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The Purification, Properties and Chemical Kinetics of Cathepsin B1

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THE PURIFICATION, PROPERTIES AND CHEMICAL KINETICS OF CATHEPSIN B1

by

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This Dissertation is dedicated to
my parents, Stanley and Helen,
and to my brothers and sisters,
Barbara, Thomas, Paul, Mark,
Mary, James and John

VITA

The author, Andrew Stanley Bajkowski, is the son of Stanley Andrew Bajkowski and Helen (Zalewski) Bajkowski. He was born July 2, 1950 in Chicago, Illinois.

His elementary education was obtained at a private school in Bellwood, Illinois, and his secondary education at Weber High School, Chicago, Illinois, where he graduated in 1968.

In September 1968, he entered Loyola University of Chicago and in June 1972, received a Bachelor of Science degree in Chemistry. He was an Illinois State Scholarship recipient between the years 1968 and 1972. He has been a member of the American Chemical Society since 1970.

In September of 1972 he joined the Department of Biochemistry and Biophysics at Loyola University of Chicago as a Ph.D. candidate in Biochemistry. In November of the same year he elected to work in the laboratory of Dr. Allen Frankfater. From January 1974 to June 1974 he served as a Graduate Teaching assistant and from July 1975 to September 1976 as a Research Fellow in that Department. In 1975 he was awarded the Presidential Medallion of Loyola University.

In 1978 he received the Masters of Management Degree from the Graduate School of Management, Northwestern University with majors in Marketing and Finance. From 1976 to

1978 he held the position of Senior Research Associate at the Center for the Interdisciplinary Study of Science and Technology, Northwestern University. His studies included R & D program development, project selection criteria, commercial feasibility of technological innovations, long-range planning, project monitoring systems, and issues involved in the management of professionals.

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BAA	alpha-N-Benzoyl-L- arginine amide
BAEE	alpha-N-Benzoyl-L- arginine ethyl ester
BANA	alpha-N-Benzoyl-L- arginine 2-naphthylamide
BAPA	alpha-N-Benzoyl-D,L-arginine p-nitroanilide
BIS	Methylenebisacrylamide
BSA	Bovine Serum Albumin
CBZ	Benzyloxycarbonyl
CGN	alpha-N-Benzyloxycarbonylglycine p-nitrophenyl ester
CLBE	alpha-N-Benzyloxycarbonyl-L-lysine benzyl ester
CLME	alpha-N-Benzyloxycarbonyl-L-lysine methyl ester
CLN	alpha-N-Benzyloxycarbonyl-L-lysine p-nitrophenyl ester
DMSO	Dimethylsulfoxide
DTNB	5,5'Dithiobis(2-nitrobenzoate)
DTT	Dithiothreitol
EDTA	(Ethylene dinitrilo)- tetraacetate
k_{cat}	Catalytic rate constant
K_m	Michaelis constant
PCMB	p-Chloromercuribenzoate
t-Boc	t-Butoxycarbonyl
TEMED	N,N,N',N'-Tetramethylethylene diamine
TNB	2-nitro-5-thiobenzoic acid
V	Velocity
V_{max}	Maximum velocity

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CHAPTER I

INTRODUCTION

A. Properties of Cathepsin B1

Cathepsin B1 is a sulfhydryl proteinase of approximately 25,000 molecular weight and is located in the lysosomes of mammalian cells (1). The enzyme requires the presence of chelating agents such as EDTA and thiols for maximal activity (2), and is inhibited by heavy metals, organomercurials, N-ethylmaleimide, and alkylating reagents such as iodoacetate and N-tosyl-L-lysyl chloromethyl ketone (2, 3). The enzyme is both an endopeptidase (4) and an esterase (1) and displays optimal activity toward both proteins and synthetic substrates in the acidic pH range. Of the various ester and amide substrates for cathepsin B1, the enzyme shows a preference for alpha-N-substituted derivatives of the basic amino acids, lysine and arginine.

Because of these properties, cathepsin B1 has been classified as an acidic sulfhydryl proteinase. Members of this class include papain and chymopapain from the papaya latex, ficin from the fig latex, bromelain from the stem and fruit of the pineapple, and streptococcal proteinase which is secreted by the Streptococcus pyrogenes bacteria (97). The plant thiol proteases have been the subjects of exten-

sive investigation and have produced a large body of information which can be useful in studies of the properties of cathepsin B1.

Cathepsin B1 was first isolated by Otto (5) from bovine spleen as a new enzyme which hydrolyzed both BAA and BAPA at an acid pH. Cathepsin B1 has subsequently been isolated from other bovine (6-8) and human tissues (9-12). Cathepsin B1 from these different tissues share some common characteristics. (1) They possess many of the properties attributable to the acidic sulfhydryl proteinases. (2) Yields of highly purified samples of cathepsin B1 are extremely low. (3) The purified enzyme appears to be a mixture of related proteins which have been classified as isoenzymes. The number of cathepsin B1 species isolated have ranged from three in bovine thyroid glands (13) to six in human liver (9). (4) Major differences between the chemical properties of the enzyme across tissue types do not appear to exist. (5) At the present time, knowledge of the physiological role of cathepsin B1 in cellular homeostasis is incomplete.

B. Measurement of the Cathepsin B1 Activity

In addition to hydrolyses of BAA and BAPA, cathepsin B1 has been detected on the basis of its ability to hydrolyze certain esters and amides of other N-substituted basic amino acids. Common synthetic substrates include BAEE, BANA

and alpha-N-benzoyl-L-lysine amide (2). Protease activity has been determined with denatured hemoglobin (14), edestin (15), azo-casein (16) and the oxidized B-chain of insulin. (4). Cathepsin B1 can also catalyze the conversion of trypsinogen to trypsin (2, 17).

Most of these substrates are not specific for cathepsin B1 (2). Many are insensitive owing to the poor reactivity of the substrate and many require prolonged incubation times at elevated temperatures. Some assays involve complicated and time consuming techniques, and several give high blanks with tissue homogenates and crude enzyme preparations.

Thus, BAA is known to be hydrolyzed by both cathepsin B1 and B2, and BAEE is hydrolyzed by a number of distinct components of tissue extracts (18). Similarly, it has recently been shown that BANA hydrolase activity does not parallel BAPA hydrolase activity during the purification of cathepsin B1 from rat liver, kidney and spleen (2). It has, therefore, been suggested that BANA hydrolase activity of cathepsin B1 preparations is due, at least in part, to the presence of a contaminant which has been termed alpha-N-benzoylarginine-2-naphthylamide hydrolase (19). Similar conclusions have been reached in recent studies with rat skin tissue extracts (20).

The specificity of cathepsin B1 against the oxidized B-chain of insulin, a polypeptide substrate, has been stud-

ied (4). The pattern of peptide bonds cleaved was different from that expected on the basis of the apparent specificity of cathepsin B1 toward small, synthetic, amino acid ester and amide substrates (2, 4). Thus, with cathepsin B1 as with papain and other proteinases, specificity toward polypeptide substrates may largely be determined by interactions between secondary binding sites on the enzyme and amino acid residues of the substrate distal from the labile peptide bond (4, 21, 22). These secondary interactions have not yet been identified for cathepsin B1.

C. Physiological Role of Cathepsin B1

In recent years a large body of evidence has accumulated directly linking tissue proteinases to a variety of normal and pathological processes. These enzymes are believed to participate in normal turnover of tissues and extracellular proteins (23-25), are involved in degenerative diseases (26-31), and are possible initiators and probable potentiators of acute and chronic inflammatory processes (30, 32-38). Such diverse disorders as hepatic fibrosis, emphysema and rheumatoid arthritis are each associated with inflamation, tissue degeneration, and concomitant formation of granulation or scar tissue. These enzymes may also contribute in the invasive behavior of malignant tumors (39, 40).

Cathepsin B1 has been demonstrated in inflamed tissues (41) and has been shown to generate vasoactive pep-

tides from inactive precursors (42, 43). It has been reported to be able to extensively digest collagen whereas purified cathepsin D, another lysosomal protease, cannot (44). Cathepsin B1 has been shown to degrade proteoglycans from adult articular cartilage (45) and can inactivate or otherwise modify several of the gluconeogenic and glycolytic enzymes from liver (2, 46). Futhermore, cathepsin B1 has been reported to be more important than cathepsin D in lysosomal protein degradation at pH 5.0 (47). Studies with WR-1339-filled lysosomes have shown that cathepsin B1 (or possibly another thiol proteinase) is the most important enzyme in the degradation of native albumin (47), native and performic acid-oxidized ribonuclease, cytochrome C, and horseradish peroxidase (48).

Inhibitors of cathepsin B1 include human alpha-2 macroglobulin and immunoglobulin G (49), leupeptin and chymostatin from Streptomyces (50), and an inhibitor from chicken egg white (51).

D. Properties of Papain

Since cathepsin B1 appears to be related to the well-studied family of thiol proteineses from plants and bacteria, information obtained with these enzymes can greatly simplify studies of cathepsin B1. Verification of a structural and functional homology between cathepsin B1 and papain would permit us to adopt this large body of information about

the plant and bacterial thiol proteinases to cathepsin B1. Since our understanding of the mechanism of thiol proteinases is still incomplete, a comparison of papain and cathepsin B1, enzymes distant from each other on the evolutionary scale, might permit abstraction of only those properties common to this class of enzymes and thus essential for catalysis. This could significantly enhance our understanding of the mechanism of action of thiol proteinases.

The primary sequence and location of disulfide bonds have been determined for papain (52). The native enzyme contains only one free cysteine residue. Evidence for the single cysteine comes from chemical modification by alkylating reagents and by heavy metal ions which show that this cysteine is essential for catalysis (53). The active site of papain also contains a histidine residue as shown by the reaction of the enzyme with the bifunctional alkylating agent, 1,3-dibromoacetone (54, 55). In this reaction, the active cysteine is intramolecularly crosslinked with the imadizole of a histidine thereby producing a catalytically inactive enzyme. The presence of a third group with a $pK_a = 4$ (56-58) was inferred from the pH dependency of the rate of enzyme-catalyzed hydrolysis. Such results suggested the presence of a ionized carboxylic acid such as aspartate or glutamate in the active site of the enzyme. A fourth residue known to be present in the active site of papain is the indole group of a tryptophan.

Fluorescence studies of papain have shown that a tryptophan residue is in sufficient proximity to the active site cysteine and histidine residues to participate in catalysis. Addition of a mercury atom to the sulfhydryl group (59) or protonation of the imidazole ring of the histidine residue (60) results in quenching of tryptophan's fluorescence.

Thus, physical and chemical evidence suggests that the side chains of a histidine, an aspartate (or glutamate), a tryptophan and a cysteine residue are present in the active site of papain. These conclusions have been confirmed by x-ray crystallography at a resolution of 2.8 A^{o} (61).

E. Mechanism of Catalysis of Papain

The mechanism of catalysis by cathepsin B1 is still uncertain. In contrast, the papain catalyzed hydrolyses of ester and amide substrates have been shown to proceed through the formation of an acyl-enzyme intermediate in which the carbonyl carbon of the substrate becomes attached via a thiolester bond to the essential cysteine residue in the enzyme (acylation step). Hydrolysis of this thiolester linkage results in the regeneration of the free enzyme and the release of the carboxylic acid product (deacylation step) (58, 62, 63). In addition to water, other nucleophiles may participate in the deacylation step (64). This mechanism is summarized by Equation 1.

$$ESH + RCOX \underset{}{\overset{K_s}{\rightleftharpoons}} ESH \cdot RCOX \xrightarrow{k_2} ES\text{-}\overset{O}{\overset{\|}{C}}\overset{+}{X}\text{-}R \xrightarrow{k_3'(H_2O)} ESH + RCOOH \qquad (1)$$

In this equation K_s is the dissociation constant for the non-covalent enzyme substrate complex, k_2 is the rate constant for the formation of the covalent complex, and k_3 is the rate constant for the decomposition of this complex.

Direct evidence for the formation of a covalent intermediate in papain catalysis has been obtained spectrophotometrically with a variety of substrates. In order to detect the pre-steady state accumulation of an acyl enzyme intermediate during substrate hydrolysis, the following conditions must be fulfilled. First, the intermediate must have a different absorption spectrum from that of the starting substrate or final product. Second, the rate of formation of the intermediate must equal or exceed its rate of decomposition, so that measurable quantities of the intermediate will accumulate during the reaction. It is also helpful if the rate of formation of the intermediate is sufficiently slow so that its accumulation may be monitored by conventional spectrophotometric methods thereby obviating the need for special techniques. This latter condition can sometimes be fulfilled by monitoring the reaction at a pH removed from the optimum pH of catalysis. Finally, if the rate of decomposition of the intermediate is sufficiently slow, it may be possible to isolate it from the reaction mixture by chrom-

atography on Sephadex G-25 and study its rate of decomposition to products independently of its rate of formation.

For papain, a variety of substrates have been shown to fulfill many of the above requirements. These include N-trans-cinnamoylimidazole and methylthionohippurate (62, 63), several nitrophenyl esters of N-substituted amino acids such as N-benzyloxycarbonyl glycinate and lysinate (18, 65), and p-nitrophenyl-p'-guanidinobenzoate, an active site titrant of trypsin (66).

