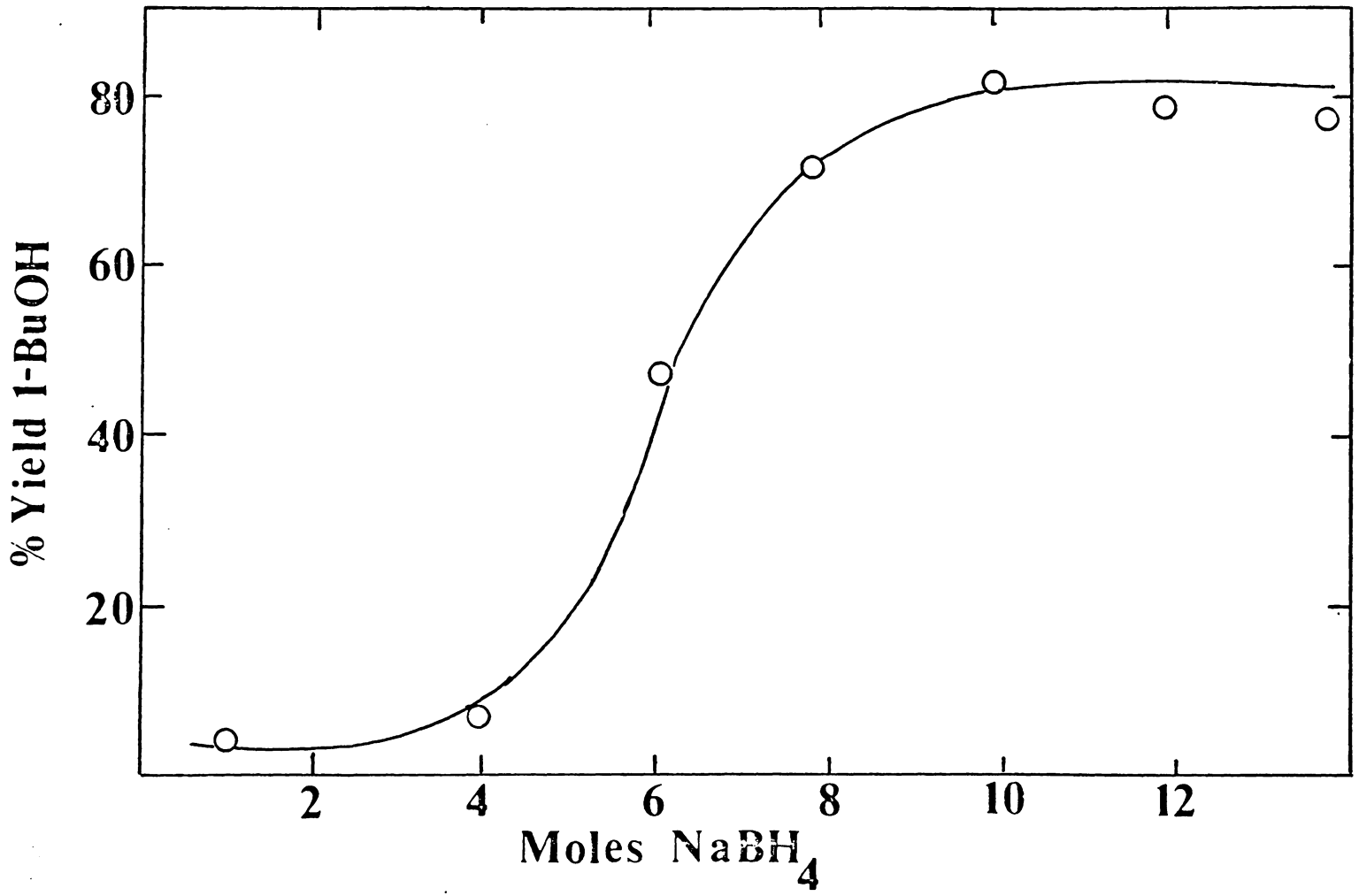

% Yield 1-BuOH
80
60
40
20
2
4
6
8
10
12
Moles NaBH4

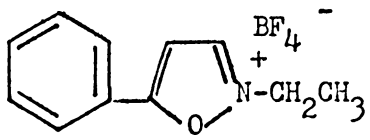
TABLE V

ACTIVATION OF 1-BUTANOIC ACID AND REDUCTION OF O-BUTYRYL-N-ETHYLCINNAMAMIDE IN WATER

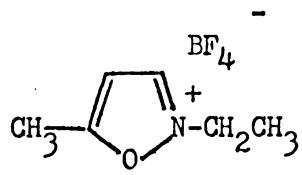
mMoles NEPIS	Overall Yield of 1-Butanol %
1	29
2	48
4	55

NEPIS, all of the accessible free carboxyl groups of a protein or enzyme could be derivatized using NEPIS, and subsequently reduced with sodium borohydride.

NEPIS is rather difficult to prepare in the laboratory, and although it is commercially available, it is costly. With these drawbacks in mind, other isoxazolium salts were synthesized in an attempt to find one salt which circumvented these difficulties and still formed enol esters which could easily be reduced by $NaBH_4$. The two other isoxazolium salts which were prepared according to published procedures were[26,134] N-ethyl-5-phenylisoxazolium tetrafluoroborate (NEPIT) and N-ethyl-5-methylisoxazolium tetrafluoroborate (NEMIT). Each of these salts was treated with a 10-fold molar excess of sodium borohydride. The results of these experiments can be seen in Table VI. When the 5-methylisoxazolium salt was reacted with 1-butanoic acid, the enol ester was isolated in only a 31% yield. Reduction of this enol ester in aqueous $NaBH_4$ provided only a 6% yield of 1-butanol based on the amount of enol ester treated with $NaBH_4$ in each case. This clearly was not acceptable as a synthetic technique.



N-Ethyl-5-phenylisoxazolium tetrafluoroborate (NEPIT)



N-Ethyl-5-methylisoxazolium tetrafluoroborate (NEMIT)

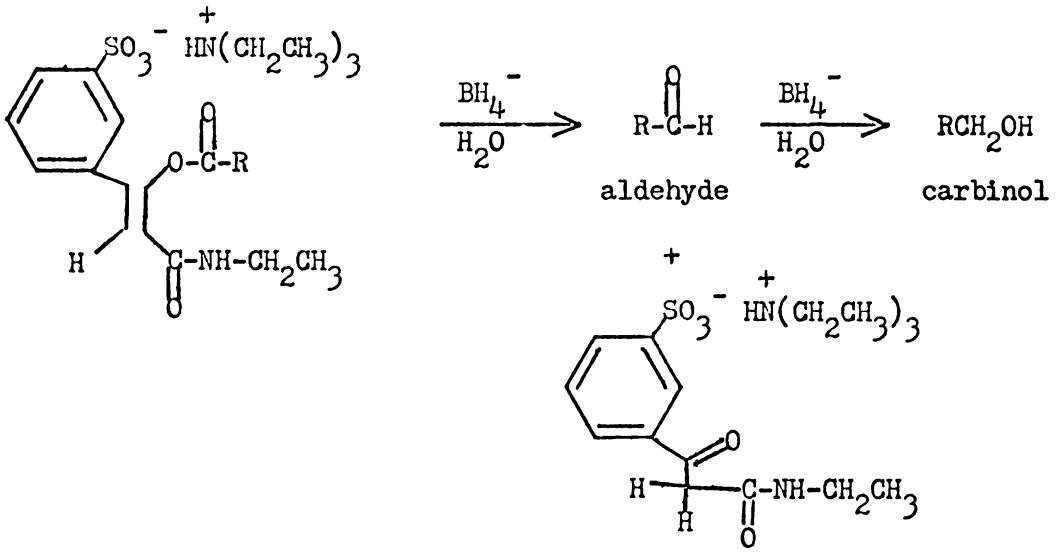
TABLE VI

SYNTHESIS AND REDUCTION OF 1-BUTANOIC ACID ENOL ESTERS OF N-ETHYL-5-PHENYLISOXAZOLIUM TETRAFLUOROBORATE AND N-ETHYL-5-METHYLISOXAZOLIUM TETRAFLUOROBORATE

Reagent	Yield of Isolated Enol Ester (%)	Reducing Conditions	% Yield of 1-Butanol
NEMIT	31 m.p. 114-115° (prepared in CH_3CN)	10:1 aq $NaBH_4$	6
NEPIT	70 m.p. 104-105° (prepared in H_2O)	10:1 aq $NaBH_4$	trace
NEPIT	--	8:1 $NaBH_4$ (50% aq CH_3CN)	10
NEPIT	--	10:1 $NaBH_4$ (50% aq CH_3CN)	8
NEPIT	--	12:1 $NaBH_4$ (50% aq CH_3CN)	13

5-Phenylisoxazolium tetrafluoroborate, on the other hand, produced a 70% yield of O-butyryl-N-ethylcinnamamide when reacted with 1-butanoic acid. While this yield was not as high as the yields of enol esters obtained with NEPIS, the 5-phenylisoxazolium salt is much easier to prepare in the laboratory and therefore an excess of the salt could be used to achieve higher yields of enol ester in a synthetic procedure, without extensive effort in preparing additional salt. However, reduction of O-butyryl-N-ethylcinnamamide with a 10-fold molar excess of aqueous $NaBH_4$ produced only a trace of 1-butanol. In addition, if the reductions were performed in 50% aqueous acetonitrile, in which the enol ester was more soluble, the yields of 1-butanol after reduction still remained low (10%) even if a 12-fold molar excess of $NaBH_4$ was used. Thus it would appear that the sulfonic acid moiety at the 3' position of the 5-phenyl group of NEPIS exerts some effect which allows the reduction of its enol esters to occur readily. This effect is probably not one of solubility since when NEPIT enol esters were completely dissolved in 50% aqueous acetonitrile the yields of 1-butanol after reduction were still very low.

Finally, the probable pathway for the reduction of NEPIS enol esters can be envisioned as shown in Equation 22. The initial step of the reaction involves production of the aldehyde and a resonance stabilized enolate anion, followed by a rapid reduction of the aldehyde to the corresponding carbinol. The β-ketoamide by-product should also be reduced by the sodium borohydride to form the corresponding β-hydroxyamide.



β-ketoamide

To summarize, evidence has been provided that NEPIS enol esters (or their corresponding imides) can be reduced by aqueous sodium borohydride to yield carbinols in reasonable yields, under relatively mild conditions. The reduction procedure involves treatment of the acid with NEPIS to form an enol ester which is then reduced with a 10-fold molar excess of aqueous sodium borohydride. This procedure has been applied to a variety of simple aromatic and aliphatic carboxylic acids with success, and, significantly, to the reduction of the γ carboxyl group of O-methyl-N-benzyloxycarbonylglutamyl glycinate.

Modification of Carboxyl Groups in Papain Using Carboxyl Group "Specific" Reagents

The goal of this phase of the research was to determine whether a carboxylate side chain in the active site of the enzyme papain was necessary for enzyme activity. Initially, a survey of the reaction

with papain of five reagents, each of which had been used previously to chemically modify carboxyl groups in enzymes, was undertaken. The general results of this survey are given in Table VII. In all cases, the conditions used were patterned after published procedures which had led to successful enzyme modifications. The five carboxyl group reagents were diazoacetamide,[7] triethyloxonium tetrafluoroborate (Meerwein's Reagent),[17] N-ethyl-5-phenylisoxazolium-3'-sulfonate (NEPIS),[26] N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ),[37] and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC).[56]

As can be seen in Table VII, when mercuripapain (see Historical section) was treated with a 300-fold molar excess of diazoacetamide for a period of 24 hours, no loss of enzymatic activity was observed, and in fact there was no evidence that any chemical modification of the enzyme had occurred at all. Thus it seems likely that the reagent was either not stable enough or was not reactive enough to modify carboxyl groups in the enzyme. It has in fact been shown that diazoacetamide is rapidly hydrolyzed in aqueous solution,[7] and therefore may have been destroyed before it could react with any free carboxyl groups in the enzyme.

Similarly, when triethyloxonium tetrafluoroborate (Meerwein's Reagent) was reacted with active or mercuripapain, no inactivation of either enzyme form was observed. Here again, one suspects that the instability of Meerwein's Reagent is the problem in these experiments. The failure to inactivate active papain, with its free unprotected thiol group, is especially revealing since it has been shown that Meerwein's Reagent reacts quite readily with thiol groups.[19] The free thiol

TABLE VII

REACTION OF PAPAIN WITH VARIOUS CARBOXYL GROUP "SPECIFIC" REAGENTS

Enzyme Form	Reagent 25°	pH	Inactivation	Probable Group(s) Modified
Mercuripapain	EDC[a]	4.75	Yes	tyrosyl, carboxyl
Active Papain	EDC	4.75	Yes	thiol, carboxyl, tyrosyl
		4.75[a]	Yes	thiol, carboxyl, tyrosyl
Papain-S-SO_3^-	EDC	4.75	Yes	tyrosyl, carboxyl
Mercuripapain	EEDQ	4.50	Yes	thiol
		6.50	Yes	thiol
		7.50	No	-
		7.00[a]	No	-
Active Papain	EEDQ	4.50	Yes	thiol
		6.70	Yes	thiol
		7.50	No	-
Mercuripapain	Diazoacetamide (10°)	5.00	No	-
Mercuripapain	$(Et)_3O^+BF_4^-$	5.00	No	-
Active Papain	$(Et)_3O^+BF_4^-$	5.00	No	-
Mercuripapain	NEPIS[b]	4.50	No	-

[a] Reaction carried out in the presence of 0.5 M glycine ethyl ester.

[b] No inactivation up to 20 minutes, then precipitation of the enzyme occurred.

group of active papain is an excellent nucleophile, and some inactivation of active papain should have been observed if the reagent remained unhydrolyzed in solution for any finite length of time. No inactivation of active papain was observed upon treatment with a 300-fold molar excess of Meerwein's Reagent. It has also been reported that Meerwein's Reagent hydrolyzes quite rapidly at room temperature at pH values around 6,[19] further evidence for the instability of this reagent.

Mercuripapain was treated with NEPIS under essentially the same conditions reported in the literature[31] for modification of carboxyl groups in carboxypeptidase A. No inactivation of the enzyme was observed within 20 minutes. However, at reaction times longer than 20 minutes extensive denaturation and precipitation of mercuripapain was observed. This was probably due either to intra- or intermolecular acylation of nucleophilic side chain moieties in the enzyme. An example of this would be acylation of the imidazole moiety of His-159 by the enol ester of Asp-158 in the active site of papain. This denaturation of the enzyme might also be caused by the rearrangement of the enol esters to the corresponding imides. Of these possibilities the intramolecular acylation of nucleophilic side chains seems most plausible, since this type of intrachain crosslinking would cause severe strain on the tertiary structure of papain.