Indirect evidence for the formation of a covalent enzyme-substrate intermediate has been inferred from the observation that substrates derived from the same amino acid and differing only in the identity of the leaving group, are hydrolyzed with identical rates. This suggests that a common acyl-enzyme intermediate is formed whose hydrolysis is in each case rate limiting (64). The observation that the papain catalyzed hydrolysis of the p-nitrophenyl, m-nitrophenyl, o-nitrophenyl, phenyl, and ethyl esters of N-benzyloxycarbonyl glycinate occur with identical rate constants has been used to infer a common benzyloxycarbonylglycine-papain covalent intermediate in the hydrolysis of these substrates (64).

Evidence for a covalent intermediate can also be inferred from steady state kinetic studies in the presence of added nucleophiles. Enzymes such as papain can transfer a portion of the substrate molecule (acyl group) from a

donor group (alcohol or amine) to an acceptor group (water or other nucleophiles such as ammonia, aliphatic and aromatic alcohols, amines, amino acids, and small peptides) (64). In the presence of nucleophiles, Equation 1 becomes

$$ESH + RCOX \underset{}{\overset{K_s}{\rightleftharpoons}} ESH \cdot RCOX \xrightarrow{k_2} \underset{+\ X}{ES\text{-}\overset{O}{\overset{\|}{C}}\text{-}R} \begin{cases} \xrightarrow{k_3'(H_2O)} ESH + RCOOH \\ \xrightarrow{k_4(NH)} ESH + RCON \end{cases} \quad (2)$$

The mechanism represented by Equation 2 predicts the following rate equations for cathepsin B1 when one monitors the release of the first product, X, (Appendix A).

$$V = \frac{\dfrac{k_2\left[k_3' + k_4(NH)\right]}{k_2 + k_3' + k_4(NH)}(E_o)}{\left[\dfrac{k_3' + k_4(NH)}{k_2 + k_3' + k_4(NH)}\right]\dfrac{K_s}{(S)} + 1} \quad (3)$$

In this equation $k_3' = k_3'(H_2O)$. From Equation 3 we can obtain the following relationships.

$$(V_{max})_{NH} = \frac{k_2\left[k_3' + k_4(NH)\right](E)_o}{k_2 + k_3' + k_4(NH)} \quad (4)$$

$$(K_m)_{NH} = \frac{k_3' + k_4(NH)}{k_2 + k_3' + k_4(NH)} K_s \quad (5)$$

$$\left[\frac{K_m}{V_{max}}\right]_{NH} = \left[\frac{K_m}{V_{max}}\right]_{NH=0} = \frac{K_s}{k_2(E)_o} \quad (6)$$

Equation 4 predicts that if substrate hydrolysis is deacylation rate limiting (i.e., $k_2 \gg k_3 + k_4(NH)$) and if the reactivity of the nucleophile is greater than that of water, then V_{max} should increase with increasing nucleophile concentration. If k_2 is very much greater than $k_3 + k_4(NH)$, Equation 4 becomes

$$(V_{max})_{NH} = k_3 (E)_o + k_4 (E)_o(NH) \qquad (7)$$

In this equation $k_3 (E)_o$ equals V_{max} in the absence of nucleophiles. In contrast to V_{max}, Equation 6 indicates that K_m / V_{max} is independent of nucleophile concentration being essentially constant.

Equations 2 through 7 predict that in the presence of added nucleophiles one may observe "ping-pong" or parallel-line kinetics in double reciprocal plots of reaction rates against substrate concentration (68). Such results were observed in the papain catalyzed hydrolysis of CGN in the presence and absence of MeOH. Hydrolysis of CGN by papain was deacylation rate limiting and in the deacylation step methanol was a 56-fold better nucleophile than water (58, 64).

Several important details about the mechanism of papain remain unclear. The suggestion that an aspartate residue participates as a general base in catalysis is highly controversial (57,58,64,67). Similarly, disagree-

ment exists about the state of ionization of the cysteine and histidine residues in the active form of papain. According to one proposed mechanism, the formation of the acyl-enzyme intermediate occurs when the unprotonated histidine residue, acting as a general base, facilitates the attack of the protonated cysteine residue on the carboxyl carbon of the substrate (54,55). An alternative mechanism proposes that the attack of the unprotonated form of the cysteine residue is facilitated by the protonated form of the histidine residue acting as a general acid (98). These mechanistic uncertainities reflect the fact that it is generally not possible to unequivocally assign a role to a particular amino acid residue at the active site on the basis of studies of the pH dependency of rate constants. Furthermore, it is not possible to know whether a particular ionization observed in such studies can be attributed to a active site residue or a residue some distance away which influences the conformation of the active site. For this reason, it may be useful to compare the properties of a variety of unrelated sulfhydryl proteinases. In this way it may be possible to abstract those catalytic properties which are common to this class of enzymes. Ideally, it is these common properties which should form the basis for any discussions of the mechanism.

Before any valid comparison of cathepsin B1 and papain can be made, it is necessary to verify that these enzymes

are functionally related. To accomplish this it is necessary to show that the reaction pathway for cathepsin B1 includes an acyl-enzyme intermediate.

F. The Goals of this Dissertation

The goals of this dissertation include the following: (1) to develop a procedure for the purification of cathepsin B1 from bovine spleen; (2) to develop new and more sensitive methods for measuring the activity of cathepsin B1; (3) to determine the specificity of cathepsin B1 toward synthetic substrates; (4) to determine the enzymatic properties and mechanism of catalysis of cathepsin B1; and (5) to verify the existence of a functional homology between cathepsin B1 and the well-studied class of plant sulfhydryl proteinases.

CHAPTER II

EXPERIMENTAL

A. Materials

1. Synthesis of Reagents and Cathepsin B1 Substrates

A number of substrates for cathepsin B1 were synthesized according to known procedures. BAEE was prepared from arginine according to the method outlined by Bergmann, et. al. (69). The melting point for BAEE-HCl was found to be 134-135°C (lit. m.p.: 135°C). Thin layer chromatography of the product on silica gel plates yielded a single spot when developed with absolute ethanol. BAA was prepared by the aminolysis of BAEE (69). The amide scintered at 120-123°C and melted at 132-133°C (lit. m.p.: 133°C). The product gave a single spot on a silica gel thin layer plate developed in absolute ethanol.

CGN was prepared by a two-step procedure. First, CBZ-glycine was prepared from glycine and benzyloxycarbonyl chloride according to Bergmann, et. al. (70). The p-nitrophenyl ester of CBZ-glycine was subsequently prepared by coupling CBZ-glycine to p-nitrophenol in dioxane at 6°C as described by Martin (71). The ester melted at 125-126°C (lit. m.p.: 126°C). Silica gel thin layer chromatography of CGN in chloroform showed a single component.

Hydrindantin, which is used in the assay of BAA activity, was prepared from ninhydrin according to the method of Moore and Stein (72). The melting point for hydrindantin was found to be 256-258°C (lit. m.p.: 255-258°C).

2. Sources of Chemicals, Tissues and Reagents

a. Substrates

BAA and CLN were obtained from Aldrich Chemical Co. CLN was also obtained from Calbiochem. t-Boc-glycine p-nitrophenyl ester, CBZ-L-isoleucine p-nitrophenyl ester, and CBZ-L-tryptophan p-nitrophenyl ester were obtained from Cyclo Chemical Co. BANA, BAPA and the p-nitrophenyl esters of t-Boc-L-asparagine, t-Boc-L-glutamine, t-Boc-L-tryptophan, CBZ-L-asparagine, and CBZ-L-tyrosine were from Schwarz Mann. BAEE, CGN, CLBE, CLME and the p-nitrophenyl esters of CBZ-L-alanine, CBZ-glycine, CBZ-L-leucine, CBZ-L-phenylalanine, and CBZ-L-valine came from Sigma Chemical Co.

b. Gel Chromatography

Sephadex G-25, Sephadex G-150, CM Sephadex C-25, DEAE Sephadex A-50 and SP Sephadex C-25 were obtained from Pharmacia Fine Chemicals. Bio Gel P-30 was from Bio Rad and CM-cellulose was from Whatman.

c. Gel Electrophoresis

Acrylamide, Alcian Blue, Bromophenol Blue, BIS, Photo-Flow 200, riboflavin and TEMED were products of Eastman Chem-

icals. Sudan Black B was obtained from Harleco; Amido Black from K and K Laboratories. Ammonium persulfate, sucrose and Trichloracetic acid were from Mallinckrodt. Fast Green Dye was obtained from Manufacturing Chemists and Coomassie Brilliant Blue from Schwarz Mann. β-alanine, Basic Fuchsin and periodic acid were products of Sigma Chemical Co.

d. Organomercurial Sepharose

Cyanogen Bromide was obtained from Aldrich Chemical Co. L-Cysteine and DTNB were products of Eastman Chemicals. Mercuric chloride was from Mallinckrodt and p-aminophenylmercuric acetate from Sigma Chemical Co. Sepharose 4B was a product of Pharmacia Fine Chemicals.

e. Molecular Weight Determination

Ribonuclease was obtained from Aldrich Chemical Co. Bovine Serum Albumin, horse myoglobin and ovalbumin were products of Nutritional Biochemical. Blue Dextran 2000 was from Pharmacia Fine Chemicals, catalase was from Sigma Chemical Co. and trypsinogen A was from Worthington.

f. Peptides

The following peptides were obtained from Aldrich Chemical Co.: L-alanylglycine, glycyl-L-glutamine, glycyl-L-methionine, glycyl-L-phenylalanine, glycyl-L-serine, and L-phenylalanylglycine. Glycylglycine was a product of Cal-

brichem. L-Glutamylglycine, glycyl-L-alanine, glycyl-L-asparagine, glycylglycine amide, glycylglycylglycine, glycyl-L-leucine, glycyl-L-lysine, glycyl-L-proline, glycyl-L-tyrosine, L-leucylglycine, D,L,-leucyl-D,L-phenylalanine, L-lysylglycine, L-methionylglycine, L-proylglycine, L-serinylglycine and L-tyrosylglycine were from Sigma Chemical Co. L-Leucyl-L-phenylalanine was obtained from Vega-Fox.

g. Other Nucleophiles

MeOH and L-tryptophan methyl ester were obtained from Aldrich Chemical Co. Hydroxylamine was a product of Fisher Scientific. Glycine ethyl ester, Methylamine, 2-phenylethylamine, tryptamine and L-tryptophan amide were from Sigma Chemical Co.

h. Miscellaneous

PCMB was obtained from J. T. Baker Co. Thin layer plates were from Brinkmann Instruments, Inc. and from Quantum Industries. Bromelain Inhibitor V was the gift of Dr. Ferenc Kézdy. Dialysis tubing and Triton X-100 were products of Fisher Scientific Co. p-Nitrophenyl-p'-guanidinobenzoate was from ICN Pharmaceuticals and EDTA was from Mallinckrodt. Tissue samples were obtained from Lincoln Packing Co. of Chicago. Ninhydrin was a product of Schwarz Mann. trans-Cinnamoylimidazole, DTT and 2-naphthylamine were from Sigma Chemical Co.

All other chemicals used in this work were analytical grade and were obtained from commercial sources. All reagents were used without further purification.

B. Methods

1. Absorbance Measurements

Absorbance measurements were performed with either a GCA/McPherson Model 707 double beam recording spectrophotometer, a Beckman DB-G grating double beam recording spectrophotometer, or with a Cary 15 double beam recording spectrophotometer. The cell compartment of each spectrophotometer was thermostated at 25 ± 0.2°C. The spectrophotometer cells had a path length of 1 cm and were made either of quartz or silica (Beckman Instruments, Inc., Pyrocell Manufacturing Company, Inc.).

2. Fluorescence Measurements

Fluorescence measurements were performed with an Aminco-Bowman ratio spectrophotofluorometer equipped with a Houston Instruments Model 2000 X-Y recorder. Cuvettes had a path length of 1 cm and were made of quartz (Beckman Instruments, Inc.).

3. Enzyme Activation

Enzyme samples were activated with DTT and EDTA in the following way. To the appropriate volume of sample (0.05-0.10 ml) at 25°C was added twice the volume of an activating solution containing 30 mM DTT and 15 mM EDTA. The activating solution was stored frozen until needed.

After a 30 min incubation the reaction was initiated by the addition of 3 ml buffer and an appropriate amount of substrate. In order to verify that a 30 min preincubation was sufficient to fully activate the enzyme, the kinetics of activation were briefly investigated. Preincubation of enzyme preparations with activating solution for 20 min at 25° and 40°C resulted in the respective expressions of 90% and 95% of cathepsin B1 activity toward CLN. After 30 min at both temperatures, cathepsin B1 was essentially fully active toward this substrate.

4. Enzyme Assay

The activity of cathepsin B1 toward a variety of substrates was determined during the course of its purification. The BAEE assays were routinely performed by the addition of 3.0 ml of a buffered substrate solution to a cuvette containing the activated sample. The substrate solution contained 0.025 M sodium acetate buffer, pH 5.1, 1 mM EDTA, and 1.10 mM BAEE. BAEE hydrolysis was monitored at 254 nm.

The CGN, CLN, BAPA and BANA assays were performed by the addition of 3.0 ml 0.025 M sodium acetate buffer, pH 5.1 containing 1 mM EDTA to the activated enzyme. The reaction was initiated by the addition of 50 μl of a stock substrate solution made up in an appropriate organic solvent. The concentration of substrates in the stock solu-

tions were typically: CLN, 5.22 mM in DMSO; CGN, 5.18 mM in acetonitrile; BAPA, 3.3 X 10^{-1} M in DMSO; and BANA, 1.71 X 10^{-1} M in N,N'-dimethylformamide. CLN and CGN hydrolyses were monitored at 326 nm and BAPA hydrolysis was monitored at 405 nm. The change in molar absorptivities for the hydrolysis of these substrates were found to be 7.58 X 10^3, 8.20 X 10^3, and 9.33 X 10^3 for CLN, CGN, and BAPA, respectively. The rates of these reactions were found to be linear with respect to enzyme concentration over a ten-fold range.

BANA was monitored spectrophotofluorometrically at an excitation wavelength of 340 nm and an emission wavelength of 404 nm. Rates of increase of fluorescence intensity were compared to a standard curve relating fluorescence intensity to 2-naphthylamine concentrations.

BAA hydrolase activity was determined by addition of a 1.0 ml solution of 5.0 X 10^{-3} M BAA in 0.025 M sodium acetate buffer, pH 5.1, containing 1mM EDTA to the activated enzyme. Ammonia was collected by the microdiffusion technique of Seligson and Seligson (73) and quantitated by the ninhydrin reaction (72). All solutions were stored at 4°C and used within two weeks except for BAA and BAEE which were made when needed.

The reactivity of cathepsin B1 toward CLN in the presence of various added nucleophiles was also investigated. All reactions were monitored by following the

release of p-nitrophenol at 326 nm below pH 7 and at 410 nm at pH 7 and above. Rate data were corrected for the spontaneous hydrolysis of CLN where necessary. Measurements above pH 7.5 were not carried out owing to a rapid spontaneous substrate hydrolysis.

The ability of cathepsin B1 to hydrolyze various esters of alpha-N-benzyloxycarbonyl-L-lysine was studied. In particular, kinetic parameters for the hydrolysis of CLN, CLME and CLBE by cathepsin B1 were determined. These assays were performed by adding 3.0 ml of 0.01 M potassium phosphate buffer, pH 6.5, containing 1 mM EDTA to the activated enzyme. The reaction was initiated by addition of 50 μl of a stock substrate solution made up in an appropriate organic solvent. The concentration of substrates in the stock solutions were: CLN, 5.36×10^{-3} M in DMSO; CLME, 2.54×10^{-1} M in acetonitrile containing 5% H_2O; and CLBE, 8.89×10^{-2} M in acetonitrile containing 5% H_2O. CLN hydrolysis was monitored at 326 nm, CLME at 224 nm and CLBE at 236 nm. For the hydrolysis of CLN the reaction was monitored on the 0-1 slidewire; for CLME on the 0-.5 slidewire, and for CLBE on the 0-.1 slidewire. The change in molar absorptivities for the hydrolysis of these substrates at pH 6.5 were found to be 5.65×10^3, 73 and 38.9 for CLN, CLME and CLBE, respectively.

Cathepsin B1 showed activity toward a number of N-substituted p-nitrophenyl esters. Table 1 shows the p-

Table 1

The Molar Absorption Coefficients of Some p-Nitrophenyl Ester Substrates

Substrates	Conc. ($\times 10^{-3}$ M)[a]	Δa_m[b]
CBZ-glycine	3.71	7816
CBZ-L-alanine	3.19	7339
CBZ-L-valine	3.41	6624
CBZ-L-leucine	2.83	6616
CBZ-L-isoleucine	2.77	6939
CBZ-L-asparagine	12.47	7726
CBZ-L-lysine	3.84	7428
CBZ-L-phenylalanine	3.21	5753
CBZ-L-tyrosine	2.84	6237
CBZ-L-tryptophan	5.08	4910
t-Boc-glycine	3.89	2577
t-Boc-tryptophan	3.70	6031
t-Boc-glutamine	3.03	7859
t-Boc-asparagine	3.38	7852

[a] Concentration of the Stock Solution.

[b] All substrates were dissolved in acetonitrile except for CLN which was dissolved in DMSO. Determined at 326 nm at 25°C. The buffer was 0.025 M sodium acetate pH 5.1, containing 1.1 mM EDTA, 0.9 mM DTT and 1.6% organic solvent.

nitrophenyl esters studied, the concentrations of the substrates in the stock solutions and the change in molar absorptivities of the substrates at pH 5.1. All substrates were dissolved in acetonitrile except for CLN which was dissolved in DMSO. To the activated enzyme was added 3.0 ml of 0.025 M sodium acetate buffer, pH 5.1, containing 1 mM EDTA. The reaction was initiated by the addition of 50 µl of the stock substrate solution. The release of p-nitrophenol was monitored at 326 nm. The rate data was corrected for the spontaneous hydrolysis of the substrate when necessary.

5. Calculation of Kinetic Constants

Kinetic constants for the cathepsin B1 catalyzed hydrolysis of N-substituted p-nitrophenyl esters and for the reaction of CLN in the presence of nucleophiles were determined by following the reactions to completion. The absorbance data were converted to rate data using the change in molar absorptivities reported in the Enzyme Assay Section. The rate data obtained were corrected for a slow spontaneous substrate hydrolysis where appropriate.

Kinetic parameters for the hydrolysis of BANA and BAPA were determined from measurements of initial rates. In each case the data were plotted according to the method of Lineweaver and Burk (74) and straight lines drawn by the method of least squares. An Olivetti Underwood Programma 101 pro-

grammable calculator and an IBM 370 Computer were used in these calculations. Programs were written by Drs. Allen Frankfater and Richard Schultz.

6. Protein Determination

Protein concentrations were generally determined by the biuret procedure (75). Occasionally, protein concentrations were also determined by the Lowry procedure (76). With highly purified samples, protein concentrations were also quantitated by measuring absorbance at 210 nm assuming an absorption coefficient of 20.5 $\pm$ 0.2 for a 1 mg/ml solution (77). Good agreement was obtained between this method and the biuret procedure. BSA was used as a protein standard.

7. pH Measurements

Measurements of pH were performed either on a Corning Model 12 Research pH Meter with a number 476051 Corning combination electrode or on a Radiometer Copenhagen Model 27 pH Meter and a number 502 combination electrode.

8. Column Chromatography

Column chromatography was performed at 4°C in jacketed columns supplied by Pharmacia Fine Chemicals. All columns were equipped with end piece adapters except for the 9 mm diameter columns for which adapters were not available.

Sephadex G-150 SP-Sephadex C-25, DEAE Sephadex A-50, and BioRad P-25 were allowed to swell at room temperature

for two days in the desired buffer. Fines were removed with suction and the gel was poured into a suitably sized column. The column was equilibrated with 2 or 3 bed volumes of degassed buffer before use.

The flow rate of the column was maintained with a Cheminert Metering Pump operating on compressed air at a pressure of 80 psi or through a Technicon Auto-Analyzer High-Pressure Micro Pump. The effluent of the column was monitored either with a Beckman DB-G spectrophotometer containing a flow cell (0.3 ml capacity and 1 cm light path, Beckman Instruments, Inc.) or with a LKB Uvicord II Fractionator. Fractions were collected with a Gilson Microfractionator.

9. Organomercurial Sepharose

An affinity adsorbent, organomercurial Sepharose, was prepared according to the method of Barrett (9). The author used the method of Axen and Ernback (78) to activate the Sepharose 4B with cyanogen bromide and the method of Sluyterman and Wijdenes (79) to couple 4-aminophenylmercuric acetate to the insoluble support.

a. Activation of Sepharose 4B

A 200 g sample of Sepharose 4B gel was washed well with water on a Buchner funnel fitted with a Whatman number 50 filter paper and then suspended in 200 ml of water with constant stirring. A solution containing 8 g of cyanogen bro-

mide in 320 ml of water was then added to the Sepharose 4B slurry. The pH was raised to 11 and then maintained at that pH for 6 min by the addition of 2 M NaOH. The gel was then washed with 2 liters of 0.10 M $NaHCO_3$ at 4°C in a Buchner funnel fitted with Whatman number 50 filter paper.

b. Coupling of 4-Aminophenylmercuric Acetate to the Activated Sepharose 4B

The activated Sepharose 4B was suspended in 400 ml of 10% (v/v) DMSO and a solution containing 0.52 g of 4-aminophenylmercuric acetate in 40 ml of DMSO was slowly added with constant stirring. The reaction was allowed to continue for 16 hr at 4°C. The coupled Sepharose was then transferred to a chromatographic column (5 cm by 100 cm) and washed with 4 liters of a 20% solution (v/v) of DMSO at a flow rate of 12 ml/hr. This procedure and subsequent work with the affinity adsorbent were performed at 4°C. The affinity adsorbent was washed with DMSO until the absorbance of the effluent at 280 nm fell to 0.10.

c. Pretreatment of the Organomercurical Sepharose

Since the adsorbent contains both reversible and irreversible binding sites, it was necessary to eliminate the irreversible binding sites by reacting them with cysteine. The adsorbent in the chromatographic column was treated with 2 bed volumes of 10 mM cysteine in 0.05 M sodium acetate buffer, pH 5.5, containing 0.2 M NaCl and 1 mM EDTA. The flow rate was 12 ml/hr. The cysteine mercaptide form of the adsorbent was then con-

verted back to its free mercurial form by passing through the column 2 bed volumes of 10 m$\underline{M}$ $HgCl_2$ in 0.05 $\underline{M}$ sodium acetate buffer, pH 4.8, containing 20 m$\underline{M}$ EDTA. Excess $HgCl_2$ was removed fron the column by washing with 4 bed volumes of 1 m$\underline{M}$ EDTA in 0.05 $\underline{M}$ sodium acetate buffer, pH 5.5, containing 0.2 $\underline{M}$ NaCl.

d. Determination of Capacity

The capacity of the organomercurial Sepharose for sulfhydryl groups was determined with DTNB. The gel was placed on a Buchner funnel fitted with Whatman number 50 filter paper and the excess buffer removed. One gram of the damp gel was resuspended in water and poured in a chromatographic column fashioned from a Pasteur pipette containing a glass wool plug. A 5 ml solution of 0.10 $\underline{M}$ KH_2PO_4-Na_2HPO_4 buffer, pH 8.0 was passed through the column followed by a 5 ml solution containing 5 mg of DTNB and 10 mg of Na_2SO_3 in the same buffer.

After saturation with TNB, the affinity adsorbent was washed with 5 ml of 0.05 $\underline{M}$ sodium acetate buffer, pH 4.8, to remove any excess TNB. The bound TNB was eluted from the column with a 10 ml solution containing 2 m$\underline{M}$ $HgCl_2$ in 0.05 $\underline{M}$ sodium acetate buffer, pH 4.8. The effluent was diluted to 25 ml, made 0.1 $\underline{M}$ in cysteine, adjusted to pH 8.5 with 1 $\underline{M}$ NaOH and then diluted to exactly 50 ml with H_2O.

The capacity of the column for thiol groups was calculated from the absorbance at 412 nm of the solution using the absorption coefficient of 13,600 $1M^{-1}cm^{-1}$ given by Ellman (80) for the anion of TNB and Equation 8:

$$\text{Capacity } (\mu\text{mol} / g) = \frac{A_{412} \times 50}{13.6 \times \text{wt of adsorbent (g)}} \quad (8)$$

The capacity of the organomercurial Sepharose was found to be 1.54 μmoles of sulfhydryl groups per gram of damp gel.

e. Use of Organomercurial Sepharose in the Purification of Cathepsin B1

Solutions of cathepsin B1 after ion-exchange chromatography were routinely concentrated to 10 ml with an Amicon Concetrator and activated with DTT and EDTA in such a manner that the final concentrations of each were 10 mM. After 30 min the activated enzyme was applied to an anaerobic column of Sephadex G-25 medium (0.9 X 27 cm) to remove excess DTT (81). The column was equilibrated and eluted with 0.01 M potassium phosphate buffer, pH 6.5, containing 1 mM EDTA which had been degassed and purged with nitrogen gas. The flow rate of the column was 24 ml/hr and 2 ml fractions were collected.

An alternative method to remove excess DTT was also used. Activated samples were dialyzed against 4 liters of 0.01 M potassium phosphate buffer, pH 6.5, containing 1 mM EDTA for 8 hr at 4°C. The buffer was made anaerobic by

bubbling nitrogen through the solution during the dialysis. Each method gave similar results; cathepsin B1 activity losses were negligible.

The activated cathepsin B1 sample was then applied to an organomercurial column (0.9 X 27 cm) that had been equilibrated with 0.01 M potassium phosphate buffer, pH 6.5, containing 1 mM EDTA. Initially, a buffer that had been purged with nitrogen was used to elute the column, but later, it was found that a degassed buffer gave similar results. The flow rate was 12 ml/hr and 2 ml fractions were collected.

Material that did not stick to the column was shown to lack cathepsin B1 activity even when assayed in the presence of additional activating solution. Cathepsin B1 was eluted from the column with 10 mM cysteine in the phosphate buffer.

Since papain is reported to be irreversibly inactivated when allowed to oxidize in the presence of cysteine (90), excess cysteine was removed by passing the enzyme through an anaerobic Sephadex G-25 column or by dialysis under nitrogen, as described previously. It was later determined that such a precautionary step had little effect on the stability of cathepsin B1. After several months in the presence of cysteine or 2-mercaptoethanol, cathepsin B1 remained fully active when assayed in the presence of activating solution.

The organomercurial Sepharose was regenerated by eluting the column with 2 bed volumes of 0.05 M sodium acetate buffer, pH 4.8, containing 10 mM $HgCl_2$ and 20 mM EDTA.

Excess $HgCl_2$ was finally removed by washing the column with several bed volumes of 0.01 M potassium phosphate buffer, pH 6.5, containing 1mM EDTA.