When active or mercuripapain was treated with EEDQ, a pH-dependent inactivation of both enzyme forms resulted. The chemical modification of papain by EEDQ will be discussed in greater detail below.

Finally, EDC also inactivated either active or mercuripapain at pH 4.75. Again, this reaction of EDC with papain will be discussed in more detail on page 71.

It should be mentioned here in the context of this discussion that when it is noted that mercuripapain is subject to "inactivation" under stated conditions, the tacit implication is that the mercuri-enzyme is rendered nonactivatable in the usual manner by thiol activator species.

Chemical Modification of Papain with N-Ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ)

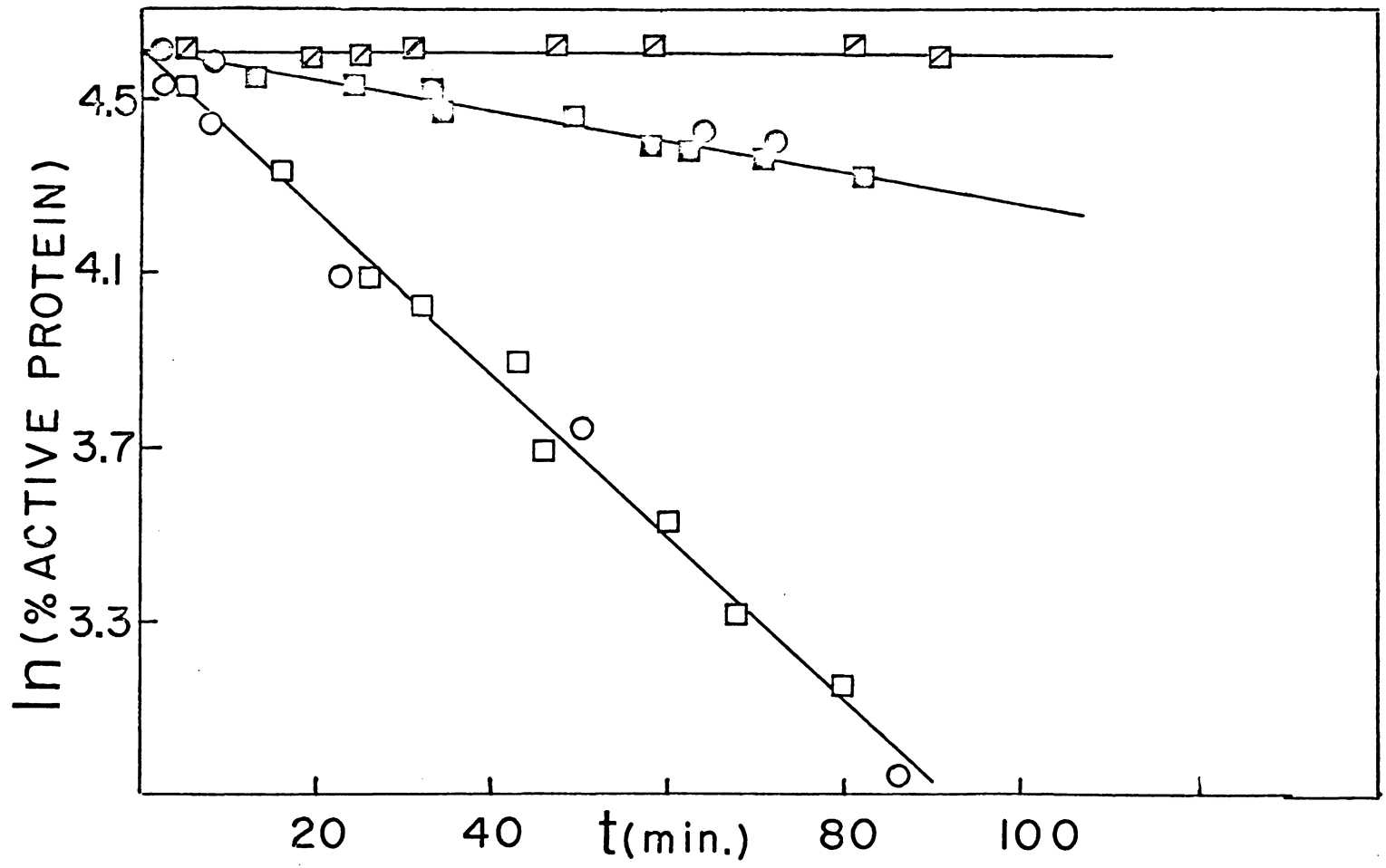
When active papain was treated with EEDQ at various pH values, pseudo-first-order inactivation rates were observed, as is illustrated in Figure 3. EEDQ has been shown to react with carboxyl groups in the serine hydrolyses, causing inactivation of these enzymes.(38) In this study, inactivation of papain was also observed. However, this inactivation appears to be due to the modification of the essential sulfhydryl group of the enzyme rather than carboxyl groups in the molecule.

As can be seen in the legend to Figure 3, the square points show the loss of enzyme activity as measured by the titrimetic α-N-benzoyl-L-arginine ethyl ester assay. This assay measures only total enzyme activity and not the chemical nature or state of any catalytically essential groups in the enzyme. The circle points in Figure 3, on the other hand, refer to the determination of the number of moles of essential thiol per mole of papain in the reaction mixture (using a thiol titration technique essentially as reported by Brocklehurst and

FIGURE 3

The Effect of EEDQ on the Rate of Inactivation of Papain at Various pH Values

Final reaction concentrations were, 0.1 mM enzyme, 12.2 mM EEDQ, in 25% acetonitrile. At pH 4.5: BzArgOEt assay (□); thiol titration (○). At pH 6.5: BzArgOEt assay (□); thiol titration (○). At pH 7.5: BzArgOEt assay (▨). Temperature was 25°.


4.5
4.1
3.7
3.3
ln (% ACTIVE PROTEIN)
20
40
t(min.)
80
100

Little;[75] see Experimental Procedure). Fully active papain should have 1 mole of active thiol per mole of protein (see Historical). Figure 3 shows that there is 1:1 correspondence between the loss of overall enzyme activity and loss of active thiol in the enzyme. Thus it would appear that the essential thiol group of papain undergoes a facile reaction with EEDQ at acid pH values, which, as expected, results in a first-order inactivation of the enzyme. Even when mercuripapain is treated with EEDQ at pH 4.5, a similar first-order inactivation with a concomitant irreversible derivatization of the essential thiol is observed (see Figure 4). Therefore, it would appear that the mercuric ion inadequately protects the essential sulfhydryl group of papain against reaction with EEDQ. It should be noted that in all cases above where inactivation of either active or mercuripapain occurred, longer reaction times (at pH 6.5) or further additions of EEDQ (at pH 4.5) could bring the enzyme activity to zero. The further addition of EEDQ was necessary at pH 4.5 because under these acidic conditions EEDQ is destroyed at an appreciable rate by hydrolysis to quinoline, carbon dioxide, and ethanol.[37]

Table VIII gives the pseudo first-order rate constants for the inactivation of active or mercuripapain at different pH values and under various conditions. It appears from the data that EEDQ reacts with the free thiol group of active papain and the protected sulfhydryl group of mercuripapain (see Figures 3 and 4) at pH 4.5 and 6.5 to inactivate the enzyme. At pH 7.5, however, essentially no inactivation of either enzyme form was observed upon treatment with EEDQ.

FIGURE 4

The Effect of EEDQ on the Rate of Inactivation of Mercuripapain at Various pH Values

Final reaction concentrations were, 0.1 mM enzyme, 12.2 mM EEDQ, in 25% acetonitrile. BzArgOEt assay: pH 4.5 (□); pH 6.5 (△); pH 7.5 (in 1M glycine methyl ester (○). Temperature was 25°.

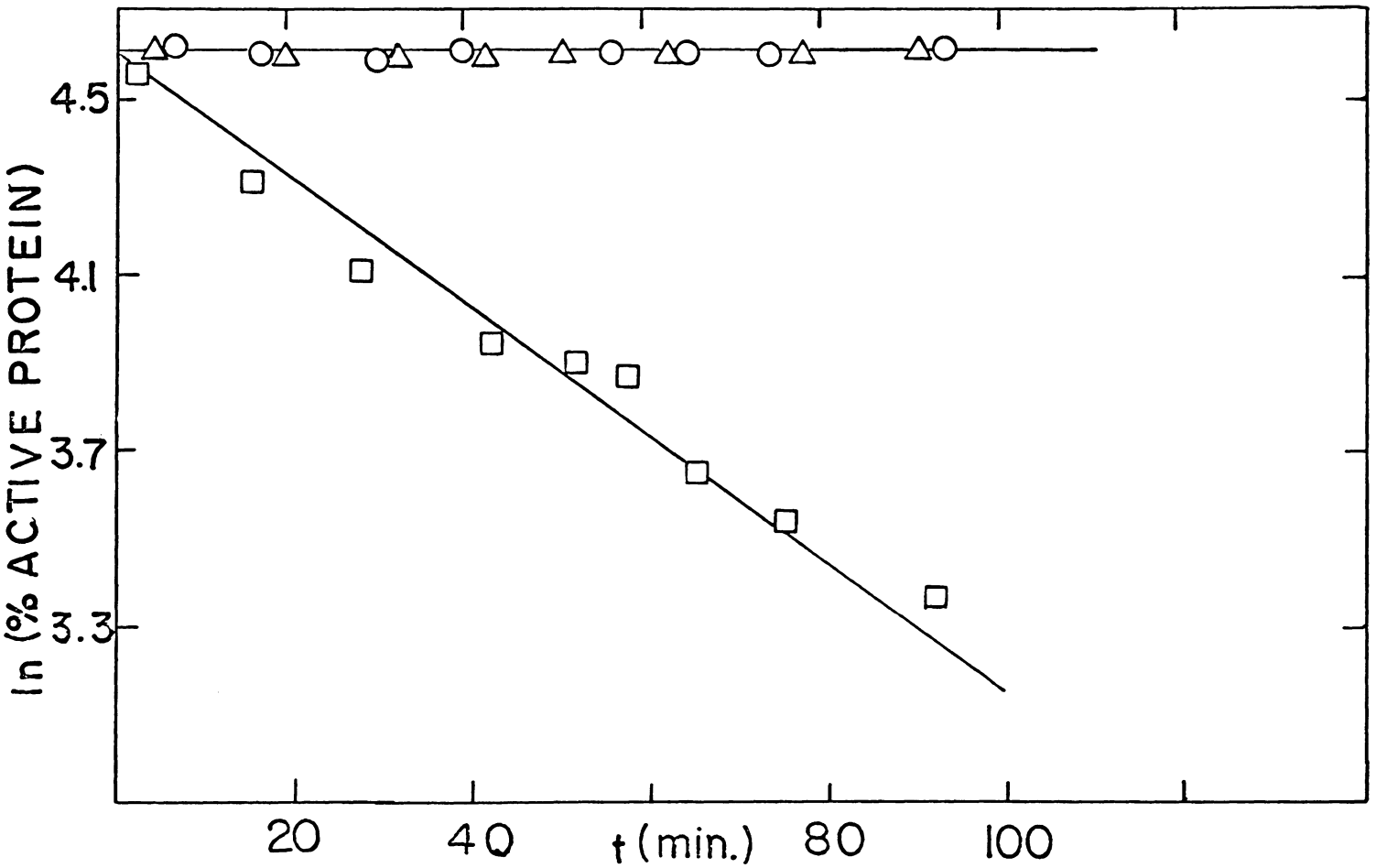

4.5
4.1
3.7
3.3
ln (% ACTIVE PROTEIN)
20
40
t (min.)
80
100

TABLE VIII

OBSERVED RATES OF INACTIVATION OF PAPAIN AND MERCURIPAPAIN BY EEDQ[a]

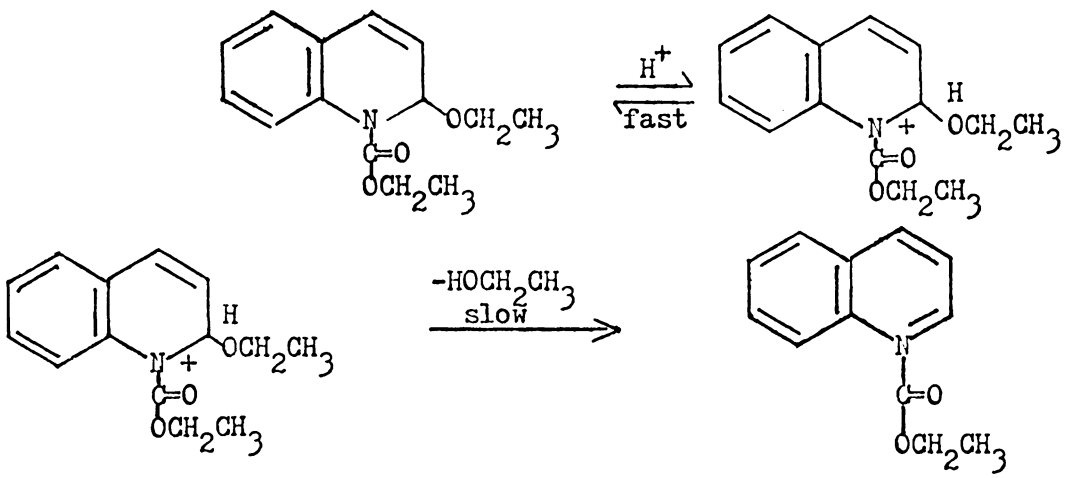
Enzyme	pH	Observed first-order rate $k \times 10^2$ (T=25°)
Activated papain	4.5	1.850 ± 0.085
	6.5	0.510 ± 0.022
	7.5	0.000
Mercuripapain	4.5	1.420 ± 0.120
	6.5	0.139 ± 0.039
	7.5	0.010 ± 0.002
Mercuripapain + glycine methyl ester	6.5	0.125 ± 0.001
	7.5	0.015 ± 0.003[b]
Mercuripapain + $\left[^{14}C_1\right]$-glycine ethyl ester	7.0	0.000[c]

[a] Enzyme concentration was 0.1 mM, EEDQ was 12.2 mM, in 25% acetonitrile.

[b] Glycine methyl ester concentration was 1 M.