10. Gel Electrophoresis

Gel Electrophoresis was used to monitor the purification of cathepsin B1, to separate various isoenzymes of cathepsin B1 and to determine some chemical properties of this enzyme. The method of Reisfeld, et. al. (82) for basic proteins was used in this study. Several minor modifications of this procedure were found to improve the resolution of cathepsin B1. These modifications included: decreasing the current to 2 mamps per tube; increasing the duration of the electrophoresis to 135 min; and changing the procedure for applying the sample as will be described.

a. Preparation of Polyacrylamide Gels

Glass Electrophoresis tubes (5 X 100 mm) were soaked overnight in either a 0.5% solution of Triton X-100 or Photo-Flow 200 and then air-dried. The lower ends of the tubes were sealed with Parafilm and the tubes were placed in a gel preparation rack. The stock solutions needed for the polyacrylamide gels are given in Table 2; buffer solutions are given in Table 3.

The separation gel was prepared by combining 6 ml of Solution A1, 3 ml of Solution B1, 12 ml of Solution C1 and 3 ml of H_2O in an Erlenmeyer flask. One ml of the mixture

Table 2

Reagents and Stock Solutions for

Polyacrylamide Gel Electrophoresis

Separation Gel:

Solution A1

Acrylamide	15 g
BIS	0.4 g
H_2O to make	50 ml

Solution B1

I N KOH	48 ml
HAC	17.2 ml
TEMED	4.0 ml
H_2O to make	100 ml

Solution C1

Ammonium Persulfate	0.28 g
H_2O to make	100 ml

Stocking Gel:

Solution A2

Acrylamide	5 g
BIS	1.25 g
H_2O to make	50 ml

Solution B2

I N KOH	48 ml
HAC	2.87 ml
TEMED	0.46 ml
H_2O to make	100 ml

Solution C2

Riboflavin	4.0 mg
H_2O to make	100 ml

Solution D2

Sucrose	25 g
H_2O to make	100 ml

Table 3

Buffer Solutions for Polyacrylamide Gel Electrophoresis

Pre-Electrophoresis Buffer Stock Solution E:

I N KOH	144 ml
HAC	51.6 ml
TEMED	12.0 ml
H_2O to make	300 ml

Electrophoresis Buffer:

β-alanine	6.24 g
HAC	1.6 ml
H_2O to make	2 L

was pipetted into each electrophoresis tube, layered with approximately 0.1 ml of H_2O and allowed to polymerize for 30 min.

In order to remove excess ammonium persulfate from the polyacrylamide, the separation gels were subjected to pre-electrophoresis. The H_2O layer was removed and the tubes were filled with buffer that was prepared by combining 300 ml of stock Solution E with 2400 ml of distilled H_2O. The tubes were placed in a Hoeffer-type cell and the upper and lower chambers filled with buffer. The anode was in the lower reservoir; the cathode in the upper reservoir. The pre-electrophoresis was performed at 4°C for 2 hr. A potential of 80 V was supplied by a Beckman Constant Voltage Duostat.

After the pre-electrophoresis the tubes were drained of buffer and returned to the gel preparation rack. The lower ends of the tubes were then resealed with Parafilm.

The stacking or spacer gel was prepared by combining 4 ml of Solution A2, 2 ml of Solution B2, 2 ml of Solution C2 and 8 ml of Solution D2 in a small Erlenmeyer flask. A 0.2 ml aliquot of the solution was added to the top of the polymerized separation gel and then overlayered with H_2O. The spacer gel was photopolymerized under an ultraviolet light for 30 min or until it became slightly opaque and kept its shape.

Following the polymerization of the spacer gel, the

distilled H_2O overlayer was removed. Protein samples were made 12.5% with respect to sucrose and a 0.2 ml aliquot of the sample was added to each electrophoresis tube. The electrophoresis tubes were then layered and filled with the electrophoresis buffer which was prepared as indicated in Table 3. The electrophoresis tubes had the following final composition: a 7.5% acrylamide separation gel, a 2.5% acrylamide stacking gel and an enzyme sample that was 12.5% in sucrose.

b. Electrophoresis of Cathepsin B1

The electrophoresis tubes were returned to the Hoeffer-type cell and the upper and lower reservoirs filled with the electrophoresis buffer. The electrodes were connected to the cell such that the anode was in the upper reservoir and the cathode in the lower reservoir. The electrophoresis was run for 135 min at 2 ma per tube and at 4°C.

c. Staining of the Gels for Protein

After the electrophoresis the gels were removed from their glass tubes and placed in capped test tubes. The gels were fixed for 1 hr with a solution consisting of 73% H_2O, 20% MeOH and 7% HAC. The gels were then stained for 3 hr in the fixing solution which in addition contained 6% TCA (w/v) and 1% Fast Green Dye (w/v). Gels were destained overnight in a Bio-Rad Diffusion Destainer. Tracings of the stained gels were recorded on a Densicord Recording Electrophoresis Densitometer. Similar electrophoretic results were

obtained with a 5% solution of Coomassie Brilliant Blue after overnight staining.

d. Detection of Enzyme Activity

Immediately following electrophoresis, some polyacrylamide gels were assayed for cathepsin B1 activity with CLN. After removal of the gels from the electrophoresis tubes, the separation gels were sliced into 1.5 mm sections. Each section was suspended in 100 μl of activating solution to which was added 100 μl of 0.025 M sodium acetate buffer, pH 5.1, containing 1 mM EDTA. After 20 min the suspensions were centrifuged and a 50 μl aliquot removed from the supernatants for assay. A typical CLN assay was then performed as previously described.

e. Staining of the Gels for Lipoprotein

In order to determine whether cathepsin B1 exists as a lipoprotein, the gels were stained with the reagent Sudan Black B according to the method of Prat, et. al. (83). Upon completion of the electrophoresis the gels were removed from their glass tubes, stained with Sudan Black B for 12 hr and destained in a Bio-Rad Diffusion Destainer against a mixture of 15% HAC, 20% acetone and 65% H_2O.

f. Staining of the Gels for Glycoprotein

Gels were stained for glycoprotein using either Alcian Blue (84) or the periodic acid-Schiff's reagent (85). Alpha-2 macroglobulin, a known glycoprotein, was used as a standard.

g. Electrophoresis in Other Gel Systems

In order to determine whether a cathepsin B1 component was homogeneous, an electrophoresis of the component was run in two different gel systems. In particular, the method of Davis (86) was used to verify whether samples exhibiting a single band in Reisfeld's cationic gel system also showed a single band in an anionic gel system. The separation gel was 7.5% acrylamide; the stacking gel was 2.5% acrylamide; and the enzyme sample was 12.5% in sucrose. The tracking dye for the anionic gel system was a 0.005% solution of Bromophenol Blue. A voltage of 2 ma per tube was applied to the gel and the electrophoresis was allowed to run until the dye had migrated three-quarters of the way down the length of the separation gel (about 2 hr). Gels were stained with either Coomassie Brilliant Blue or Fast Green Dye and destained as previously described.

11. Titration of Cathepsin B1 with DTNB

A sample of purified enzyme was activated with two volumes of a solution containing 30 mM DTT and 15 mM EDTA. After 30 min the activated enzyme was applied to an anaerobic column of Sephadex G-25 (0.9 X 27 cm) (81). The column was equilibrated and eluted with 0.01 M potassium phosphate buffer, pH 6.5, containing 1 mM EDTA. The buffer had been degassed and was continuously purged with nitrogen during the chromatography. The column had a flow rate of 24 ml/hr and

2 ml fractions were collected.

The titration was initiated by mixing 1 ml of the effluent with 50 μl of a 1 mM solution of DTNB in 0.01 M potassium phosphate buffer, pH 6.5. The absorbance was measured at 412 nm and corrected with a suitable blank. Allowance was also made for the very slow spontaneous hydrolysis of DTNB at this pH.

12. Amino Acid Analysis

Samples for amino acid analysis were hydrolyzed for 24 hr at 110°C in 1-2 ml of 6N HCl (87). The hydrolysis tubes were evacuated by freezing the material and subsequently applying a vacuum for 5-10 minutes. The tubes were then brought to room temperature and the process repeated. The tubes were sealed with a flame and placed in an oven. After hydrolysis the samples were placed in a dessicator that had contained NaOH pellets and dried under a vacuum. The residue was dissolved in 0.02 M citrate buffer, pH 2.2.

Hydrolyzed samples were analyzed for amino acids on a Beckman Amino Acid Analyzer. The column contained Beckman Resin PA No. 35.

CHAPTER III

RESULTS

A. Development of Specific Spectrophotometric Assays for Cathepsin B1

Before undertaking the purification of cathepsin B1 we endeavored to develop a rapid and highly sensitive assay for this enzyme which would be free of interference by extraneous components present in tissue homogenates. Since cathepsin B1 shows striking similarities to the well-studied plant sulfhydryl proteases, papain, bromelain and ficin, CLN and CGN were investigated as potential substrates for cathepsin B1. In order to demonstrate that the CLN and CGN activities present in tissue extracts were due to cathepsin B1, the enzyme was partially purified according to procedures described previously (7, 9). These procedures are described in greater detail in section III-B of this Dissertation. As will be shown in Figures 4 and 6, CLN and CGN activity paralleled that of BAPA, a known substrate for cathepsin B1 (2), during chromatography on Sephadex G-150 and DEAE Sephadex A-50. In addition, studies with the partially purified enzyme confirmed that CLN and CGN were more rapidly hydrolyzed by tissue homogenates than BAPA and other substrates previously described for cathepsin B1.

The catalyses of a number of ester and amide substrates by partially purified samples of cathepsin B1 were investigated. Figure 1 represents a typical Lineweaver-Burk plot for the hydrolysis of CLN by cathepsin B1 preparations prepared by the method of Barrett (9). A similar plot for the hydrolysis of BAPA is shown in Figure 2. It is evident that the hydrolysis of CLN and BAPA by cathepsin B1 obeys Michaelis-Menten Kinetics. Similar results were also seen with CGN and BANA. The kinetic constants obtained with these various substrates are collected in Table 4.

The K_m values reported here for the hydrolysis of BAPA and BANA by this cathepsin B1 preparation are in good agreement with the values obtained by other workers with comparable enzyme preparations (2, 88). From Table 4 it is also apparent that BANA is hydrolyzed 11 times faster than BAPA. A comparable ratio of reactivities of cathepsin B1 toward these two amides has been seen by others who have similarly purified this enzyme (2, 88).

The kinetic parameters observed for the hydrolysis of CGN and CLN are also shown in Table 4. It is apparent from their K_m values that CGN and CLN bind 100 to 1000 times more strongly to cathepsin B1 than BANA and BAPA. Similarly, a comparison of V values reveals that CGN and CLN are hydrolyzed 5 and 30 times more rapidly than BANA and 60 and 400 times more rapidly than BAPA. The fact that activated esters are hydrolyzed more rapidly than amides and with K_m values

Figure 1. Lineweaver and Burk Plot of Rate Data for the Hydrolysis of CLN at 25°C. The buffer was 0.025 M sodium acetate, pH 5.1, containing 1.1 mM EDTA and 0.9 mM DTT.

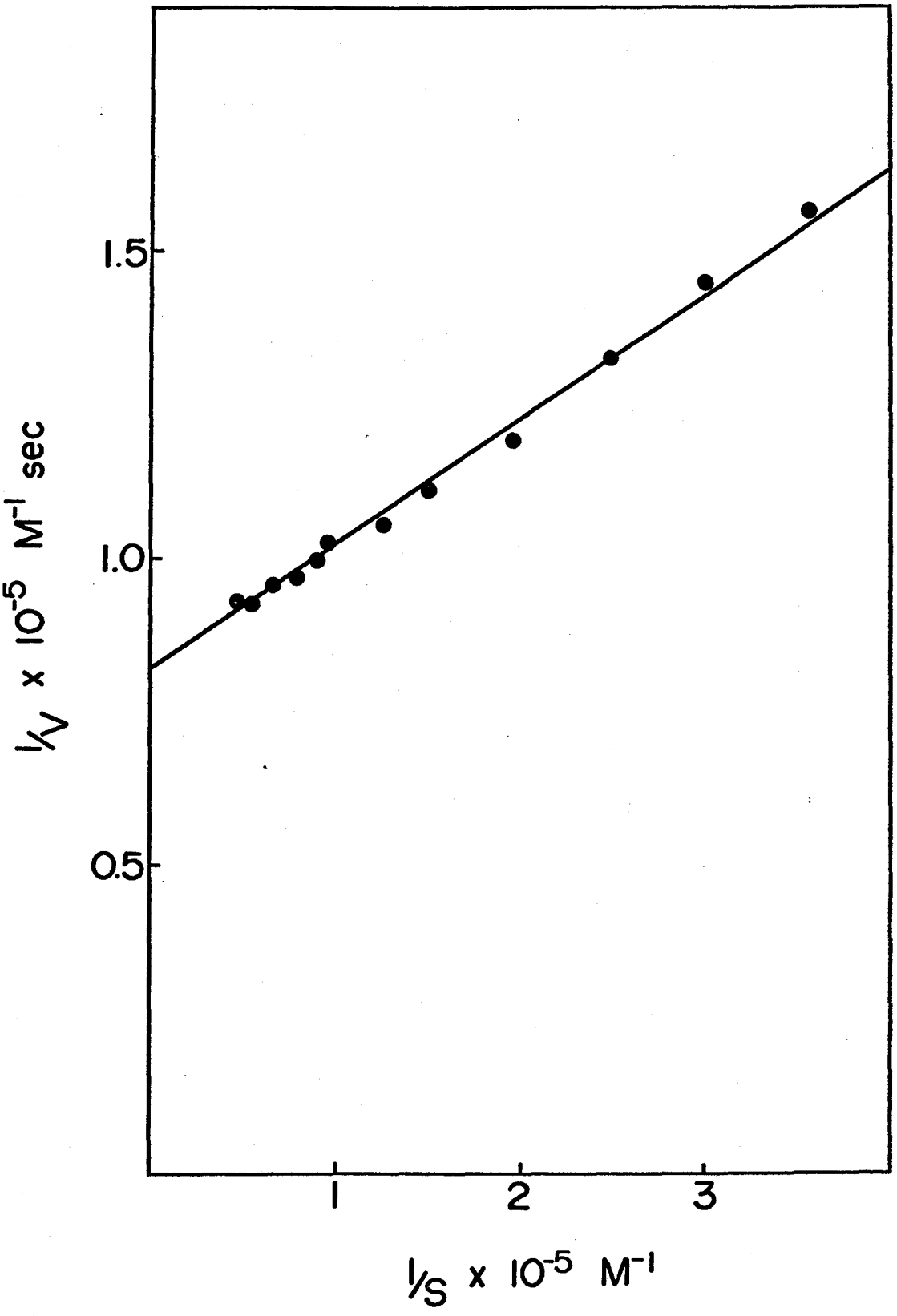

1.5
1.0
0.5
1/V x 10-5 M-1 sec
1
2
3
1/S x 10-5 M-1

Figure 2. Lineweaver and Burk Plot of Rate Data for the Hydrolysis of BAPA at 25°C. The buffer was 0.025 M sodium acetate, pH 5.1, containing 1.1 mM EDTA and 0.9 mM DTT.

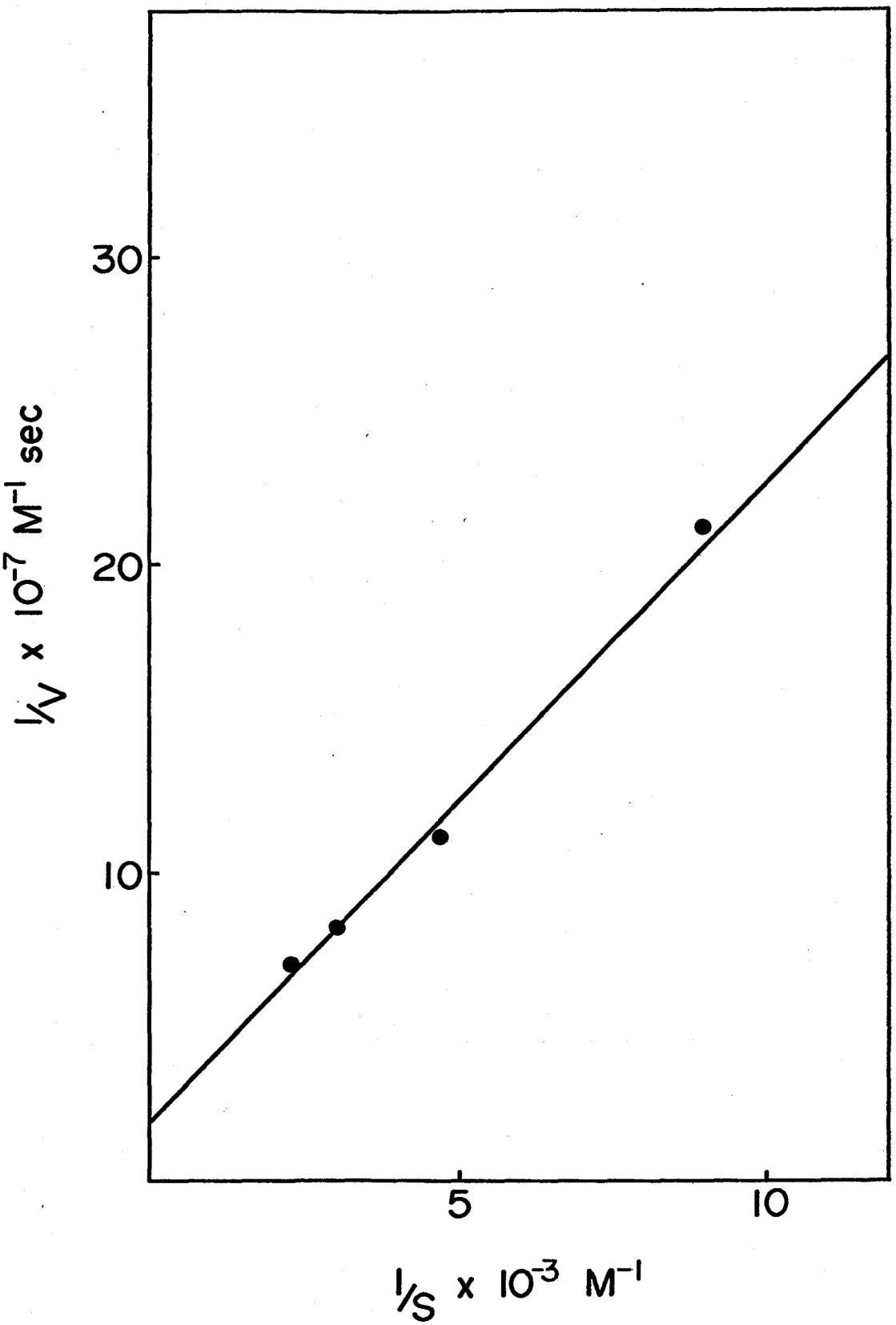

30
20
10
5
10
1/V x 10^-7 M^-1 sec
1/S x 10^-3 M^-1

Table 4

Kinetic Constants for Some

Cathepsin B1 Substrates at pH 5.1

Substrate	V[a] ($\underline{M}sec^{-1} \times 10^{8}$)	K_m ($\underline{M} \times 10^{6}$)	V/K_m[a] ($sec \times 10^{5}$)
CLN[b]	2080 ± 203	2.7 ± .30	771,000 ± 43,900
CGN[c]	337 ± 33	30.1 ± 5.2	11,200 ± 1,840
BANA[d]	61.2	1560	39.2
BAPA[d]	5.38	1300	4.14

[a] Data corrected for differences in E_0.

[b] Average of four determinations ± s.d.

[c] Average of three determinations ± s.d.

[d] Represents only a single determination.

several orders of magnitude smaller has been interpreted for other esterases and peptidases in terms of the formation of an acyl–enzyme intermediate (89).

The ratio V/K_m, which has been used as a measure of the specificity of an enzyme for a substrate, is also presented in Table 4. The values of V/K_m for CGN and CLN are 290 and 20,000 times greater than the corresponding values for BANA and 2700 and 190,000 times greater than the same values for BAPA.

B. Purification of Cathepsin B1

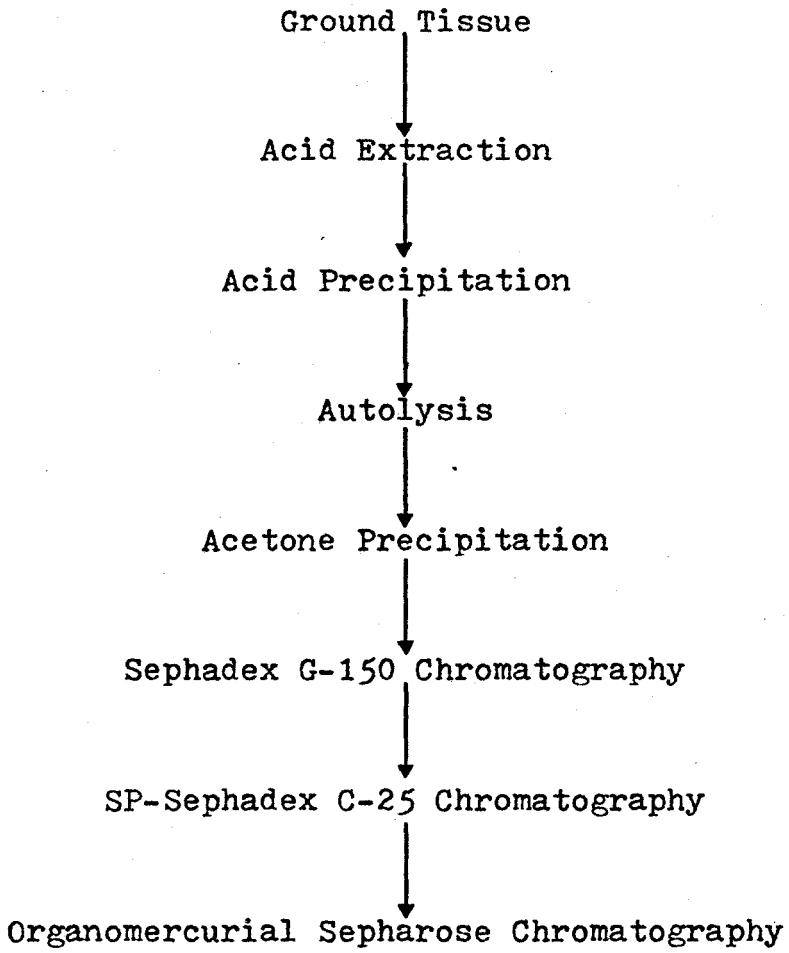
In order to purify cathepsin B1, we undertook a systematic analysis of various purification techniques (some of which had been applied previously to this enzyme). Our principle goal in this analysis was the development of a purification scheme that would not only optimize protein yield but also produce a homogeneous enzyme sample for future study. An outline of the final purification scheme for cathepsin B1 is given in Figure 3.

1. Grinding of Tissue

Fresh bovine spleen was obtained from the slaughterhouse, immediately frozen at -21°C, and stored at this temperature until needed. Cathepsin B1 was extracted and partially purified by a modification of the procedure of Barrett (9). All purification steps were performed at 4°C unless otherwise stated.

Figure 3. Purification Scheme for Cathepsin B1.

Purification Scheme for Cathepsin BI



The frozen spleen(3-4 kg total) was thawed, stripped of fasciae and the larger blood vessels and ground once in a meat grinder. The fasciae and blood vessels typically accounted for 35% of the wet weight of the spleen.

2. Acid Extraction

The ground tissue was dispersed in two parts (v/w) of a cold solution of 1% NaCl, 2% n-butanol, and 1 mM EDTA with a Waring Blender. Triton X-100 was added to a final concentration of 0.2% and the pH adjusted to 6.1 with 5 M sodium formate-formic acid buffer, pH 2.8. The homogenate was stirred for 2 hr and then centrifuged at 14,000g for 45 minutes.

Samples were centrifuged in a RC-2 Sorvall Centrifuge refrigerated at 4°C. Depending upon the volumes of the samples to be centrifuged, either an SS-34 or a GSA centrifuge head was used. Centrifuge tubes were made either of polypropylene or polycarbonate.

3. Acid Precipitation and Autolysis

The pH of the supernate was adjusted to 4.4 with 5 M formic acid-sodium formate buffer, pH 2.8, and then subjected to autolysis (9). Autolysis was carried out either at 40°C for 24 hr or at 4°C for 12 hours. No significant differences were observed between these two methods in terms of the quantity of cathepsin B1 recovered. The autolysis at 4°C for 12 hr was, therefore, adopted. After autolysis, the extract

was clarified by centrifugation at 14,000g for 45 minutes. In the case of the 40°C autolysis, the extract was cooled to 4°C before centrifugation.

4. Acetone Precipitation

To the clear supernate was slowly added with stirring 1.3 volumes of acetone which was pre-chilled to 4°C. The precipitate that formed was found to contain most of the cathepsin B1, B2 and D activity originally present in the extract.

The precipitate was collected by centrifugation in a RC-2 Sorvall Centrifuge refrigerated at 4°C and equipped with a Szeynt-Georgy Continuous Flow system. Cathepsin B1 was collected at 12,500g at a flow rate of 4 L/hr. The precipitate was then resuspended in a minimum volume of 0.025 M sodium acetate buffer, pH 5.1, containing 0.1 M NaCl and 1 mM EDTA. The suspension was stirred for 2 hr at 4°C, centrifuged to remove insoluble material, and dialyzed overnight against two 4-liter changes of 0.025 M sodium acetate buffer, pH 5.1, containing 1 mM EDTA.

The dialysis was performed at 4°C in tubing supplied by Fisher Scientific Co. The dialysis tubing was prepared by boiling it in the presence of EDTA for 30 min, rinsing it with distilled-deionized water, and repeating the boiling in the presence of EDTA for another 30 minutes. The tubing was then rinsed well with distilled-deionized water

and stored in the refrigerator until needed.

Precipitation of cathepsin B1 by ammonium sulfate, a procedure previously used by other workers (7), was found to be unsatisfactory. Cathepsin B1 precipitated over a wide range of ammonium sulfate concentration and none of the fractions obtained showed significant enrichment of the enzyme. In contrast, acetone fractionation was found to be reproducible and resulted in a 4.5 fold increase in the specific activity of cathepsin B1.

5. Separation of Cathepsin B1 on Sephadex G-150

The previous dialysate was concentrated at 4°C in either an Amicon Model 40L Concentrator fitted with an Amicon PM-10 Membrane or, for larger samples, in an Amicon Model 402 Concentrator fitted with an Amicon PM-10 Membrane. A pressure of 70 lbs. of nitrogen was applied to the stirring cell. The liquid that passed through the membrane was found not to contain cathepsin B1 when assayed with CLN or BAPA.