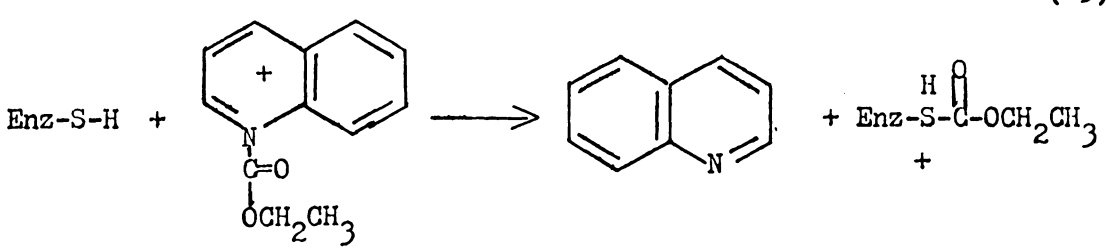
[c] $\left[^{14}C_1\right]$-Glycine ethyl ester concentration was 0.5 M.

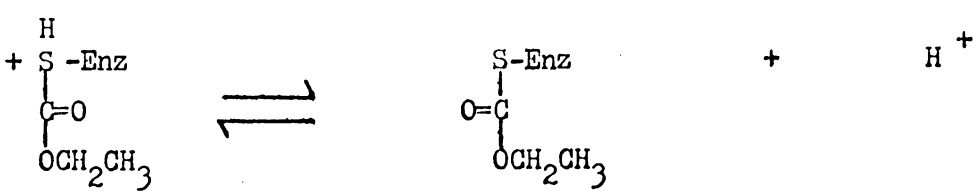
The pH dependence of the reaction of active papain with EEDQ is unexpected in that, as the pH of the reaction approaches the pK_a of the essential sulfhydryl group of Cys-25 (pK_a=8.5,[96]), the observed rates of inactivation of the enzyme decrease. If the reaction pathway involved a simple S_N2 attack of the thiol group at the 2-position of EEDQ, the thiolate anion should be a far better nucleophile than its neutral conjugate acid. One plausible way to account for the observed pH dependence is to invoke acid catalysis as shown in Equation 23:



4

(23)





5

The initial step of the reaction involves rapid and reversible protonation of the 2-ethoxy group followed by the rate determining loss of ethanol to form the intermediate 4 above. Once this intermediate is formed it can then be attacked by the active thiol group of papain in a rapid step to form the enzyme derivative 5. Thus as the pH of the reaction is increased the rate of inactivation of the enzyme by EEDQ would decrease as is observed. Further support for a mechanism of this type is found in the reaction of EEDQ with mercuripapain, which has the same sort of pH dependence observed for the active enzyme. In this case the metallated thiol group could not undergo protonation or deprotonation within the range of pH employed here, but pre-equilibrium protonation of the EEDQ would not be affected. The rates of inactivation at pH 4.5 and 6.5 are smaller for mercuripapain than for active papain as expected due to a decrease in the nucleophilic character of the sulfur atom of the essential thiol group, caused by the presence of the positive, electron withdrawing mercuric ion.

Table VIII also gives the pseudo first-order rate constants of inactivation of mercuripapain by EEDQ in the presence of 1 M glycine methyl ester. Belleau et al.[38] proposed that EEDQ activates carboxyl groups towards attack by a suitable nucleophile, resulting in formation of the appropriate carboxylate derivative. Therefore, if any of the observed loss of catalytic activity from active or mercuripapain were due to carboxyl group modification via formation of a mixed carbonic anhydride intermediate, then addition of an added nucleophile to effect irreversible carboxyl group modification should increase the observed rate of inactivation. That is, in the absence of an added nucleophile,

water will attack the mixed carbonic anhydride intermediate and regenerate free carboxyl groups. In the presence of an added nucleophile, however, irreversible modification should occur to some extent and should increase the rate of enzyme inactivation if a carboxyl group is needed for catalytic activity. The data in Table VIII show that the rates of inactivation of mercuripapain by EEDQ in 1 M glycine methyl ester at pH 6.5 and 7.5 are essentially identical to the rates in the absence of methyl glycinate, indicating either than no carboxyl group modification is occurring or that there are no catalytically essential carboxyl groups in papain. In order to determine which of these two possibilities was more likely, mercuripapain was reacted with EEDQ at pH 7.0 in the usual manner in the presence of $\left[^{14}C_1\right]$-glycine ethyl ester. Over reaction times of 90 minutes and 5 hours, no inactivation of the enzyme was observed and furthermore, no incorporation of labeled ethyl glycinate was found. Thus it would appear that EEDQ is not a useful reagent for the activation of any carboxyl groups in papain toward reaction with added ethyl glycinate in aqueous solution. No report has been found in the literature where EEDQ has been employed to modify carboxyl groups in enzymes or proteins in aqueous solutions of an added nucleophile. Belleau et al.[38] used EEDQ to modify a catalytically essential group in α-chymotrypsin. However, in this case he concluded that the nucleophile which reacted with the anhydride intermediate was the imidazole moiety of His-57 in the active site of α-chymotrypsin. This explanation is reasonable for α-chymotrypsin since the active site of this enzyme is known to be hydrophobic (water largely excluded).[150] Thus the imidazole moiety of His-57 could compete quite readily with water for reaction with

a mixed carbonic anhydride intermediate. However, in the case of carboxyl groups exposed on the surface of a protein or in a hydrophilic active site of an enzyme, no added nucleophile could compete successfully with water of a 55 M concentration for the anhydride intermediate and cause irreversible carboxyl group modification. The failure of EEDQ to cause irreversible modification of any carboxyl groups in papain probably indicates that the accessible carboxyl groups of this enzyme are all in hydrophilic regions where the anhydride intermediate is hydrolyzed before any reaction with an added nucleophile can occur. Further support for this hypothesis is given on page 87 where evidence is presented that water-soluble carbodiimide, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, does successfully activate carboxyl groups toward reaction with added ethyl glycinate via a relatively more stable O-acylisourea intermediate.

Finally, Table VIII does not contain the pseudo first-order rate constant for the inactivation of mercuripapain by EEDQ in the presence of glycine methyl ester at pH 4.5. This is due to the fact that, under these conditions, the glycine methyl ester catalyzed the hydrolysis of EEDQ, as was shown by a complete loss of EEDQ and a quantitative production of quinoline within 15 seconds after addition of EEDQ to a 1 M glycine ethyl ester solution at pH 4.5 in the absence of any papain.

It should be pointed out here, that there appears to be no inactivation of papain by the hydrolysis products of EEDQ, ethanol, CO_2, or quinoline. This is demonstrated by the fact that no inactivation of the enzyme was observed at pH 4.5 in the presence of glycine ethyl ester. As stated above, under these conditions EEDQ is almost immediately

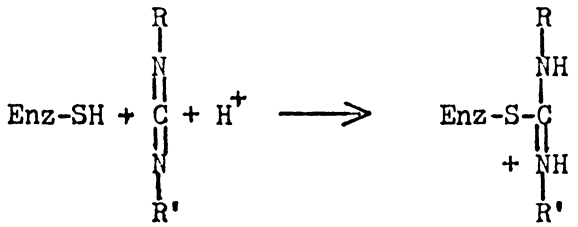
hydrolyzed to ethanol, CO_2 and quinoline, making their concentration in this reaction mixture higher than in any of the other reactions. Therefore, any loss of enzymatic activity due to these products would have been observed in these reactions and this was not the case.

In conclusion, while inactivation of the serine hydrolases was proposed to be due to the reaction of EEDQ exclusively with carboxyl groups,[38] it has been shown that EEDQ undergoes a facile, acid-catalyzed reaction with the essential thiol group of papain at acid pH values. Thus the possible reaction of EEDQ with other thiol groups should be considered in any use of this reagent on other proteins and enzymes. In addition, it is apparent that EEDQ was unable to incorporate ethyl glycinate residues into papain at pH 7.0 in aqueous solution, seriously limiting the usefulness of this reagent in any investigation of the number and nature of carboxyl groups in many other proteins and enzymes.

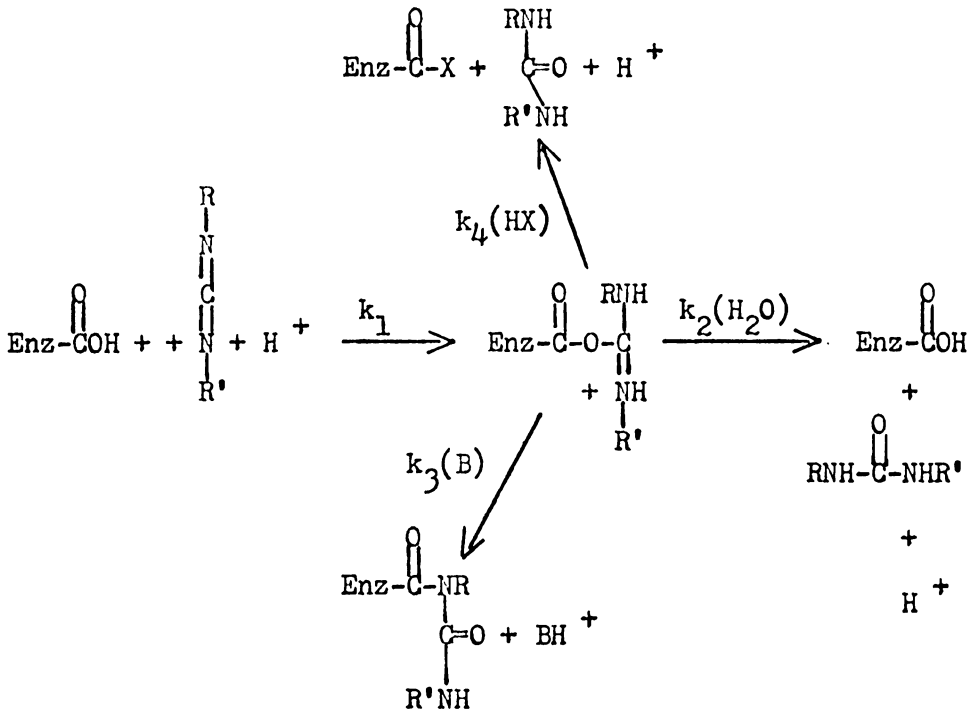
Chemical Modification of Papain with 1-Ethyl-3-(3-dimethylaminopropyl) Carbodiimide (EDC)

Treatment of active papain with EDC under mildly acidic aqueous conditions may be expected *a priori* to result in the modification of several types of amino acid residues in the enzyme. These residues are: first, the single cysteine residue (Cys-25) of papain as indicated in Equation 24 below;[47] second, any accessible carboxyl groups from among the fifteen present in papain (6 Asp and 8 Glu plus the C-terminal residue) *via* the pathways outlined in Equation 25;[45] and third, any accessible phenolic hydroxyl groups among the nineteen tyrosyl residues in the enzyme will react according to Equation 26 below.[50] Amine

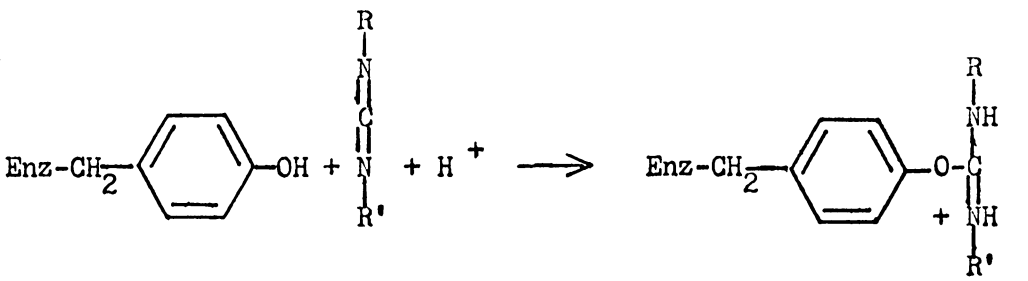
residues would not be expected to be subject to reaction with EDC except under more alkaline conditions.[48,49]



(24)



(25)



(26)

Any derivatization of the catalytically essential thiol group of Cys-25 should lead to the inactivation of papain, as has been demonstrated in a variety of ways.[96,102,108,109,110,111,113-115] As can be seen in Table IX, and Figure 5, treatment of active, activator-free papain (0.103 mM) with a 600-fold molar excess of EDC at pH 4.75, 25°, resulted in a nearly complete inactivation of the enzyme toward the assay substrate, α-N-benzoyl-L-arginine ethyl ester, within 60 minutes. Dialysis of this inactivated enzyme resulted in some recovery of activity (see Table IX) but there was still a close correspondence between activity loss and thiol derivatization, presumably via Equation 24, during the reaction of active papain with EDC. Furthermore, when the inactivation was carried out in the presence of GlyOEt, the rate and extent of inactivation were similar to that found in the absence of any added nucleophile (see Figure 5). Again, dialysis resulted in some recovery of activity, but again a close correspondence between ultimate irreversible activity loss and loss of active thiol was observed (Table X).

Benzamidoacetonitrile, a good competitive inhibitor of papain, (K_i = 2.4 x 10^{-4} M at pH 3.8 and 1.3 x 10^{-4} M at pH 6.),[3] afforded substantial protection of active papain against inactivation by EDC in the presence of GlyOEt (see Table X). Two successive 1 hour treatments of the sample of active papain by EDC in 600:1 molar excess resulted in a modified papain which, after dialysis was still 80% active and had retained about 80% of its original thiol titre.

As can be seen in Figure 6, mercuripapain is also subject to inactivation by EDC. However, the time course of inactivation of the mercurienzyme is slower and much less complete under given conditions

TABLE IX

INACTIVATION OF PAPAIN AND MERCURIPAPAIN BY EDC[e]

Enzyme[a]	Initial (EDC)/(Enzyme) Number of additions	Reaction time before dialysis (min)	% Activity before dialysis	% Activity after dialysis[b]	% Free thiol[c] after dialysis
Active papain	600(1)	60	6.1	26.0	28
Mercuri-papain	300(1)	60	51.6	68.0	100
	600(1)	60	46.0	70.8	94
	600(1)[d]	60	23.5	54.0	90
	600(2)	90	14.2	26.8	100
	1200(1)	60	18	61.1	100
	1200(2)	90	12.6	31.6	100

[a]Initial enzyme concentration was 0.103 mM.

[b]Dialysis was carried out for 18-24 hours against 5 x 300 ml of 10 mM KCl, at 4°.

[c]Determined by spectrophotometric titration using 2,2'-dipyridyldisulfide (see Experimental).

[d]Inactivation carried out in the presence of 0.5 M triethylamine.

[e]See Experimental Procedure for reaction conditions.

FIGURE 5

The Inactivation of Active Papain by EDC

Active, activator-free papain in the presence (O), or absence (Δ) of 0.5 M GlyOEt. The initial EDC concentration was 61.8 mM, a 600-fold molar excess over initial active papain concentration. For other reaction conditions see Tables IX and X.