Chromatography of cathepsin B1 on Sephadex G-150 was carried out in either a 5 cm diameter by 90 cm length column or, for small scale purifications, in a 2.6 cm X 90 cm column. In both cases the column was equilibrated with 0.025 M sodium acetate buffer, pH 5.1, containing 1 mM EDTA. The flow rate was 24 ml/hr. For the larger column 8 ml fractions were collected; with the smaller column 4 ml fractions were collected. In each case the volume of the protein solution

applied was equal to 5% of the bed volume of the column.

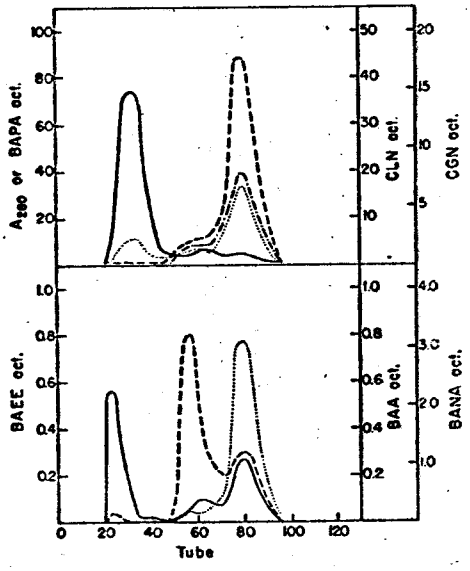
Figure 4 shows the results obtained on chromatography of the acetone precipitable fraction on Sephadex G-150. Fractions were assayed for protein at 280 nm and for BAA, BAEE, BAPA, BANA, CLN and CGN hydrolase activity as described in the experimental section.

The elution profile reveals the presence of two components with BAA amidase activity. One component produced a major peak at tube 56 and the second component produced a minor peak at tube 80. These undoubtedly correspond to cathepsin B2 and B1 with approximate molecular weights of 50,000 and 25,000, respectively (2).

There were at least three components with BAEE esterase activity. One component produced a peak at tube 80 and most likely corresponds to cathepsin B1. A second component produced a minor peak at tube 60, and a third component produced a major peak at tube 23 corresponding to the void volume of the column. This high molecular weight sulfhydryl dependent BAEE esterase activity was observed in all our preparations and has not yet been characterized. The majority of protein eluted from the column in either the void volume of the column or in fractions of high molecular weight.

Assays with BAPA, BANA, CLN, and CGN in each case revealed only a single major peak of activity under the conditions of the assay. The position of the peak in each case

Figure 4. Chromatography of a Solubilized Acetone Precipitate on Sephadex G-150. The column (2.6 X 90 cm) was thermostated at 4°C. The buffer was 0.025 M sodium acetate and 1 mM EDTA, pH 5.1. The flow rate was 24 ml/hr. Top: (——) absorbance at 280 nm; (—●—●) BAPA hydrolase activity in nmoles/ml/min; (— — —) CLN hydrolase activity in μmoles/ml/min; and (● ● ●) CGN hydrolase activity in μmoles/ml/min. Bottom: (——) BAEE hydrolase activity in μmoles/ml/min; (— — —) BAA hydrolase activity in μmoles/ml/min; and (● ● ●) BANA hydrolase activity in nmoles/ml/min.


A_{280} or BAPA act.
CLN act.
CGN act.
BAEE act.
BAA act.
BANA act.
Tube
0
20
40
60
80
100
120

corresponded to the location of cathepsin B1. A small, unknown peak with activity toward these substrates also occurred at tube 56.

Cathepsin B1 fractions having greater than 20 μmoles/ml/min of CLN activity were pooled for further study. In order to determine the number of CLN hydrolase species present in the pool, a gel electrophoresis at pH 4.5 was performed. Some gels were stained for protein with Fast Green Dye while other gels were sliced into 1.5 mm sections and assayed with CLN. The results are shown in Figure 5.

The pool contained at least 4 proteins as shown by the densitometer tracings. There were two major bands present and two minor ones. The two major bands reacted with CLN and coincided with the protein peaks. The two minor bands showed no activity toward CLN. Thus, it appears that CLN is hydrolyzed by at least two enzyme species in a bovine spleen homogenate.

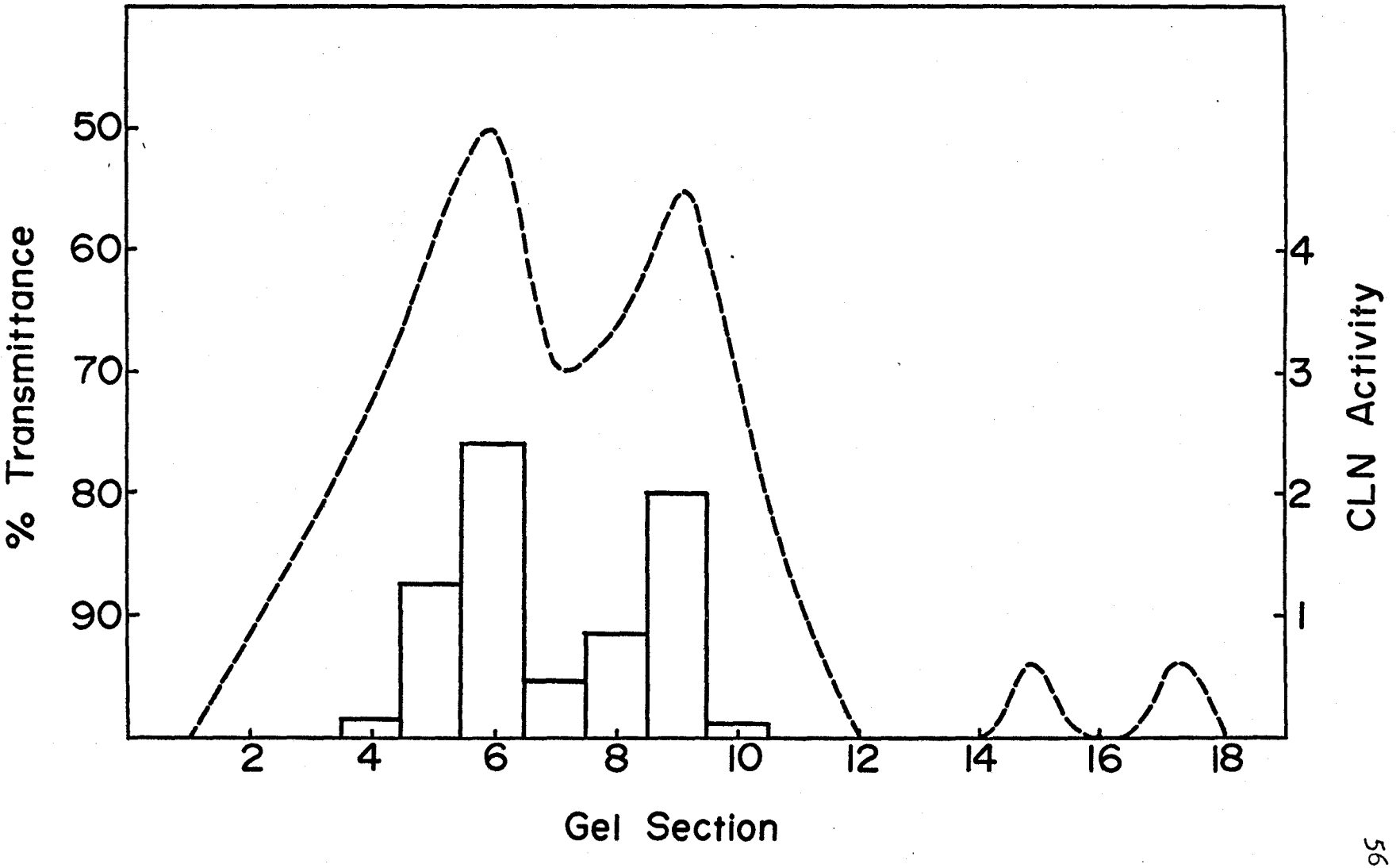
6. Ion-Exchange Chromatography

a. DEAE Sephadex A-50

The Sephadex G-150 pool was dialyzed against 0.01 M sodium phosphate buffer, pH 6.8, containing 1 mM EDTA and applied to a DEAE Sephadex A-50 column (1.5 X 25 cm) which was equilibrated in the same buffer. The flow rate was 12 ml/hr.

After the sample was applied, the column was washed

Figure 5. Electrophoresis of a Sephadex G-150 Pool at pH 4.5. Gels were stained for protein with a 1% Fast Green dye solution containing 6% TCA. The densitometer tracing (————) is in % Transmittance. CLN hydrolase activity (——) was measured as the change in absorbance/min at 326 nm. The buffer was 0.025 M sodium acetate and 1 mM EDTA, pH 5.1.


% Transmittance
50
60
70
80
90
CLN Activity
4
3
2
1
2
4
6
8
10
12
14
16
18
Gel Section

with 2 bed volumes of 0.01 M phosphate buffer, pH 6.8, containing 1 mM EDTA. Protein was eluted by the stepwise application of 2.5 bed volumes of the initial buffer containing 0.1 M NaCl and 3.0 bed volumes of the initial buffer containing 0.15 M NaCl. Cathepsin B1 was eluted with the 0.15 M NaCl step.

Fractions were assayed for protein at 280 nm and for BAPA, CLN and CGN activity. The resulting elution profiles are shown in Figure 6. As can be seen the three activity profiles are coincident suggesting that they are due to the same enzyme, cathepsin B1.

b. SP-Sephadex C-25

The Sephadex G-150 pool was concentrated to 22 ml, adjusted to pH 4.1 with dilute HCl, and applied to a SP-Sephadex C-25 column (1.6 X 32 cm) equilibrated with 0.025 M acetic acid-sodium acetate buffer, pH 4.1, containing 1 mM EDTA. After the sample was applied, the column was washed with 3 bed volumes of the initial buffer. The flow rate was 60 ml/hr and 4 ml fractions were collected. Cathepsin B1 was eluted with 800 ml of a 0 to 0.2 M sodium chloride gradient in that same buffer.

Figure 7 shows an elution profile obtained after chromatography of pooled fractions from the Sephadex G-150 column on a column of SP-Sephadex C-25. Fractions were assayed for protein at 280 nm and for BAPA and CLN activity.

Figure 6. Chromatography of Active Fractions from the Sephadex G-150 Column on DEAE Sephadex A-50. The column (1.5 X 25 cm), equilibrated with 0.01 M potassium phosphate and 1 mM EDTA, pH 6.8, was thermostated at 4°C. After application of the sample the column was washed with 2 volumes of buffer. The activity was then eluted by the stepwise addition of buffer plus 0.10 M NaCl (first arrow) and buffer plus 0.15 M NaCl (second arrow). The flow rate was 12 ml/hr. (——) Absorbance at 280 nm; (—●—●) BAPA hydrolase activity in nmoles/ml/min; (— — —) CLN hydrolase activity in µmoles/ml/min; and (● ● ● ●) CGN hydrolase activity in µmoles/ml/min.

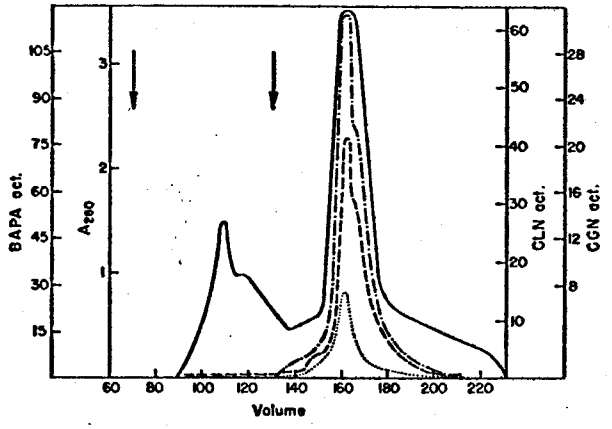
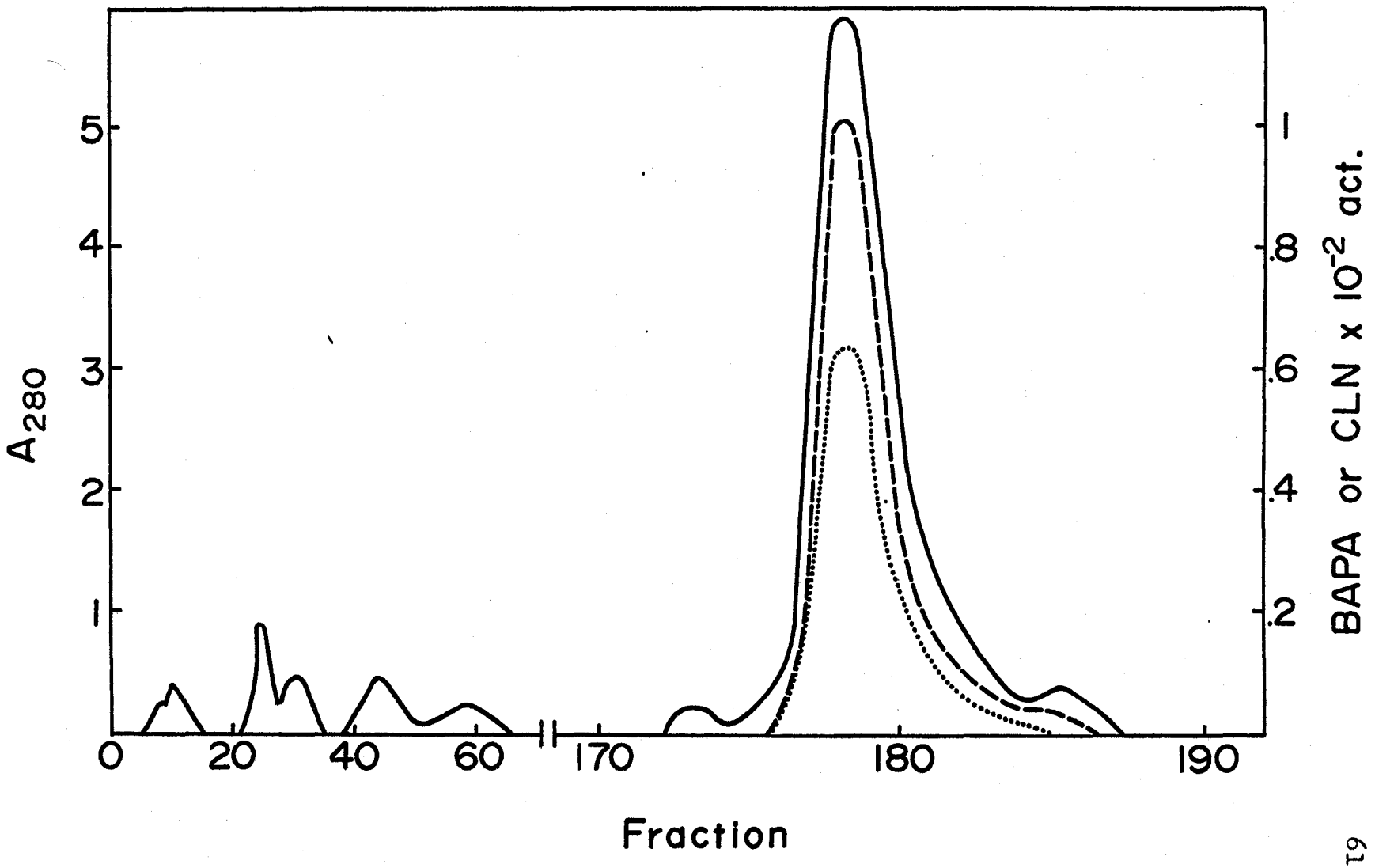

BAPA act.
A280
CLN act.
CGN act.
Volume
105
90
75
60
45
30
15
3
2
1
60
50
40
30
20
10
28
24
20
16
12
8
60
80
100
120
140
160
180
200
220

Figure 7. Chromatography of Active Fractions from the Sephadex G-150 Column on SP-Sephadex C-25. The column (1.6 X 32 cm) was equilibrated at 4°C with 0.025 M sodium acetate buffer, pH 4.1, containing 1 mM EDTA. After application of the sample, the column was washed with three column volumes of buffer. The activity was eluted with 800 ml of a 0 to 0.2 M sodium chloride gradient (applied at tube 85). The flow rate was 60 ml/hr and 4 ml fractions were collected. (———) absorbance at 280 nm; (— — —) CLN activity in μmoles/ml/min; and (• • • •) BAPA activity in μmoles/ml/min.


A280
5
4
3
2
1
0
20
40
60
170
180
190
Fraction
1
.8
.6
.4
.2
BAPA or CLN x 10-2 act.

Both protein and activity were eluted as a single, slightly skewed peak.

Aliquots were removed from various fractions under the activity peak and subjected to polyacrylamide gel electrophoresis at pH 4.5 (82). The peak fractions (tubes 177 through 179) showed only a single protein band. This band was shown to possess cathepsin B1 activity. A second smaller band was present in tube 180 and it also possessed activity. Two additional minor bands which appeared inactive were found in tubes 181 and 182. The proportions of the various components under the peak were determined by densitometer measurements of the gels and found to be approximately 70: 20:5:5. Only the first two bands were active.

These findings are in contrast to results obtained previously by us and others (6, 7, 9) which showed the presence of 4 to 6 major active components in purified preparations of cathepsin B1 from bovine spleen. Differences between current and earlier findings may reflect the omission of the 40°C autolysis step in the present purification procedure. Further work is needed to clarify this point.

One possible explanation for the presence of multiple species of cathepsin B1 may be that the enzyme exists as a mixture of both oxidized and reduced forms. To test this hypothesis, another SP-Sephadex C-25 column was run under reducing conditions.

Before the sample was applied to the column, the pooled

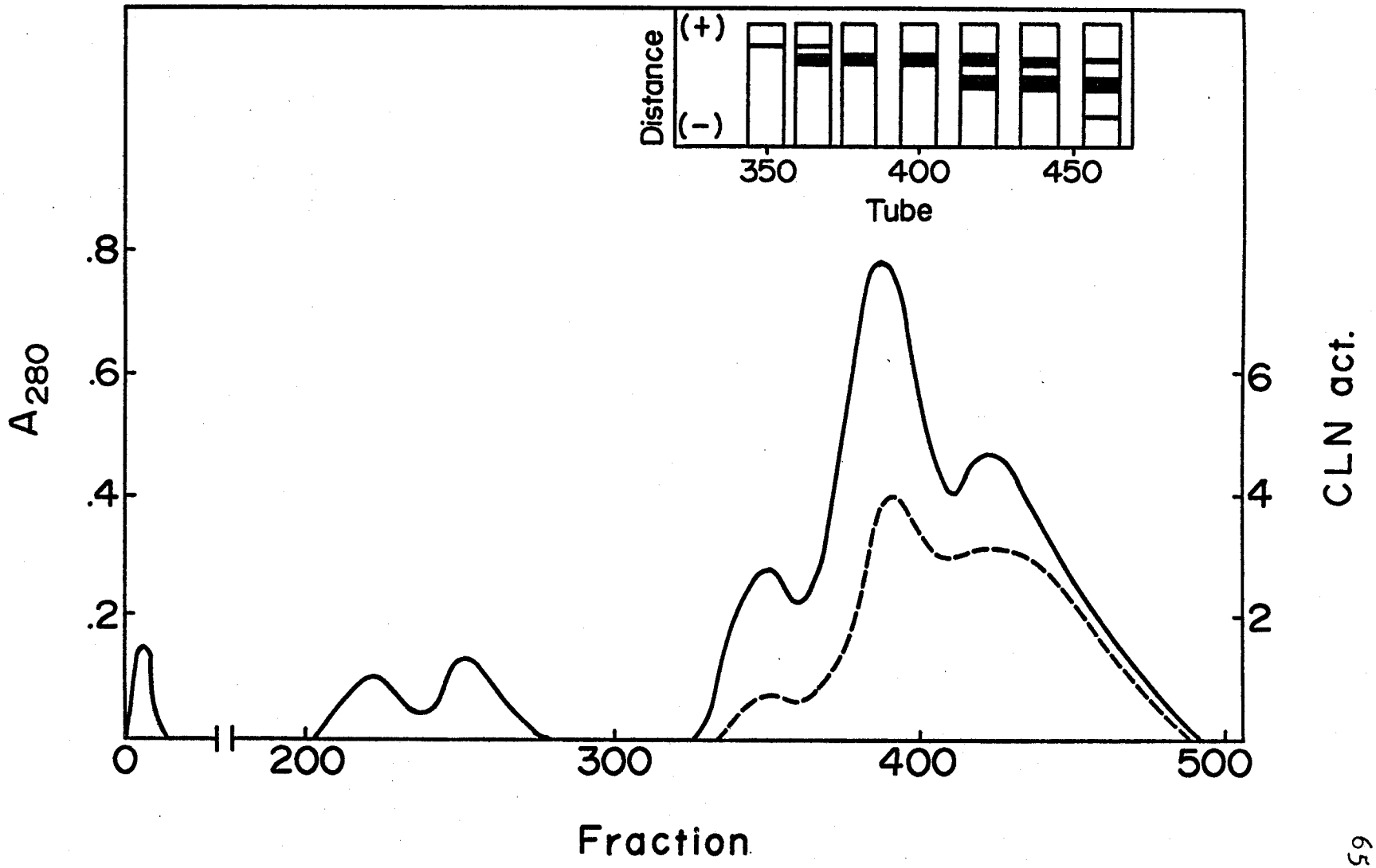
fractions from the Sephadex G-150 were activated with DTT and EDTA for 30 minutes. The buffer used to elute the protein from the SP-Sephadex C-25 column was identical to the one used previously except that it contained 10 mM 2-mercaptoethanol. The results showed that the elution profiles with and without 2-mercaptoethanol were identical. Thus, the multiplicity of cathepsin B1 must be due to some other factor than the oxidation state of the enzyme.

Cathepsin B1 was also eluted from the SP-Sephadex C-25 column under slightly different conditions. In particular, the volume of the column was increased, the NaCl gradient was made less steep and the flow rate of the column decreased. Such changes improved the resolution of cathepsin B1.

The Sephadex G-150 pool was concentrated to 43 ml, adjusted to pH 4.1 with dilute HCl, and applied to a SP-Sephadex C-25 column (1.6 X 93 cm) equilibrated with 0.025 M acetic acid-sodium acetate buffer, pH 4.1, containing 1 mM EDTA. After the sample was applied, the column was washed with 3 bed volumes of the initial buffer. The flow rate was 24 ml/hr and 4 ml fractions were collected. Cathepsin B1 was then eluted with 1600 ml of a 0 to 0.2 M NaCl gradient in that same buffer.

Figure 8 shows an elution profile obtained after chromatography of pooled fractions from the Sephadex G-150 column on a column of SP-Sephadex C-25. Fractions were assayed for protein at 280 nm and subjected to polyacrylamide

Figure 8. Chromatography of Active Cathepsin B1 Fractions from the Sephadex G-150 Column on SP-Sephadex C-25. The column (1.6 X 90 cm) was equilibrated at 4°C with 0.025 M sodium acetate buffer, pH 4.1, containing 1 mM EDTA. After application of the sample, the column was washed with 1 column volume of buffer. The activity was eluted with 1600 ml of a 0 to 0.2 M NaCl gradient (applied at tube 80). The flow rate was 24 ml/hr and 4 ml fractions were collected. (——) absorbance at 280 nm; (— — —) CLN activity in umoles/ml/min. Insert: polyacrylamide gel electrophoresis at pH 4.5. Protein was stained with Fast Green Dye.


Distance
(+)
(−)
350
400
450
Tube
.8
.6
.4
.2
A_{280}
0
200
300
400
500
Fraction
6
4
2
CLN act.

gel electrophoresis at pH 4.5 (82).

Cathepsin B1 eluted from the column between tubes 325 and 490. The protein and activity elution profile shows the presence of three cathepsin B1 species. Investigation of these peaks with polyacrylamide gel electrophoresis at pH 4.5 confirmed the presence of three components. The first protein peak at tube 350 gives a single band on gel electrophoresis and is active toward CLN. The second protein peak at tube 390 represents the major cathepsin B1 species present in the elution profile, is active toward CLN and is present in all fractions between tubes 360 and 460. Between tubes 375 and 405, it is the only cathepsin B1 component present. The third protein peak at tube 420 is electrophoretically distinct from the preceeding two peaks and is active toward CLN. At the end of the elution profile a fourth inactive species appeared on polyacrylamide gel electrophoresis.

Thus, by changing the elution parameters of the SP-Sephadex C-25 column, it was possible to resolve the species responsible for the hydrolysis of CLN. There are, therefore, at least three distinct cathepsin B1 species present in bovine spleen which hydrolyze CLN at pH 5.1. Based on the electrophoretic analyses of the elution profile in Figure 8, they are present in the relative proportions of approximately 5:70:25. According to their relative position in the elution profile, they will be referred to as cathepsin B1 (I), cathepsin B1 (II) and cathepsin B1 (III). Through careful study of the electrophoretic patterns of each fraction, tubes containing only cathepsin B1 (II) were pooled (tubes 375 to 405).