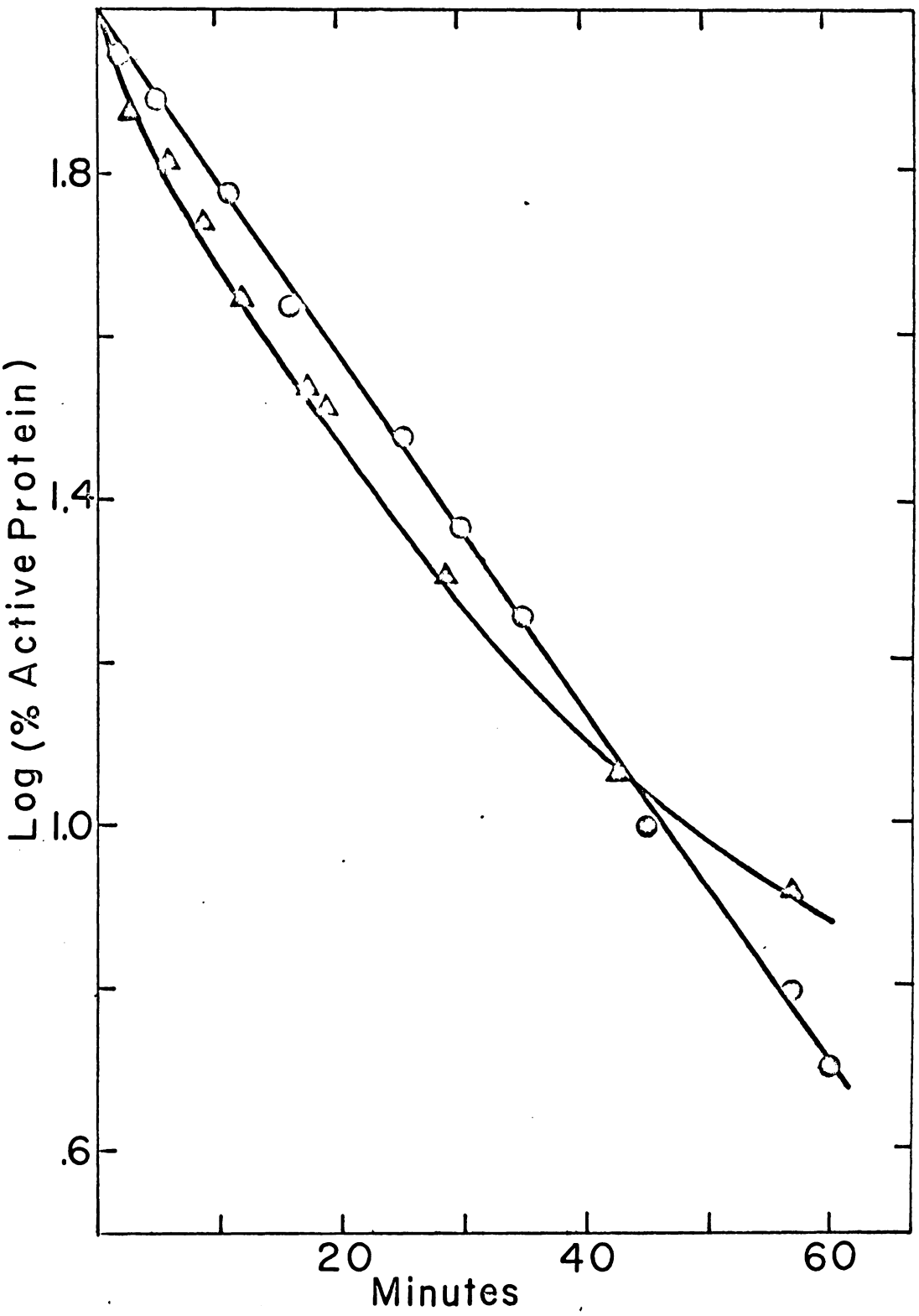

1.8
1.4
1.0
.6
Log (% Active Protein)
20
40
60
Minutes

TABLE X

INACTIVATION OF PAPAIN AND MERCURIPAPAIN BY EDC IN THE PRESENCE OF GLYCINE ETHYL ESTER[f]

Enzyme[a]	Inhibitor[b] present	Initial (EDC / Enzyme) Number of additions	Reaction time before dialysis (min)	% Activity before dialysis	% Activity after dialysis	% Free thiol[d] after dialysis
Active papain	-	600(2)	90	4.9	17	21
	+	600(2)	90	70.1	80	79.5
Mercuri-papain	-	600(1)	60	9.0	16.5	92
	-	600(2)	90	6.4	10.0	98
	+	600(2)[e]	90	5.7	16.2	100
	+	600(2)	90	9.3	16.5	100

[a]Initial enzyme concentration was 0.103 mM.

[b]The inhibitor, when present, was benzamidoacetonitrile (18.7 mM).

[c]Dialysis was carried out against 5 x 300 ml of 10 mM KCl for 18-24 hours at 4°.

[d]Determined by spectrophotometric titration using 2,2'-dipyridyldisulfide (see Experimental).

[e]Benzamidoacetonitrile concentration was 0.85 mM.

[f]See Experimental Procedure for reaction conditions.

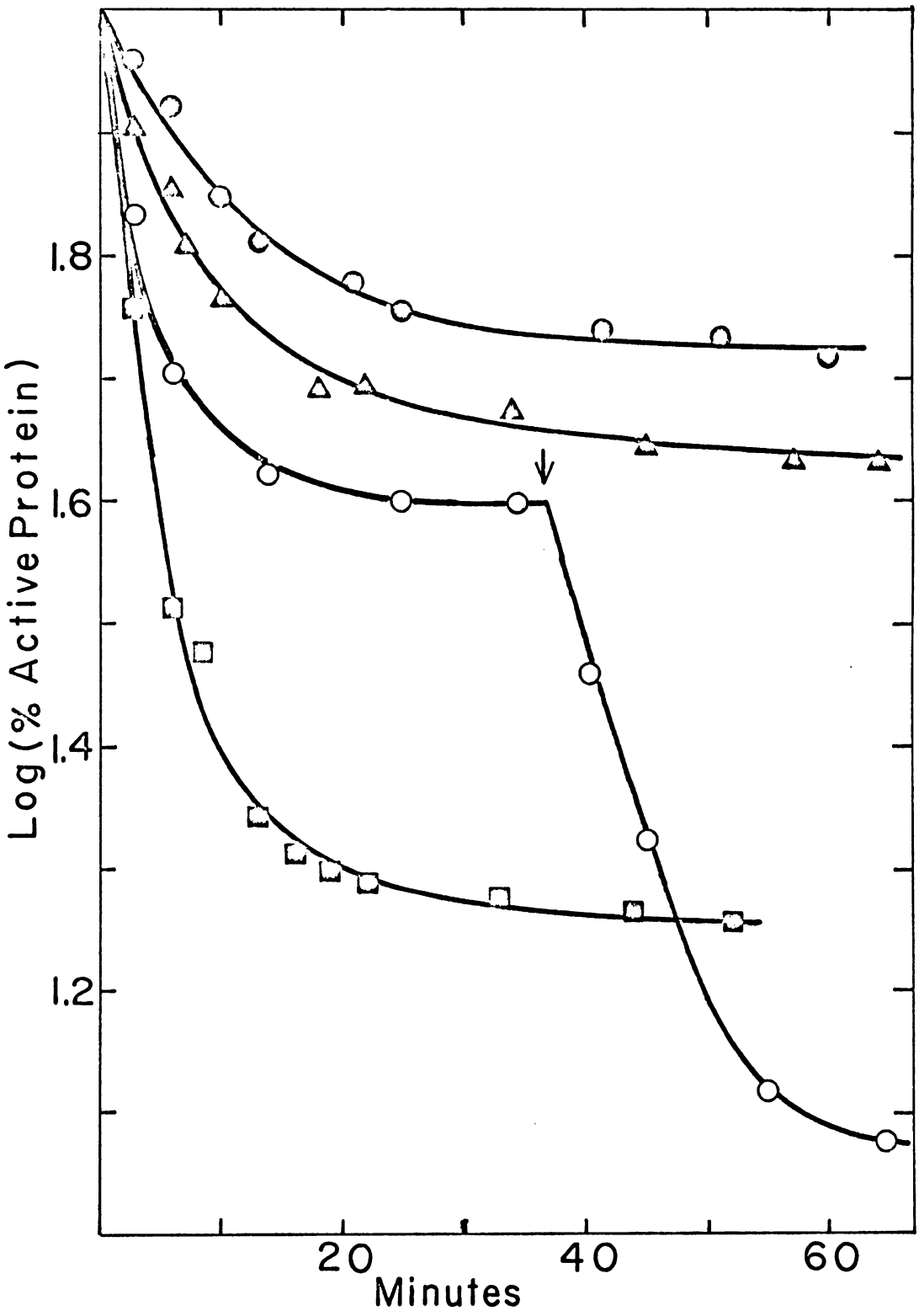
than that for the active enzyme. Significantly, no apparent modification of thiol groups accompanies inactivation of the mercurienzyme, even under forcing conditions, where the extent of irreversible inactivation is similar to that achieved with active papain (see Table IX). Again, as with active papain, dialysis of the inactivated enzyme leads to some recovery of activity. This recovery is more extensive if the dialysis is carried out at a slightly elevated pH (see Table IX). It might be mentioned here that 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-p-toluene sulfonate exhibited the same type of inactivation of mercuripapain as was observed with EDC. It appears therefore, that while mercuripapain is effectively immune to thiol derivatization by EDC, it is nonetheless vulnerable to irreversible inactivation by this water-soluble carbodiimide to a considerable extent. This inactivation of the mercurienzyme then, must be due to modification of some carboxyl group(s) and/or tyrosyl residue(s), most likely one or more such groups near the Cys-25 in the active site of papain.

Turning first to the consideration of tyrosyl modification by EDC, Carraway and Koshland[50] have shown that EDC does indeed convert accessible tyrosine residues in proteins to O-acylisourea derivatives which are resistant to acid hydrolysis. However, they have also shown that hydroxaminolysis of the modified protein residues quantitatively reverses this tyrosine modification. Table XI summarizes the results of analyses of various modified and unmodified papain preparations for their amino acid composition. Values are given only for tyrosine, histidine and glycine. Values obtained for all other amino acids were unaffected by modification under any of the conditions noted in Table XI, and in all

FIGURE 6

The Inactivation of Mercuripapain by EDC in the Absence of Added Nucleophile or Base

The carbodiimide was added initially in 300-fold (O), 600-fold (Δ), 780-fold (○), or 1200-fold (□) molar excess over enzyme. The arrow indicates the addition of a second 780-fold excess of EDC to the reaction mixture. For reaction conditions see Table IX.


Log (% Active Protein)
1.8
1.6
1.4
1.2
20
40
60
Minutes

cases their nearest integral values, normalized to an assumed value of 14 for alanine, agreed precisely with the established composition of native papain.[74] As can be seen from the results presented in Table XI, obtained using the procedures described by Carraway and Koshland,[50] at least six and up to ten tyrosyl residues can be derivatized during the course of inactivation of papain or mercuripapain by EDC. These derivatizations presumably occur via Equation 26. The competitive inhibitor benzamidoacetonitrile, which does afford substantial protection of active papain against activity loss (Table IX), does not afford any concurrent protection against tyrosyl modification. Furthermore, complete regeneration of free tyrosyl residues by hydroxaminolysis of extensively inactivated mercuripapain failed to restore this enzyme to its activatable state. Thus it seems unlikely that tyrosine modification by EDC, which takes place along with inactivation of papain, is itself in any way responsible for the actual inactivation observed. Therefore, it appears that among the tyrosyl residues accessible to attack by EDC, none is associated as a catalytically essential group in the active site of papain.

Let us next consider the modification of carboxyl groups by EDC and the subsequent effects of this treatment on enzymic activity. Hoare and Koshland have shown that when a protein is treated with an appropriate water-soluble carbodiimide, such as EDC, in the presence of an added nucleophile, such as glycine ethyl ester, under the conditions employed in the experiments with papain, a virtually complete derivatization by the nucleophile of any carboxyl groups accessible to attack by the carbodiimide should result.[45] Although cysteine residues and tyrosine

TABLE XI

CHANGES IN AMINO ACID COMPOSITION OF PAPAIN UPON REACTION OF THE ENZYME WITH EDC IN THE PRESENCE OF GLYCINE ETHYL ESTER[a]

Experiment[c]	Amino Acid Composition[b] Tyr	His	Gly	% Enzymic Activity Remaining after Modification
A	19.26(19)	1.74(2)	27.88(28)	100
B	10.20(10)	1.78(2)	32.31(32)	71
C	10.03(10)	1.81(2)	34.09(34)	10
D	19.98(20)	1.82(2)	27.56(28)	100
E	19.74(20)	2.05(2)	33.75(34)	6
F	19.99(20)	1.73(2)	27.56(28)	100
G	13.17(13)	1.95(2)	27.50(28)	31

[a]For reaction conditions, see Experimental Procedure.

[b]Values tabulated are residues per molecule of papain calculated relative to an assumed value for alanine of 14.00 (Mitchel *et al.*[82]). For reference, values for these amino acids from the most recent published primary sequence of papain[82] are Tyr, 19; His, 2; Gly, 28.

[c]Experiment A: unmodified stock papain; B: active papain modified in the presence of 18.7 mM benzamidoacetonitrile; C: modified mercuripapain; D: (control for experiment C) mercuripapain treated as in experiment C but with EDC omitted; E: mercuripapain modified with EDC/GlyOEt followed by exhaustive hydroxaminolysis to regenerate tyrosyl residues; F: (control for experiment E) mercuripapain treated as in experiment E but with EDC omitted; G: mercuripapain modified with EDC alone (two additions of EDC in 1200:1 molar excess over enzyme).

residues are also subject to modification by water-soluble carbodiimides, as in the case of papain, only carboxyl group modification involves incorporation of the added nucleophile by the protein. Comparison of Figure 7 and Table X with Figure 6 and Table IX shows that the inactivation of mercuripapain by EDC in the presence of GlyOEt is more rapid and more extensive than inactivation in the absence of the added nucleophile. Again, no loss of free thiol groups is observed. The implication is that inactivation of mercuripapain by EDC in the presence of GlyOEt is at least partially attributable to modification of carboxyl groups in the enzyme. Triethylamine, a base which cannot react with activated carboxyl groups in the k_4 (HX) step (see Equation 25) to form stable derivatives, does, in fact, enhance both the rate and extent of mercuripapain inactivation, but not nearly as effectively as GlyOEt. This is expected from the known susceptibility of O-acylisourea isomerization to catalysis by organic bases.(151)

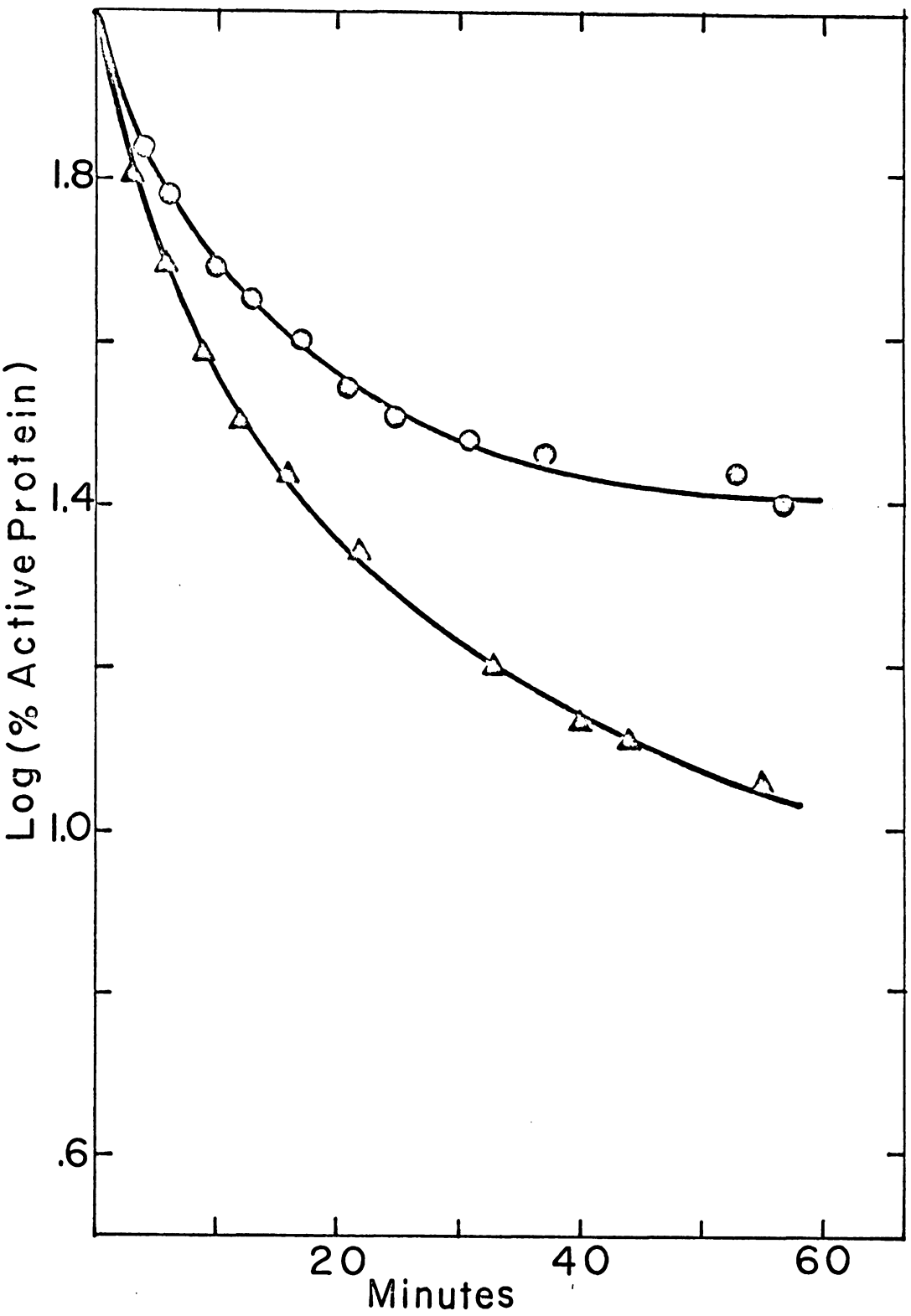
Finally, the competitive inhibitor benzamidoacetonitrile does not significantly protect mercuripapain against inactivation. This is presumably the result of the failure of this inhibitor to associate appreciably with the metallated active site of the enzyme. It is known that mercuripapain does not bind to a tetrapeptide competitive inhibitor ligand attached to agarose.(79)

It should be mentioned here, that the urea derivative formed by hydrolysis of EDC does not cause inactivation of papain to any measurable extent. This can be seen in Table IX where treatments of 300, 600, and 1200 to 1 molar excesses of EDC gave essentially the same

FIGURE 7

Inactivation of Mercuripapain by EDC in Glycine Ethyl Ester

The EDC was in a 600-fold molar excess over the enzyme, in the presence of 0.5 M triethylamine (O) or 0.5 M glycine ethyl ester (△). For reaction conditions see Experimental Procedure.


1.8
1.4
1.0
.6
Log (% Active Protein)
20
40
60
Minutes

amount of inactivation after dialysis. This would not be the case if the urea of EDC were causing any inactivation of the enzyme, since then, the amount of inactivation should be greater for the 1200 to 1 treatment, where the concentration of the urea would be 4 times greater than that for the 300 to 1 molar excess treatment of papain.

Table XII presents the results of experiments designed to determine the extent of carboxyl group modification accompanying inactivation of papain by EDC. These inactivations were carried out in the usual way with two additions (at 0 and at 60 minutes) of EDC in 600-fold molar excess over the enzyme. Radioactive $\left[^{14}C_1\right]$ - glycine ethyl ester (0.5 M) was present as a nucleophile to derivatize activated carboxyl groups. It was found that both active, activator-free papain and mercuripapain incorporate six ethyl glycinate residues per molecule of enzyme, with the concomitant irreversible loss, in both cases, of approximately 85% of their original enzymatic activity toward α-N-benzoyl-L-arginine ethyl ester.

Table XII also shows that the protection afforded active papain by benzamidoacetonitrile against inactivation and against thiol modification (see Table X) includes substantial protection against carboxyl group modification as well. Only four ethyl glycinate residues are incorporated per molecule of active papain in the presence of benzamidoacetonitrile, as opposed to six residues incorporated in the absence of the inhibitor. It should be noted here that the glycine data of Table XI are nicely consistent with, and thus confirm the data of Table XII for experiments with radioactive GlyOEt.

TABLE XII

REACTION OF PAPAIN WITH EDC IN THE PRESENCE OF [^{14}C]-GLYCINE ETHYL ESTER[b]

Enzyme	Benzamido-acetonitrile (18.7 mM)	No. residues incorporated per molecule of enzyme[a]	% Activity remaining after dialysis
Mercuripapain	-	6.0	15
Active Papain	-	6.0	17
Active Papain	+	4.1	71

[a]Mean of several experiments.

[b]The [^{14}C] -glycine ethyl ester concentration was 0.5 M and the reaction was run at pH 4.75, 25°, in 10 mM KCl.

In the case of mercuripapain, the inactivation which accompanies modification can be attributed solely to carboxyl group modification since tyrosyl modification is without effect and sulfhydryl group modification does not occur. For active papain, it appears that virtually all of the observed inactivation can be accounted for in terms of sulfhydryl group modification. However, it is readily apparent that the presence of the competitive inhibitor benzamidoacetonitrile protects the active enzyme not only against loss of free thiol groups, but also against derivatization of two of the six otherwise accessible carboxyl groups. Although it has not been demonstrated that the six modified carboxyl groups in active papain constitute the same set of six which are modified in the mercurienzyme, if this is assumed to be the case, then it can be concluded that the inactivation of mercuripapain must result from the derivatization of just one or two carboxyl groups, which presumably are present at the enzyme's active site, where a competitive inhibitor could afford them protection against attack by EDC.

From the description of the papain active site by Drenth et al.,[87] a tentative assignment of one of the two protected carboxyl groups as Asp-158 can be made. However, if residue-64 of papain, also known to belong to the active site,[87] is indeed an asparagine rather than an aspartic acid residue[117] it is difficult to assign a tentative identity to the second protected carboxyl group. It may be that the binding of benzamidoacetonitrile to the active site of papain results indirectly, through conformational changes that involve amino acid residues without as well as within the active site, in a reduction of carboxyl group

accessibility at some other locus on the protein surface. Or it may be that the inhibitor binds significantly at one or more alternative sites, in addition to the active site, where it could provide protection against EDC attack. Indeed, evidence exists, though none specifically for benzamidoacetonitrile, which suggests that effectors of papain catalysis may in fact bind at remote sites on the enzyme.(152,153)

It is indeed interesting to note that in both active papain and mercuripapain, the numbers of carboxyl groups subject to labeling by EDC/GlyOEt treatment is the same. It might be expected that the simultaneous labeling of two groups very close to one another in the active site of an enzyme would be unlikely on simple steric grounds. Such seems to be the case, for example, in the exclusive modification of one or the other, but not both, of the two active-site histidine residues of ribonuclease by carboxyalkylation.(154) The sulfhydryl group modification by EDC in active papain apparently neither prevents nor is prevented by the derivatization of any carboxyl group. For, if either were the case, one would expect fewer carboxyl groups to be derivatized in active papain than in mercuripapain, or a less than 1:1 correlation between loss of thiol and loss of enzymatic activity in active papain, or both. This leads one to the conclusion that while a carboxyl group in the active site can be derivatized leading to a reduction or loss of enzymatic activity, that carboxyl group is not in the immediate vicinity of the catalytically essential thiol and thus is unlikely to be involved along with that thiol group in catalysis per se.

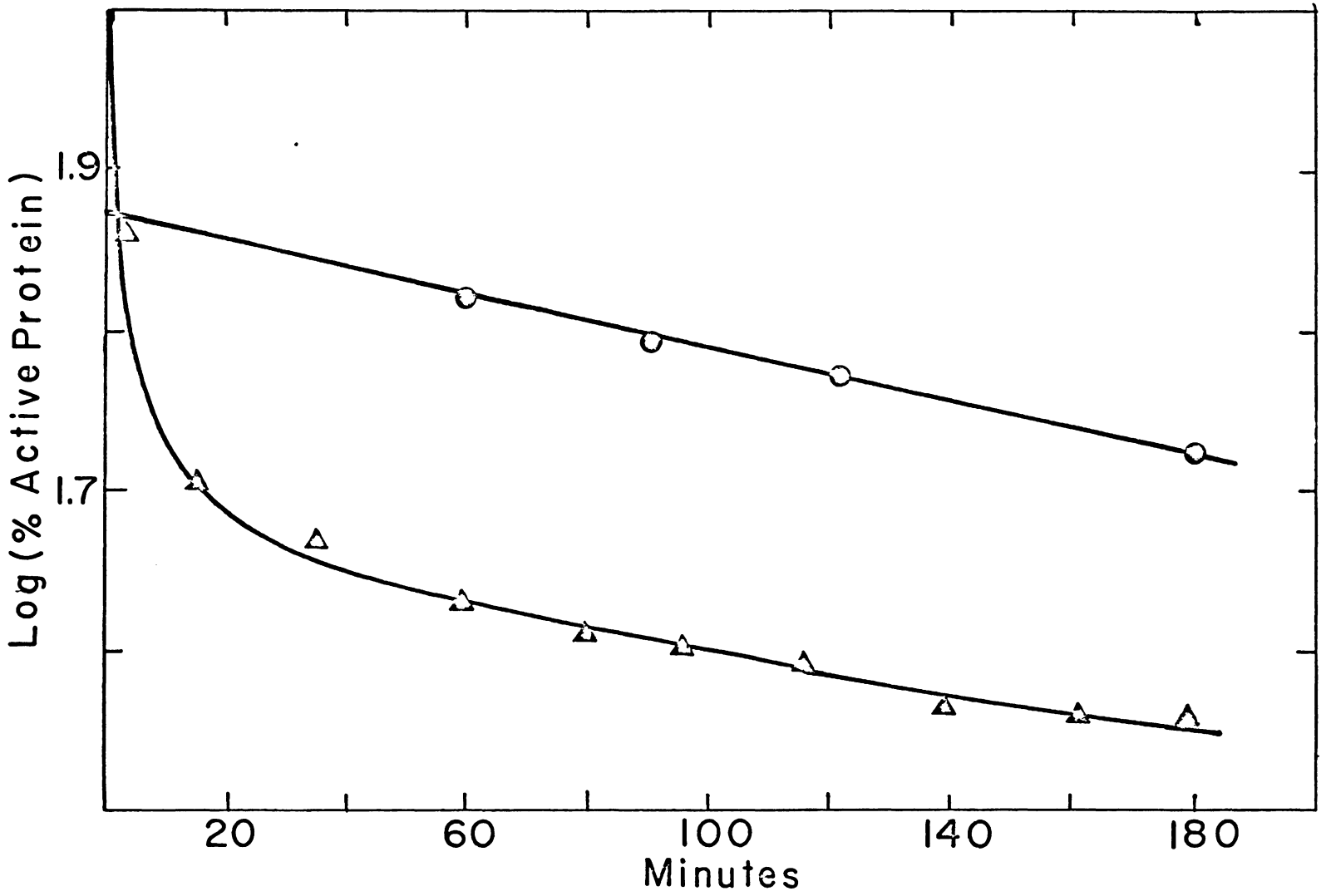
To this point in the discussion of carboxyl group modification, the fact that mercuripapain can be inactivated to some extent with

large excesses of EDC in the absence of any added nucleophile (Table IX, Figure 6), has been ignored. Since the possibility that this inactivation is due to tyrosyl modification, which does not involve any added nucleophile, has been eliminated, the irreversible inactivation can tentatively be accounted for in terms of Equation 25 without the $k_4(HX)$ step. The initial relatively rapid rate of inactivation of mercuripapain is attributed to the build-up of the labile, but nonetheless enzymatically inactive, O-acylisourea enzyme derivative in the k_1 step. The concentration of this species reaches a maximum at the point in time when its second-order rate of formation is equaled by its pseudo first-order rate of disappearance via the $k_2(H_2O)$ and k_3 steps. Subsequent inactivation proceeds at a very slow rate with the irreversible formation of the N-acyl urea derivative of the enzyme in the k_3 step. Support for this hypothesis is provided in Figure 8. If indeed Equation 25 without the $k_4(HX)$ step is the pathway for the reaction of carboxyl groups of mercuripapain with EDC in the absence of any nucleophile, then at long times, as the concentration of EDC decreases and the O-acylisourea enzyme intermediate continues to partition itself between the irreversibly inactivated N-acyl urea enzyme derivative and regenerated enzyme having a free carboxyl group, there should be an overall decrease in the absolute amount of active enzyme. Figure 8 shows that this is in fact the case. The total amount of active enzyme in the reaction mixture does decrease at long times when the EDC concentration is approaching zero. Furthermore, when, after inactivation has proceeded to a certain extent, the reaction is terminated by the addition of excess acetic acid buffer and the modified

FIGURE 8

Inactivation of Mercuripapain by EDC at Long Reaction Times

Mercuripapain treated with a 600-fold molar excess of EDC (Δ). Total enzyme activity found in the reaction mixture (see Experimental Procedure) (○).


Log (% Active Protein)
1.9
1.7
20
60
100
140
180
Minutes

enzyme dialyzed. The labile O-acylisourea component in the mixture of inactive enzyme species is hydrolyzed back to fully activatable mercuripapain. This is shown in Tables IX and X by the discrepancies between percent activity before and after dialysis.

Several other experiments were performed to further elucidate the unusual kinetics observed in the reaction of EDC with mercuripapain. As can be seen in Figure 6, after approximately 40 minutes, the inactivation reaction appears to stop. One explanation for this behavior would be that, after this length of time the EDC concentration has become so low that no further inactivation occurs, however, while Figure 9 shows that EDC is rapidly hydrolyzed in the presence of mercuripapain, Figure 10 demonstrates that even after 60 minutes, when the inactivation curve for mercuripapain levels off, there is still in fact, sufficient EDC present to cause the inactivation of active papain by reaction with the free thiol group of Cys-25.

In order to determine the exact nature of the hydrolysis of EDC in the presence of mercuripapain, several kinetic experiments were performed. It has been shown previously, that carboxyl groups catalyze the hydrolysis of certain carbodiimides to their corresponding ureas,[155] and indeed it was found that this was also the case for EDC. Figure 11 shows that the rate of hydrolysis of EDC at pH 4.75, 25° is increased by about 26% in the presence of a catalytic amount of acetic acid. The pseudo first-order rate constants for the hydrolysis of EDC to its corresponding urea at pH 4.75, 25° under various conditions are given in Table XIII. As can be seen in Table XIII the acetic acid catalyzed hydrolysis of EDC is slowed by either addition of triethylamine or

FIGURE 9

Hydrolysis of EDC in the Presence of Mercuripapain

Mercuripapain treated with a 600-fold molar excess of EDC (O). The change in concentration of EDC (Δ).

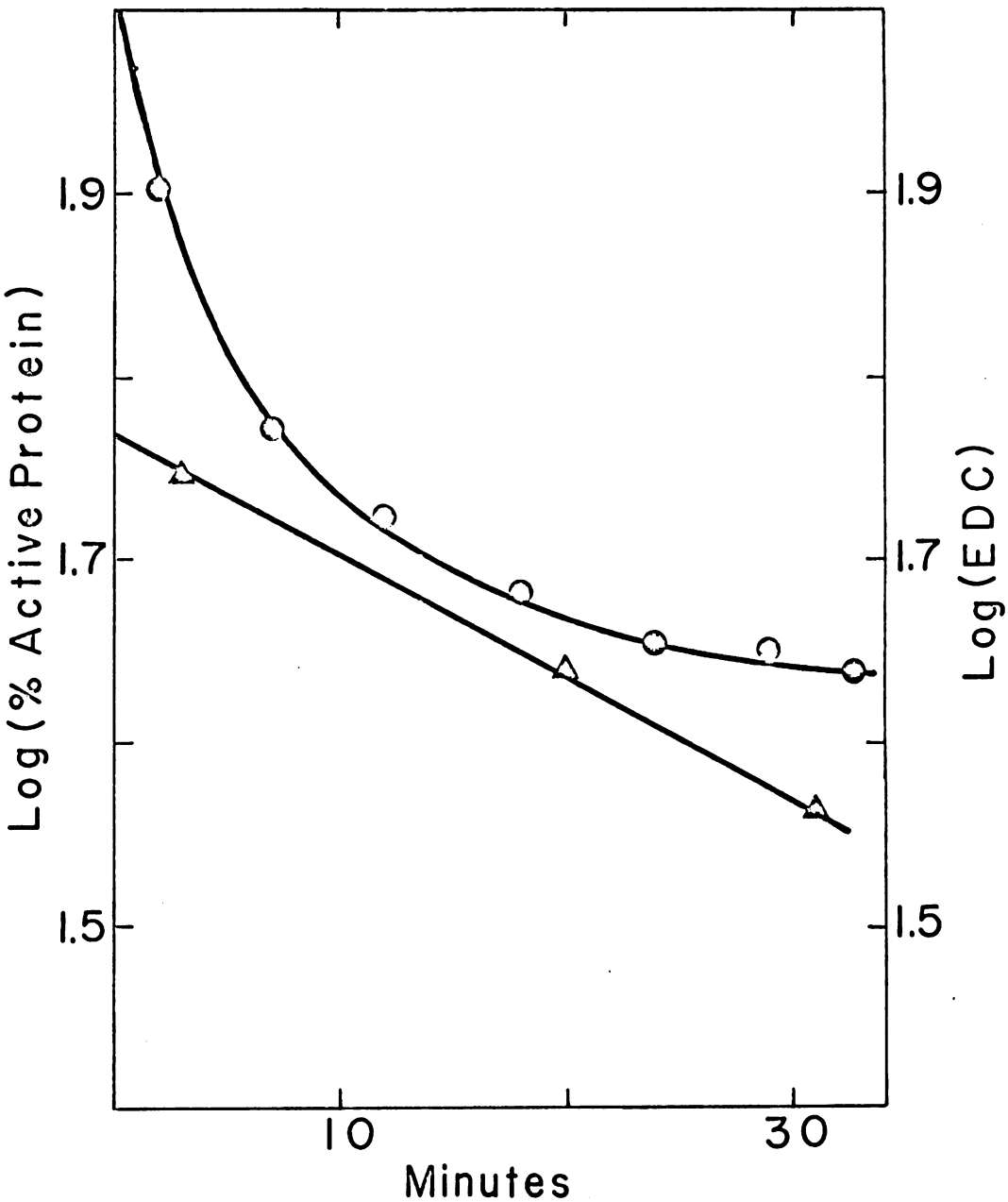
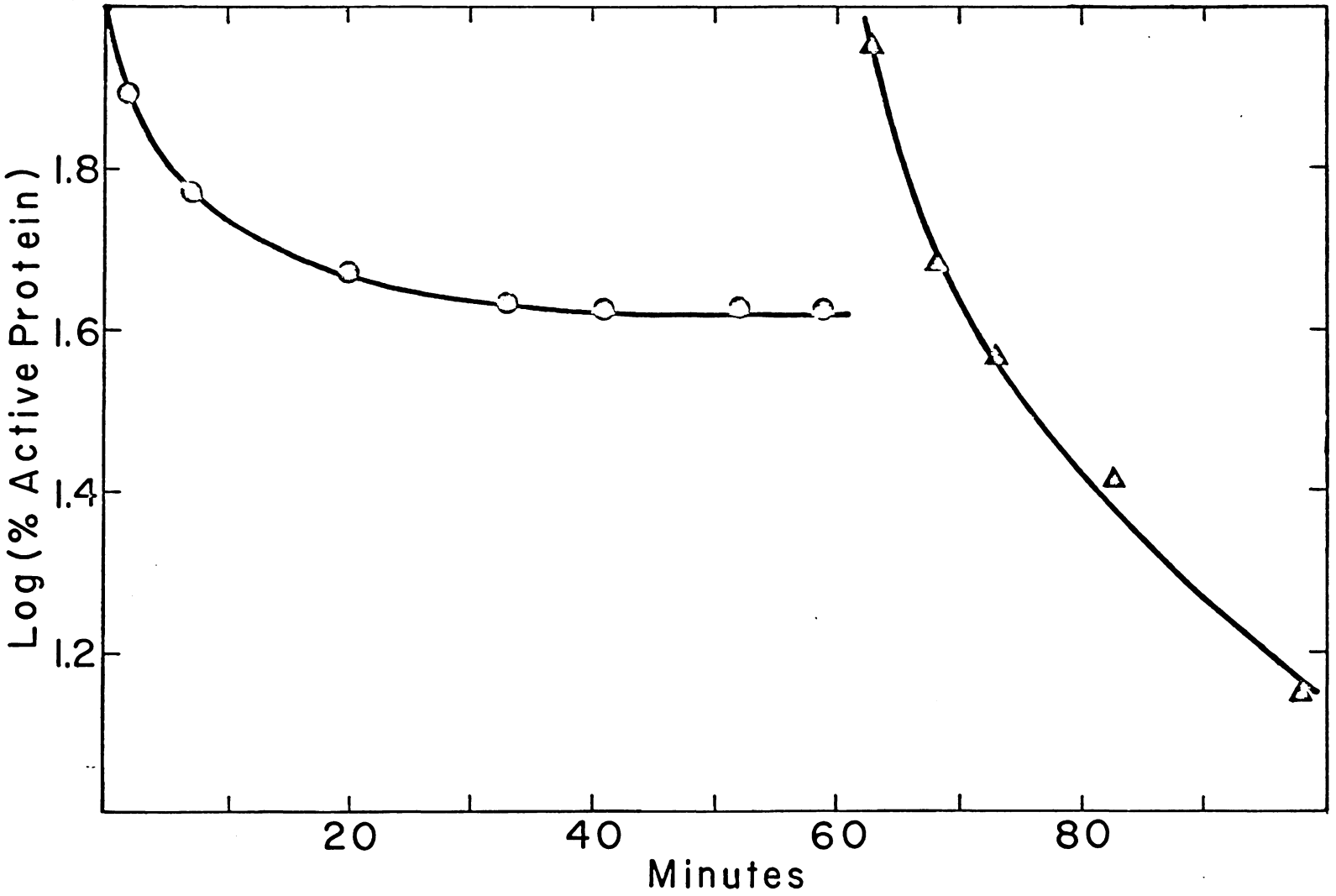

1.9
1.7
1.5
Log (% Active Protein)
1.9
1.7
1.5
Log (EDC)
10
30
Minutes

FIGURE 10

Inactivation of Active and Mercuri-papain by EDC

Mercuripapain treated with a 600-fold molar excess of EDC (○). Active papain added at 60 minutes (▲) (see Experimental Procedure).


Log (% Active Protein)
1.8
1.6
1.4
1.2
20
40
60
80
Minutes

glycine ethyl ester. This can be attributed to modification and in effect removal of the acetic acid catalyst either by triethylamine-catalyzed rearrangement of the O-acylisourea to an N-acyl urea (Equation 25, k_3 (B) step) or by reaction of the acetic acid O-acylisourea with glycine ethyl ester to form N-acetyl glycine ethyl ester (Equation 25, k_4(HX) step).

The above data are in agreement with the results presented by Hoare and Koshland[45] for the reaction of 1-benzyl-3-dimethylamino-propyl carbodiimide and EDC with aqueous solutions of carboxylic acids. Referring to Equation 25, Hoare and Koshland found that after the initial formation of the O-acylisourea derivative of a carboxylic acid, two main reactions can occur in the absence of a strong nucleophile. The O-acylisourea derivative can rearrange to the N-acyl urea (Equation 25, k_3(B) step) or it can hydrolyze to regenerate free carboxylic acid (Equation 25, $k_2(H_2O)$ step). Hoare and Koshland found that for the O-acylisourea formed from acetic acid and EDC, hydrolysis proceeded about 20 times faster than rearrangement.[45] Keeping these possible reactions of carbodiimides with carboxylic acids in mind, let us look at a possible explanation for the unusual inactivation curves which were found during reaction of mercuripapain with EDC, in either the presence (Figure 7) or absence (Figure 6) of any added nucleophile or base. While no analytical solution to the kinetic data could be obtained to completely establish the validity of the proposed cyclic pathway for the reaction of mercuripapain with EDC, several qualitative observations supporting this type of reaction mechanism can be made. First, the kinetic behavior of mercuripapain when treated with EDC in the absence

FIGURE 11

Hydrolysis of EDC

EDC in water at pH 4.75, 25° (○). EDC in water at pH 4.75, 25°, in the presence of a catalytic amount of acetic acid (△) (see Experimental Procedure).

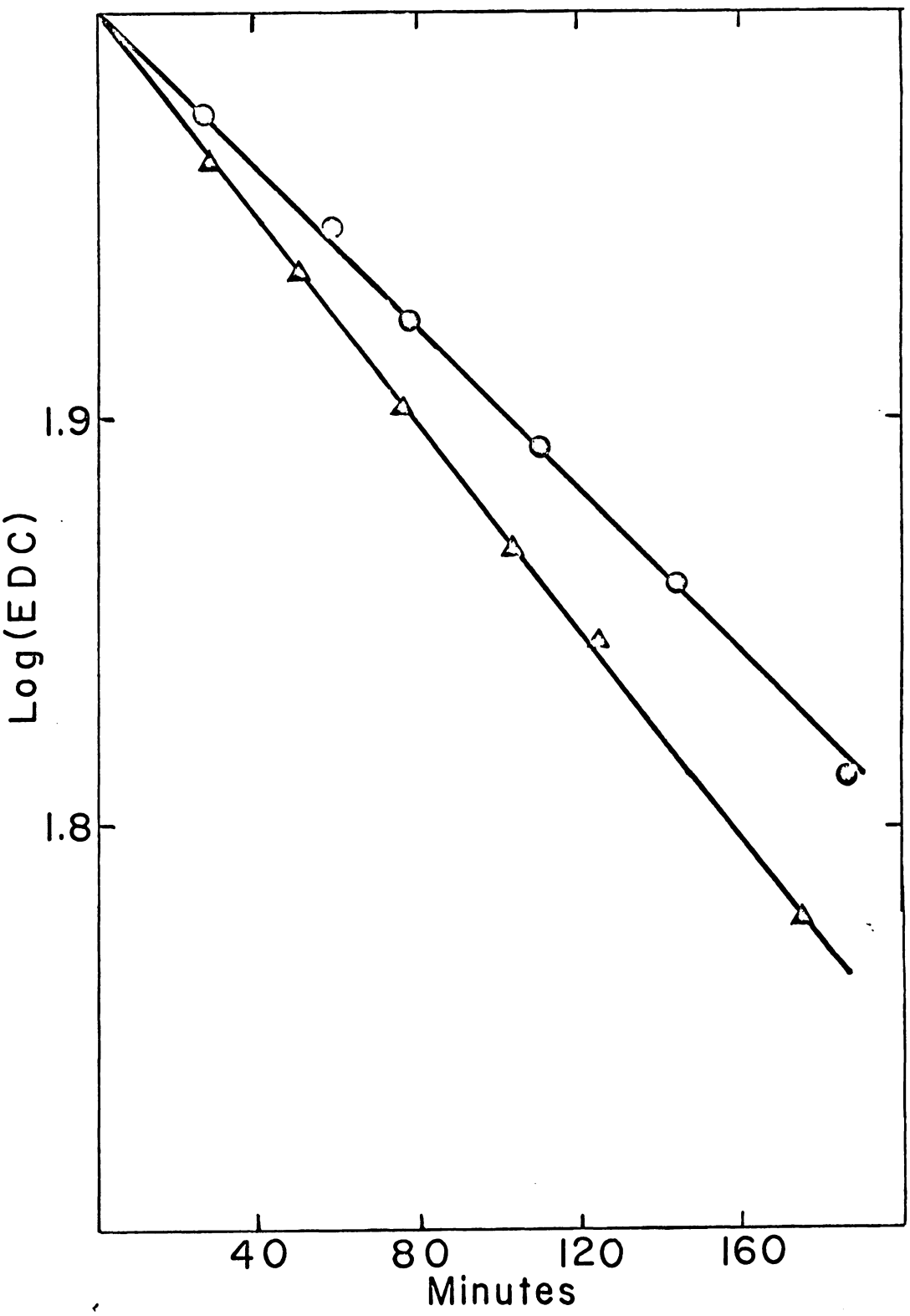

1.9
1.8
Log(EDC)
40
80
120
160
Minutes

TABLE XIII

PSEUDO FIRST-ORDER RATE CONSTANTS FOR HYDROLYSIS OF EDC UNDER VARIOUS CONDITIONS[a,b]

Conditions	$k \times 10^3$ (min^{-1})[c]
EDC, H_2O	0.987 ± .001
EDC, H_2O, and Acetic Acid	1.244 ± .011
EDC, H_2O, Acetic Acid and Triethylamine	1.096 ± .007
EDC, H_2O, Acetic Acid and Glycine Ethyl Ester	1.219 ± .007

[a] pH 4.75, 25°

[b] Ratios of all reactants were the same as those found in reactions with active or mercuripapain (see Experimental Procedure)

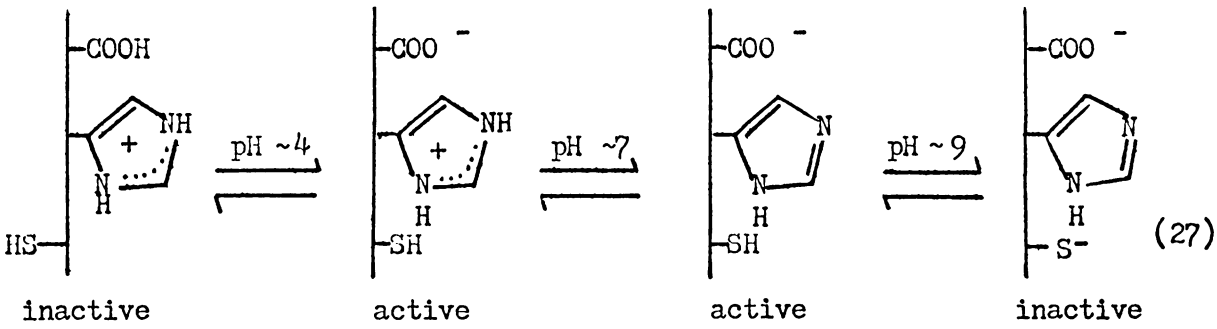
[c] Linear least square values (see Experimental Procedure)

of any added nucleophile or base has been discussed previously, and it was pointed out that the inactivation curves observed (see Figure 6) were qualitatively in agreement with the pathway given in Equation 25, without the $k_4(HX)$ step. Second, in the presence of the nucleophile glycine ethyl ester or the base triethylamine, the inactivation curves again showed a rapid initial decrease in enzyme activity, but no leveling off of the inactivation curves was observed as was the case in the absence of any nucleophile. This is consistent with the reaction pathway proposed in Equation 25 since now after the initial buildup of the O-acylisourea derivative there is a third competing pathway for reaction, the $k_4(HX)$ step. Hoare and Koshland have shown that the reaction of the O-acylisourea derivative is much faster than the rearrangement to the N-acyl urea.(45) Thus in the presence of glycine ethyl ester one would not expect the inactivation curve for mercuripapain and EDC to level off but it should continue to decrease to essentially zero activity. Figure 7 shows that this is indeed the case. Figure 7 also shows that there is a larger amount of inactivation of the enzyme in the presence of triethylamine, presumably due to the previously mentioned catalysis of the k_3 step of Equation 25 by organic bases.(152) Thus it appears that the kinetics of the reaction of carboxyl groups in papain are, at least qualitatively, consistent with the reaction pathway proposed in Equation 25.

Finally, it is conceivable that the EDC inactivation of papain or mercuripapain might have been due, at least in part, to a reaction of some kind between the carbodiimide and the imidazole moiety of His-159, a known constituent of the papain active site.(87) Such a reaction must

be regarded as unlikely in view of the fact that carbodiimides do not react with the nitrogen function of the imidazole moiety of histidine during peptide formation *via* carbodiimides.[130] Furthermore, histidine residues in ribonuclease were found to be unaffected by treatment with a water-soluble carbodiimide at pH 4.5.[12] Accordingly, the data for histidine in Table XI reveal no significant loss of histidine in papain or mercuripapain upon treatment with EDC at pH 4.75. This is consistent with the recent data of Murachi and Okumura,[125] in which they provide evidence that the pK_a of His-159 is ~6.7. If this assignment of pK_a is correct then the imidazole moiety of His-159 would be protonated at pH 4.75 and no reaction with EDC would be expected to occur. In addition, a number of experiments with the model compound N-*t*-butoxycarbonyl-L-histidine methyl ester were carried out by treating the model compound with EDC under a variety of conditions, including non-aqueous solvents and high pH. In all of these experiments a quantitative amount of the unmodified histidine derivative was recovered intact, and analysis of reaction mixtures by thin layer chromatography revealed no trace of unidentifiable components which might be attributable to the sought after product with a modified imidazole moiety.

A recent publication by Murachi *et al.*[157] has provided further evidence that the pK_a of His-159 of papain is not perturbed and has a value of ~6.7. Murachi and co-workers have therefore proposed a modified mechanism of action for the thiol proteinases papain and stem bromelain, in which a carboxyl group in the active site of the enzyme, presumably Asp-158, induces a conformational change which exposes the catalytic sulfhydryl group as shown Equation 27:



The data presented by Murachi et al. are explained by assuming that there is not an intimate electronic interaction between the sulfhydryl group of Cys-25 and the imidazole ring of His-159, but that catalysis can be accomplished by the sulfhydryl group alone if all the other environmental conditions are favorable for the substrate to be properly oriented toward it.

In conclusion, while the evidence provided here does not completely establish or discount an essential role for a carboxyl group in papain catalysis, this investigation has provided the first definitive chemical modifications of carboxyl groups and of tyrosyl residues in papain. In addition, the evidence provided keeps a carboxyl group, probably Asp-158, very much in contention for an active role in papain catalysis.

SUMMARY

In summary, it has first been shown that enol esters of the 3-unsubstituted isoxazolium salt N-ethyl-5-phenylisoxazolium-3'-sulfonate can be reduced to carbinols corresponding to the original carboxylic acid employed in the coupling with NEPIS, using a 10-fold molar excess of aqueous $NaBH_4$. This procedure was also extended to the model peptide O-methyl-N-benzyloxycarbonyl-α-L-glutamyl glycinate, and the free carboxyl group of glutamate was reduced by this procedure in a 50% yield.

Second, a survey of five carboxyl group "specific" reagents resulted in no inactivation of the sulfhydryl proteinase papain (EC 3.4.4.10) with diazoacetamide, triethyloxonium tetrafluoroborate, or N-ethyl-5-phenylisoxazolium-3'-sulfonate. Conversely, inactivation of papain was observed with N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline. This inactivation involved reaction of EEDQ with the free sulfhydryl group of the enzyme. It was further found that when active papain was converted to mercuripapain only slight protection from reaction with EEDQ was afforded the free thiol group of the enzyme by converting it to its mercuric derivative with $HgCl_2$.

In addition, reaction of EEDQ with papain in the presence of the nucleophile glycine ethyl ester yielded no incorporation of ethyl glycinate residues into papain. This lack of incorporation is attributed to a very rapid hydrolysis of the mixed carbonic anhydride formed upon reaction of EEDQ with a carboxyl group.

Thirdly, chemical modification of papain with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide in the absence or presence of glycine ethyl ester resulted in almost complete inactivation of the enzyme. Subsequent studies showed that the free sulfhydryl group of papain could be essentially completely protected from reaction with EDC by converting the active sulfhydryl group to its mercuric derivative with $HgCl_2$. The modification of tyrosyl residues in papain by EDC was shown to have no effect on enzyme activity. Finally, reaction of papain with EDC in the presence of $\left[^{14}C_1\right]$-glycine ethyl ester resulted in nearly complete inactivation of the enzyme with the incorporation of 6 ethyl glycinate residues into either active or mercuripapain. Treatment of active papain with EDC and $\left[^{14}C_1\right]$-glycine ethyl ester in the presence of the competitive inhibitor benzamidoacetonitrile prevented inactivation of the enzyme to the extent of about 80% and allowed incorporation of only four ethyl glycinate residues to the enzyme molecule. This indicates that at least one and possibly two of the carboxyl groups modified in the enzyme were located in the active site. The identity of one of these carboxyl groups has been assigned to Asp-158, allowing a tentative assignment of a direct mechanistic role in catalysis to this amino acid residue.

LITERATURE CITED

1. Lowe, G. and Yuthavong, Y., Biochem. J., 124, 117 (1971).

2. Kirsch, J.F. and Igelström, M., Biochemistry, 5, 783 (1966).

3. Lucas, E.C. and Williams, A., Biochemistry, 8, 5125 (1969).

4. Campbell, P. and Kaiser, E.T., J. Amer. Chem. Soc., 95, 3735 (1973).

5. Fraenkel-Conrat, H. and Olcott, H.S., J. Biol. Chem., 161, 259, (1945).

6. Means, G.E. and Feeny, R.E., "Chemical Modification of Proteins," Holden-Day, Inc., San Francisco, p. 139 (1971).

7. Wilcox, P.E., "Methods in Enzymology", Vol. 11, 605 (1967).

8. Frieden, E.H., J. Amer. Chem. Soc., 78, 961 (1956).

9. Doscher, M.S. and Wilcox, P.E., J. Biol. Chem., 236, 1328 (1961).

10. Broomfield, C.A., Riehm, J.P. and Scheraga, H.A., Biochemistry, 4, 751 (1965).

11. Ram, J.S. and Maurer, P.H., Arch. Biochem. Biophys., 85, 512 (1959).

12. Riehm, J.P. and Scheraga, H.A., Biochemistry, 4, 772 (1965).

13. Lundblad, R.L. and Stein, W.H., J. Biol. Chem., 244, 154 (1969).

14. Rajagopalan, T.G., Moore, S. and Stein, W.H., J. Biol. Chem., 241, 4940 (1966).

15. Chibnall, A.C., Mangan, J.L. and Rees, M.W., Biochem. J., 68, 114 (1958).

16. Wilcox, P.E., Abstracts of the 12th International Congress of Pure and Applied Chemistry, New York, pp. 60,61.

17. Meerwein, H., Org. Syn., 46, 113 (1966).

18. Meerwein, H., Hinz, G., Hofmann, P., Kronig, E. and Pfeil, E., J. Prakt. Chem., 147, 257 (1937).

19. Wilcox, P.E., "Methods in Enzymology," Vol. 25, 596 (1972).

20. Yonemitsu, O., Hamada, T. and Kanaoka, Y., Tett. Lett., 23, 1819 (1969).

21. Parsons, S.M., Jao, L., Dalquist, F.W., Borders, Jr., C.L., Groff, T., Racs, J., and Raftery, M.A., Biochemistry, 8, 700 (1969).

22. Nakayama, H., Tanizawa, K. and Kanaoka, Y., Biochem. Biophys. Res. Commun., 40, 537 (1970).

23. Paterson, A.K. and Knowles, J.R., Eur. J. Biochem., 31, 510 (1972).

24. Fölsch, G., Chem. Scr., 6, 32 (1974).

25. Mumm, O., Dissertation, Kiel, 1902.

26. Woodward, R.B. and Olofson, R.A., Tetrahedron Suppl., 7, 415 (1966).

27. Kemp, D.S. and Woodward, R.B., Tetrahedron, 21, 3019 (1965).

28. Woodward, R.B., Olofson, R.A. and Mayer, H., Tetrahedron Suppl., 8, 321 (1966).

29. Hall, P.L. and Perfetti, R.B., J. Org. Chem., 39, 111 (1974).

30. King, J.W., Masters Thesis, Va. Poly. Instit. and State Univ., 1974.

31. Petra, P.H., Biochemistry, 10, 3163 (1971).

32. Petra, P.H. and Neurath, H., Biochemistry, 10, 3171 (1971).

33. Bodlaender, P., Feinstein, G. and Shaw, E., Biochemistry, 8, 4941 (1969).

34. Feinstein, G., Bodlaender, P. and Shaw, E., Biochemistry, 8, 4949 (1969).

35. Blake, A.J. and Weber, B.H., J. Biol. Chem., 249, 5452 (1974).

36. Belleau, B., Martel, R., Lacasse, G., Menard, M., Weinberg, N.L. and Perron, Y.G., J. Amer. Chem. Soc., 90, 823 (1968).

37. Belleau, B. and Malek, G., J. Amer. Chem. Soc., 90, 1651 (1968).

38. Belleau, B., DiTullio, V. and Godin, D., Biochem. Pharmacol., 18, 1039 (1969).

39. Yajima, H. and Kawatani, Chem. Pharm. Bull., 19, 1905 (1971).

40. Mercer, J.F.B., and Symons, R.H., Biochim. Biophys. Acta, 238, 27 (1971).

41. Chang, K. J., Moran, J.F. and Triggle, D.J., Pharmacol. Res. Commun., 2, 63 (1970).

42. Sheehan, J.C. and Hlavka, J.J., J. Org. Chem., 21, 439 (1956).

43. Sheehan, J.C. and Hlavka, J.J., J. Amer. Chem. Soc., 79, 4528 (1957).

44. Hoare, D.G. and Koshland, Jr., D.E., J. Amer. Chem. Soc., 88, 2057 (1966).

45. Hoare, D.G. and Koshland, Jr., D.E., J. Biol. Chem., 242, 2447 (1967).

46. Carraway, K.L. and Koshland, Jr., D.E., "Methods in Enzymology," Vol. 25, 616 (1972).

47. Carraway, K.L. and Triplett, R.B., Biochim. Biophys. Acta, 200, 566 (1970).

48. Ho, N.W.Y. and Gilham, P.T., Biochemistry, 10, 3651 (1971).

49. Riehm, J.P. and Scheraga, H.A., Biochemistry, 5, 99 (1966).

50. Carraway, K.L. and Koshland, Jr., D.E., Biochim. Biophys. Acta, 160, 274 (1968).

51. Lewis, S.D. and Shafer, J.A., Biochim. Biophys. Acta, 303, 284 (1973).

52. Lin, T.-Y. and Koshland, Jr., D.E., J. Biol. Chem., 244, 505 (1969).

53. Horinishi, H., Nakaya, K., Tani, A. and Shibata, K., J. Biochem. (Tokyo), 63, 41 (1968).

54. Atassi, M.Z. and Rosenblatt, M.C., J. Biol. Chem., 249, 4802 (1974).

55. Wilchek, M., Frensdorff, A. and Sela, M., Biochemistry, 6, 247 (1967).

56. Ozawa, H., Biochemistry, 9, 2158 (1970).

57. Banks, T.E., Blossey, B.K. and Shafer, J.A., J. Biol. Chem., 244, 6323 (1969).

58. Eyl, A. and Inagami, T., Biochem. Biophys. Res. Commun., 38, 149 (1970).

59. Radhakrishnan, T.M., Walsh, K.A. and Neurath, H., Biochemistry, 8 4020 (1969).

60. Roufogalis, B.D. and Wickson, V.M., J. Biol. Chem., 248, 2254 (1973).

61. Lin, T.-Y., Biochemistry, 9, 984 (1970).

62. Armstrong, J.M. and McKenzie, H.A., Biochim. Biophys. Acta, 147, 93 (1967).

63. Swaisgood, H. and Natake, M., J. Biochem. (Tokyo), 74, 77 (1973).

64. Riordan, J.F. and Hayashida, H., Biochem. Biophys. Res. Commun., 41, 122 (1970).

65. Gray, C.J. and Jolley, M.E., FEBS Lett., 29, 197 (1973).

66. DeToma, F. and Abeles, R.H., Fed. Prod. Fed. Amer. Soc. Exp. Biol., 29, 461 (1970).

67. Franzblau, C., Gallop, P.M. and Seifter, S., Biopolymers, 1, 79 (1963).

68. Wurtz, A. and Bouchut, E., Compt. Rend., 89, 425 (1879).

69. Hwang, K. and Ivy, A.C., Ann. N.Y. Acad. Sci., 54, 161 (1951).

70. Willstätter, R. and Grussman, W., Z. Physiol. Chem., 138, 184, (1924).

71. Kimmel, J.R. and Smith, E.L., Federation Proc., 13, 241 (1954).

72. Kimmel, J.R. and Smith, E.L., J. Biol. Chem., 207, 515 (1954).

73. Balls, A.K. and Lineweaver, H.J., J. Biol. Chem., 130, 669 (1939).

74. Glazer, A.N. and Smith, E.L. in P.D. Boyer, ed., "The Enzymes," 3rd ed., Academic Press, New York, Vol. 3, p. 501 (1971).

75. a. Brocklehurst, K. and Little, G., Biochem. J., 133, 67 (1973). b. Liener, I.E., Adv. in Chem. Series, No. 136, "Food Related Enzymes," p. 202 (1974). c. Brocklehurst, K. and Kierstan, P.J., Nature New Biol., 242, 167 (1973).

76. Finkle, B.J. and Smith, E.L., J. Biol. Chem., 230, 669 (1958).

77. Glazer, A.N. and Smith, E.L., J. Biol. Chem., 240, 201 (1965).

78. Klein, I.B. and Kirsch, J.F., Biochem. Biophys. Res. Commun., 34, 575 (1969).

79. Blumberg, S., Schechter, I. and Berger, A., Eur. J. Biochem., 15, 97 (1970).

80. Sluyterman, L.A. AE. and Wijdenes, J., Biochim. Biophys. Acta, 200, 595 (1970).

81. Hall, P.L., Anderson, C.D. and Crawford, Jr., G.D., Arch. Biochem. Biophys., 153, 162 (1972).

82. Mitchel, R.E.J., Chaiken, I.M. and Smith, E.L., J. Biol. Chem., 245, 3485 (1970).

83. Drenth, J., Jansonius, J.N., Koekoek, R., Sluyterman, C.A.A., and Wolthers, B.J., Phil. Trans. Roy. Soc. London, Ser. B., 257, 231 (1970).

84. Stockell, A. and Smith, E.L., J. Biol. Chem., 227, 1 (1957).

85. Lowe, G., Phil. Trans. Roy. Soc. London, Ser. B., 257, 247 (1970).

86. Sluyterman, L.A. AE. and Wolthers, B.G., Proc. Konjukl. Ned. Akad. Wetensch. Proc. Ser. B., 72, 14 (1969).

87. Drenth, J., Jansonius, J.N., Koekoek, R. and Wolthers, B.G., in P.D. Boyer, ed., "The Enzymes," 3rd ed., Academic Press, New York, Vol. 3, p. 485 (1971).

88. Wolthers, B.G., Drenth, J., Jansonius, J.N., Koekoek, R. and Swen, H.M. in P. Desnuelle, H. Neurath, and M. Ottesen, eds., "Structure-Function Relationships of Proteolytic Enzymes," Academic Press, New York, p. 272 (1970).

89. Berger, A. and Schechter, I., Phil. Trans. Roy. Soc. London B 257, 249 (1970).

90. Glazer, A.N. and Smith, E.L., J. Biol. Chem., 236, 2948 (1961).

91. Husain, S.S. and Lowe, G., Biochem. J., 114, 279 (1969).

92. Schechter, I. and Berger, A., Biochem. Biophys. Res. Commun., 32, 898 (1968).

93. a. Alecio, M.R., Dann, M.L. and Lowe, G., Biochem. J., 141, 495 (1968). b. Lowbridge, J. and Fruton, J.S., J. Biol. Chem., 249, 6754 (1974).

94. Smith, E.L. and Parker, M.J., J. Biol. Chem., 233, 1387 (1958).

95. Whitaker, J.R. and Bender, M.L., J. Amer. Chem. Soc., 87, 2728 (1965).

96. Williams, A., Lucas, E.C., Rimmer, A.R. and Hawkins, H.C., J. Chem. Soc. Perkin Trans., 627 (1972).

97. Sluyterman, L.A. AE., Biochem. Biophys. Acta, 85, 305 (1964).

98. Smith, E.L., Chavré, V.J. and Parker, M.J., J. Biol. Chem., 230, 283 (1958).

99. Williams, D.C. and Whitaker, J.R., Biochemistry, 6, 1047 (1967).

100. Finkle, B.J. and Smith, E.L., J. Biol. Chem., 207, 551 (1958).

101. Light, A., Frater, R., Kimmel, J.R. and Smith, E.L., Proc. Natl. Acad. Sci. U.S., 52, 1276 (1964).

102. Light, A., Biochem. Biophys. Res. Commun., 17, 781 (1964).

103. Sluyterman, L.A. AE., Biochim. Biophys. Acta, 151, 178 (1968).

104. Chaiken, I.M. and Smith, E.L., J. Biol. Chem., 244, 5087 (1969).

105. Whitaker, J.R., Biochemistry, 8, 4591 (1969).

106. Shapira, E. and Arnon, R., J. Biol. Chem., 244, 1026 (1969).

107. Kimmel, J.R., Rogers, H.J. and Smith, E.L., J. Biol. Chem., 240, 266 (1965).

108. Wallenfels, K. and Eisele, B., Eur. J. Biochem., 3, 267 (1968).

109. Anderson, B.M. and Vasini, E.C., Biochemistry, 9, 3348 (1970).

110. Whitaker, J.R. and Perez-Villaseñor, J., Arch. Biochem. Biophys., 124, 70 (1968).

111. Shaw, E., Cuia, M.M. and Cohen, W., Biochemistry, 4, 2219 (1965).

112. Wolthers, B.G., FEBS Lett., 2, 143 (1969).

113. Bender, M.L. and Brubacher, L.J., J. Amer. Chem. Soc., 88, 5880 (1966).

114. Husain, S.S. and Lowe, G., Chem. Commun., 345 (1965).

115. Husain, S.S. and Lowe, G., Biochem. J., 108, 855 (1968).

116. Husain, S.S. and Lowe, G., Biochem. J., 110, 53 (1968).

117. Husain, S.S. and Lowe, G., Biochem. J., 117, 333 (1970).

118. Lowe, G. and Williams, A., Biochem. J., 96, 194 (1965).

119. Drenth, J., Jansonius, J.N., Koeboek, R., Swen, H.M. and Wolthers, B.G., Nature (London), 218, 929 (1968).

120. Shields, G.S., Hill, R.L. and Smith, E.L., J. Biol. Chem., 234, 1747 (1959).

121. Sluyterman, L.A. AE., Wijdenes, J. and Wolthers, B.G., Biochem. Biophys. Acta, 173, 392 (1969).

122. Chaiken, I.M. and Smith, E.L., J. Biol. Chem., 244, 4247 (1969).

123. Silman, I.H. and Katchalski, E., Ann. Rev. Biochem., 35, 873 (1966).

124. Menecko, G. and Günzel, G., Naturwissenschaften, 54, 647 (1967).

125. Murachi, T. and Okumura, K., FEBS Lett., 40, 127 (1974).

126. Klages, A. and Haak, O., Ber. Deut. Chem. Ges., 36, 1646 (1903).

127. Fieser, L.F. and Fieser, M., "Reagents for Organic Synthesis," Vol. II, John Wiley and Sons Inc., New York, p. 191 (1969).

128. Sheehan, J.C., Cruickshank, P.A. and Boshart, G.L., J. Org. Chem., 26, 2525 (1961).

129. Harris, G. ed. "Dictionary of Organic Compounds," 4th ed., Vol. 5, Oxford University Press, New York, N.Y., p. 3126 (1965).

130. Greenstein, J.P. and Winitz, M., "Chemistry of the Amino Acids," Vol. 2, John Wiley and Sons, New York, N.Y., p. 1023, Vol. 3, (1961).

131. Hay, R.W. and Morris, P.J., J. Chem. Soc. A, 1518 (1971).

132. Schröder, E. and Gibian, H., Ann., 656, 190 (1962).

133. Hofmann, K., Haas, W., Smithers, M.J. and Zanetti, G., J. Amer. Chem. Soc., 87, 631 (1965).

134. Wilson, B.D. and Burness, D.M., J. Org. Chem., 31, 1565 (1966).

135. Yoon, N.M., Pak, C.S., Brown, H.C., Krishnamurthy, S., and Stocky, T.P., J. Org. Chem., 38, 2786 (1973).

136. Hammick, Jr., P.S. and Hawser, C.R., J. Org. Chem., 26, 4199 (1961).

137. Le Quesne, W.J. and Young, G.T., J. Chem. Soc., 1954 (1950).

138. Bray, G., Anal. Biochem., 1, 279 (1960).

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

140. Beckman Instruments, Inc., Model 121 Automatic Amino Acid Analyzer Instruction Manual, Sect. 5-6, Spinco Division, Palo Alto, California.

141. Ford, S.G. and Marvel, C.S., Org. Syn., 10, 62 (1930).

142. Huisgen, R. and Reinert-Shober, J., Justus Liebigs Ann. Chem., 575, 174 (1952).

143. Limaye, D.B. and Pause, T.B., Rasayanam, 2, 32 (1950).

144. Uhle, F.C., J. Org. Chem., 26, 2998 (1961).

145. Kondo, Y. and Witkop, B., J. Org. Chem., 33, 206 (1968).

146. Brown, H.C., Mead, E.J. and Subba Rao, B.C., J. Amer. Chem. Soc., 77, 6209 (1955).

147. Bowden, K. and Hardy, M., Tetrahedron, 22, 1169 (1966).

148. Yonemitzu, O., Hamada, T. and Kanaoka, Y., Tetrahedron Lett., No. 32, 3775 (1968).

149. Yonemitzu, O., Hamada, T. and Kanaoka, Y., Chem. Pharm. Bull., 17, 2075 (1969).

150. Sigler, P., Matthews, B.W., Henderson, R. and Blow, D.M., J. Mol. Biol., 35, 143 (1968).

151. Kurzer, F. and Douraghi-Zadeh, K., Chem. Rev., 67, 107 (1967).

152. Hall, P.L. and Anderson, C.D., Biochemistry, 13, 2082 (1974).

153. Fink, A.L. and Gwyn, C., Biochemistry, 13, 1190 (1974).

154. Hendrickson, R.L., Stein, W.H., Crestfield, A.M. and Moore, S.J., J. Biol. Chem., 240, 2921 (1965).

155. Knorre, D.G., Kurbatov, V.A., Mushinskaya, G.S. and Sailovich, E.G., Siberian Chemistry Journal, 3, 395 (1967).

156. Murachi, T., Tsudzuki, T. and Okumura, K., Biochemistry, 14, 249 (1975).

The vita has been removed from the scanned document

CHEMICAL MODIFICATION OF CATALYTICALLY ESSENTIAL FUNCTIONAL GROUPS IN THE ACTIVE SITE OF PAPAIN

by

Randolph B. Perfetti

(ABSTRACT)

A new technique for conversion of carboxylic acids to carbinols under mild conditions has been developed. This technique requires first formation of enol esters by coupling N-ethyl-5-phenylisoxazolium-3'-sulfonate with an appropriate carboxylic acid in acetonitrile, followed by reduction with a 10-fold molar excess of $NaBH_4$.

In addition five carboxyl group "specific" reagents were surveyed for their ability to inactivate the sulfhydryl proteinase papain (EC 3.4.4.10). No inactivation of the enzyme was observed with three of the reagents, diazoacetamide, triethyloxonium tetrafluoroborate, and N-ethyl-5-phenylisoxazolium-3'-sulfonate. The remaining two reagents, N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide did indeed inactivate papain. Inactivation by N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline appears to be the result of reaction of this reagent with the free thiol group of the enzyme. Treatment of papain with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide resulted in modification of the free thiol group, in 6 to 10 of the nineteen tyrosyl groups, and in six of the fifteen free carboxyl groups. The free thiol group could be essentially completely protected from reaction by converting it to its mercuric derivative

with $HgCl_2$, and the tyrosyl modification was shown to have no effect on enzymatic activity. Reaction of active or mercuripapain with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide in the presence of glycine ethyl ester resulted in incorporation of 6 ethyl glycinate residues per molecule of enzyme, with essentially complete inactivation. In the presence of benzamidoacetonitrile, a competitive inhibitor of papain, only four ethyl glycinate residues were incorporated into active papain with retention of ~ 80% of enzymatic activity, thus establishing that at least one and perhaps two of the six modified carboxyl groups were located in the active site of the enzyme. The identity of one of these carboxyl groups was postulated to be Asp-158, and thus some evidence was provided for the tentative assignment of a direct mechanistic role in catalysis for this amino acid residue.