7. Organomercurial Sepharose Chromatography

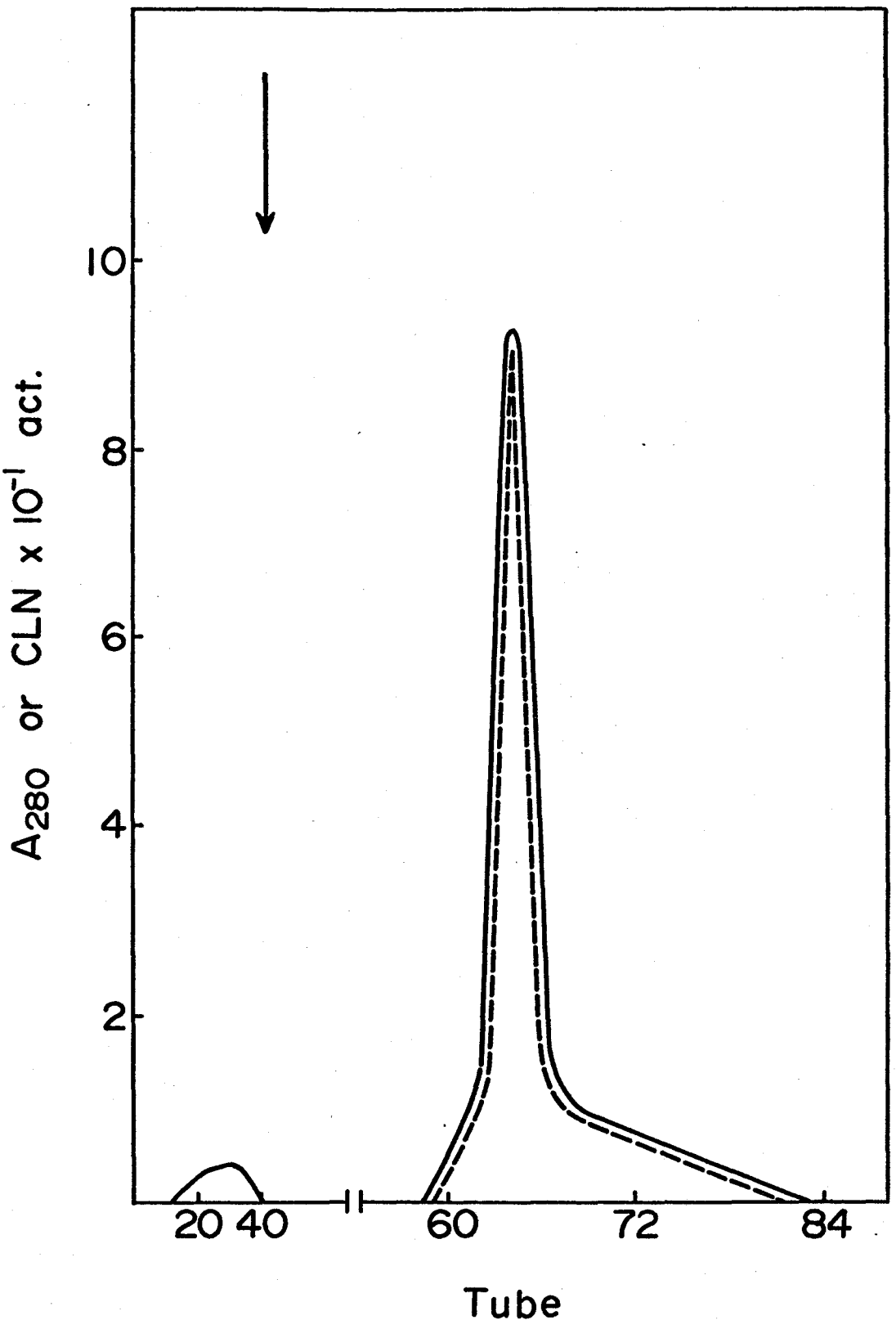
The cathepsin B1 (II) pool was concentrated to 20 ml and both DTT and EDTA were added so that the final concentrations of each was 1 mM. The activated cathepsin B1 (II) was dialyzed under nitrogen against 0.05 M sodium acetate buffer, pH 5.5, containing 0.2 M NaCl and 1 mM EDTA for 6 hr to remove excess DTT. The enzyme was then applied to an organomercurial Sepharose column (0.9 X 28 cm) and eluted with 10 mM L-cysteine as described previously in the Methods Section.

Figure 9 shows the results obtained on chromatography of cathepsin B1 (II) on Organomercurial Sepharose. Fractions were assayed for portein at 280 nm and for CLN hydrolase activity. Although not shown, fractions were also assayed for CGN, BAPA and BANA hydrolase activities.

Cathepsin B1 (II) eluted from the Organomercurial Sepharose column as a single protein peak with coincident CLN hydrolase activity. A small amount of protein did not stick to the column and represented inactive enzyme. Assays with BAPA, BANA and CGN in each case produced a peak which was coincident with both the protein peak and CLN activity peak. Polyacrylamide gel electrophoresis at both pH 4.5 (3) and pH 7.5 (86) showed cathepsin B1 (II) to be homogeneous. The single band observed after electrophoresis at pH 4.5 was active toward CLN.

Fractions containing cathepsin B1 (II) (tubes 59-80

Figure 9. Chromatography of Cathepsin B1 (II) on Organomercurial Sepharose. The column (0.9 X 28 cm) was equilibrated at 4°C with 0.05 M sodium acetate buffer, pH 5.5, containing 0.2 M NaCl and 1 mM EDTA. Active cathepsin B1 (II) fractions were eluted from the column by 10 mM L-cysteine in the above buffer (arrow). The flow rate was 12 ml/hr and 2 ml fractions were collected. (——) absorbance at 280 nm and (— ——) CLN activity in µmoles/ml/min.


10
8
6
4
2
A_{280} or CLN x 10^{-1} act.
20 40
60
72
84
Tube

in Figure 9) were pooled and dialyzed against 0.025 M sodium acetate buffer, pH 5.1, containing 1 mM EDTA to remove the cysteine. The enzyme was stored at 4°C. Fractions 470 through 490 in Figure 8, which appeared to contain primarily cathepsin B1 (III), were also pooled and subjected to further purification on the Organomercurial Sepharose column as described above.

8. Purification of Cathepsin B1: Summary

Cathepsin B1 from bovine spleen homogenates was shown to be composed of at least three distinct isoenzymes. Purification and partial resolution of these species from ground bovine spleen was accomplished by acid extraction, acid precipitation, autolysis at 4°C for 12 hr, acetone precipitation, chromatography on Sephadex G-150, chromatography on SP-Sephadex C-25, and finally, chromatography on Organomercurial Sepharose. One of these species, referred to as cathepsin B1 (II), was purified to homogenity as determined by polyacrylamide gel electrophoresis in two different buffer systems. Cathepsin B1 (II) represents the major CLN, CGN, BAPA, and BANA hydrolase recoverable from SP-Sephadex C-25 chromatography.

Table 5 summarizes the purification of cathepsin B1 (II). The distribution of both CLN and BAPA in the various fractions follow each other rather closely during the course of the purification. The final yield for cathepsin B1 (II) was

Table 5. Purification of Cathepsin B1 (II). Data is presented for both CLN (top) and BAPA (bottom). Total activity for CLN is expressed as ΔA_{326}/min/ml of stock solution assayed times total volume; total activity for BAPA is expressed as ΔA_{410}/min/ml of stock solution assayed times total volume. Both substrates were assayed at pH 5.1. Protein was determined by the Biuret method (75). The starting material was 3 kg of bovine spleen.

Table 5

Purification of Cathepsin B1 (II)

	Protein (mg)	Total Activity	Activity Mg Protein	Yield %	Purification
Homogenate	215,760	198,569 (1137)	0.92 (.005)	(100)	--
Resolubilized Acid PPT	74,120	141,490 (607)	1.91 (.008)	71 (53)	2 (2)
Resolubilized Acetone PPT	14,391	119,676 (580)	8.32 (.040)	60 (51)	9 (8)
Sephadex G-150 Pool	1,288	68,992 (369)	53.6 (.286)	35 (32)	58 (57)
SP Sephadex C-25 Pool	107	5,143 (29)	47.9 (.269)	3 (3)	53 (54)
Organomercurial Sepharose Pool	45	4,064 (19)	90.8 (.422)	2 (2)	99 (85)

2%. Major losses occurred during the SP-Sephadex C-25 chromatography. No other species of cathepsin B1 could be eluted from the column even after washes with very high salt concentrations or high pHs. Calculation of protein recoveries indicated that the protein became bound to the column irreversibly. This could be prevented by using smaller columns to purify the enzyme. This change resulted in a poorer resolution of the isoenzymes but with a greater yield. For example, the SP-Sephadex C-25 chromatography depicted in Figure 7 had a yield greater than 80%.

The remainder of the cathepsin B1 fractions from the SP-Sephadex column were pooled, concentrated and applied to an Organomercurial Sepharose column. The protein was eluted with L-cysteine and then dialyzed. This pool contained all three cathepsin B1 species. It contained 24 mg of protein and had 2037 units of CLN activity. Since cathepsin B1 (II) was the major species purified from bovine spleen, it will be referred to as simply cathepsin B1 in the remainder of this dissertation unless otherwise specified.

C. Physical and Chemical Characterization of Cathepsin B1

1. Stability of Cathepsin B1

Purified cathepsin B1 is extremely stable at 4°C in 0.025 M sodium acetate buffer, pH 5.1, containing 1 mM EDTA. After 3 months cathepsin B1 samples retained 95% of their original activity when assayed in the presence of activating

solution. Partially purified samples of cathepsin B1, however, rapidly lost both BAPA and CLN hydrolase activity with time. It therefore, became imperative that cathepsin B1 was purified as rapidly as possible in order to minimize these losses.

Since thiols can irreversibly inactivate papain (90), the stability of cathepsin B1 in 10 mM 2-mercaptoethanol was also investigated. The buffer was 0.025 M sodium acetate, pH 5.1, containing 1 mM EDTA. After 72 hr cathepsin B1 lost 17% of its activity. This loss of activity, however, could be recovered with the addition of activating solution to the enzyme sample.

2. Determination of Cathepsin B1 Concentrations

Protein concentrations were generally determined by the biuret procedure (75). With highly purified samples, protein was also determined by measuring the absorbance at 210 nm. For a 1 mg/ml solution of cathepsin B1 the molar absorption coefficient was assumed to be 20.5 $\pm$ 0.2 (77). Using both knowledge of protein concentration from absorbance data at 280 nm and from the biuret procedure, the molar absorption coefficient of cathepsin B1 at 280 nm was calculated. These results are presented in Table 6. Both the biuret procedure and the measurements of absorbance at 210 nm gave similar results. The absorption coefficient for cathepsin B1 at 280 nm, calculated using a molecular weight of 26,000 (see below), was 4.03 $\pm$ .13 X 10^4 lm^{-1} cm^{-1}.

3. Determination of the Molecular Weight of Cathepsin B1

The molecular weight of cathepsin B1 has been deter-

Table 6

Determination of the Absorption

Coefficient of Cathepsin B1 at 280 nm

[Protein] by Biuret[a]	[Protein] by A_{210}[a]	A_{280}	Calculated[b] a_m 1% 280	Calculated[c] a_m 1% 280
0.40	0.43	0.69	1.60	1.73
1.21	1.26	1.92	1.52	1.59
2.30	2.32	3.57	1.54	1.55
3.04	3.08	4.67	1.52	1.54
			1.55 ± .03[d]	1.60 ± .08[d]

[a] Expressed as mg/ml.

[b] Calculated using Biuret procedure.

[c] Calculated using A_{210} data.

[d] Average of four determinations ± s.d.

mined by a number of investigators with enzymes obtained from a variety of tissues. The molecular weight of cathepsin B1 from bovine spleen has been reported to be between 24,000 and 27,000 (5, 6). Because of this wide range of molecular weights, it was decided to redetermine the molecular weight of cathepsin B1 with highly purified samples of the enzyme.

The molecular weight of cathepsin B1 was determined by the method of molecular exclusion chromatography. Bio-Rad PM 30 was swollen in 0.025 M sodium acetate buffer, pH 5.1, containing 1 mM EDTA for three days. Fines were removed from the degassed gel slurry before pouring it into a (1.6 X 33.3 cm) jacketed Pharmacia column. The column had two A-26 adapters at each end and was thermostated at 4°C. The outlet adapter was connected to a Beckmann flow cell (0.3 ml capacity and 1.0 cm light path) and the effluent monitored at A_{280} with a Beckman DB-G spectrophotometer. The elution profile was recorded on a Beckman 10-inch recorder. The inlet adapter was connected to a sample injection valve and a Cheminert Metered Pump. The column was equilibrated for 24 hr with degassed 0.025 M sodium acetate buffer, pH 5.1, containing 1 mM EDTA. The flow rate was 24 ml/hr.

Protein standards were applied to the column by means of the sample injection valve containing a 2.5 ml sample loop. Since the sample injection valve permitted sample

application to occur without interruption of flow, it was possible to determine the elution volume directly from the recorded elution profile with an accuracy of 1 to 2%.

There exists a relationship between the sieve coefficient of a series of pure proteins and their respective molecular weights. This relationship, which is only valid over a relatively narrow range of molecular weights, is expressed by Equation 9.

$$\sigma = - A \ln M + B \tag{9}$$

In Equation 9, σ is the sieve coefficient, M is the molecular weight of a protein and A and B are column calibration constants determined experimentally with standard proteins of known molecular weights. The value of σ can be determined from the elution volumes of individual proteins according to Equation 10.

$$\sigma = \frac{V_e - V_o}{V_T - V_o} \tag{10}$$

In Equation 10, V_e is the elution volume of the protein species, V_o is the void volume of the column, and V_T is the total volume of the column. The void volume of the column can be approximated from the elution volume of Blue Dextran 2000. The total volume of the column is to a good approximation equal to the total bed volume of the column and can be calculated from the geometry of the particular column in question. Alternatively, V_T can be determined

from the elution volume of a small molecular weight standard such as potassium chromate.

Table 7 lists the protein standards, their molecular weights, their concentration in stock solutions, their elution volumes, V_e, and their calculated sieve coefficients, σ. Data is also included for cathepsin B1. A plot of σ against the log molecular weight is given in Figure 10. If one assumes that the symmetry of cathepsin B1 and the symmetry of the protein standards are similar, one can determine the molecular weight of cathepsin B1 from its sieve coefficient. From the elution profile, the molecular weight of bovine spleen cathepsin B1 was found to be approximately 26,000. This molecular weight falls in the range found by other investigators.

4. Polyacrylamide Gel Electrophoresis

A modification of the cationic polyacrylamide gel electrophoresis system as developed by Reisfeld, et. al. at pH 4.5 (82) and described in the Methods Section, appeared to effectively separate the isoenzymes of cathepsin B1. The direct assay of the gels for CLN hydrolase activity was found to be especially useful. Not only did it identify those species with activity toward CLN but it also permitted their quantification. Such a procedure may be useful for measuring small amounts of the enzyme in tissue samples.

It has been shown previously that cathepsin B1 could

Table 7

Determination of the Molecular Weight of

Cathepsin B1 Through Molecular Exclusion Chromatography[a]

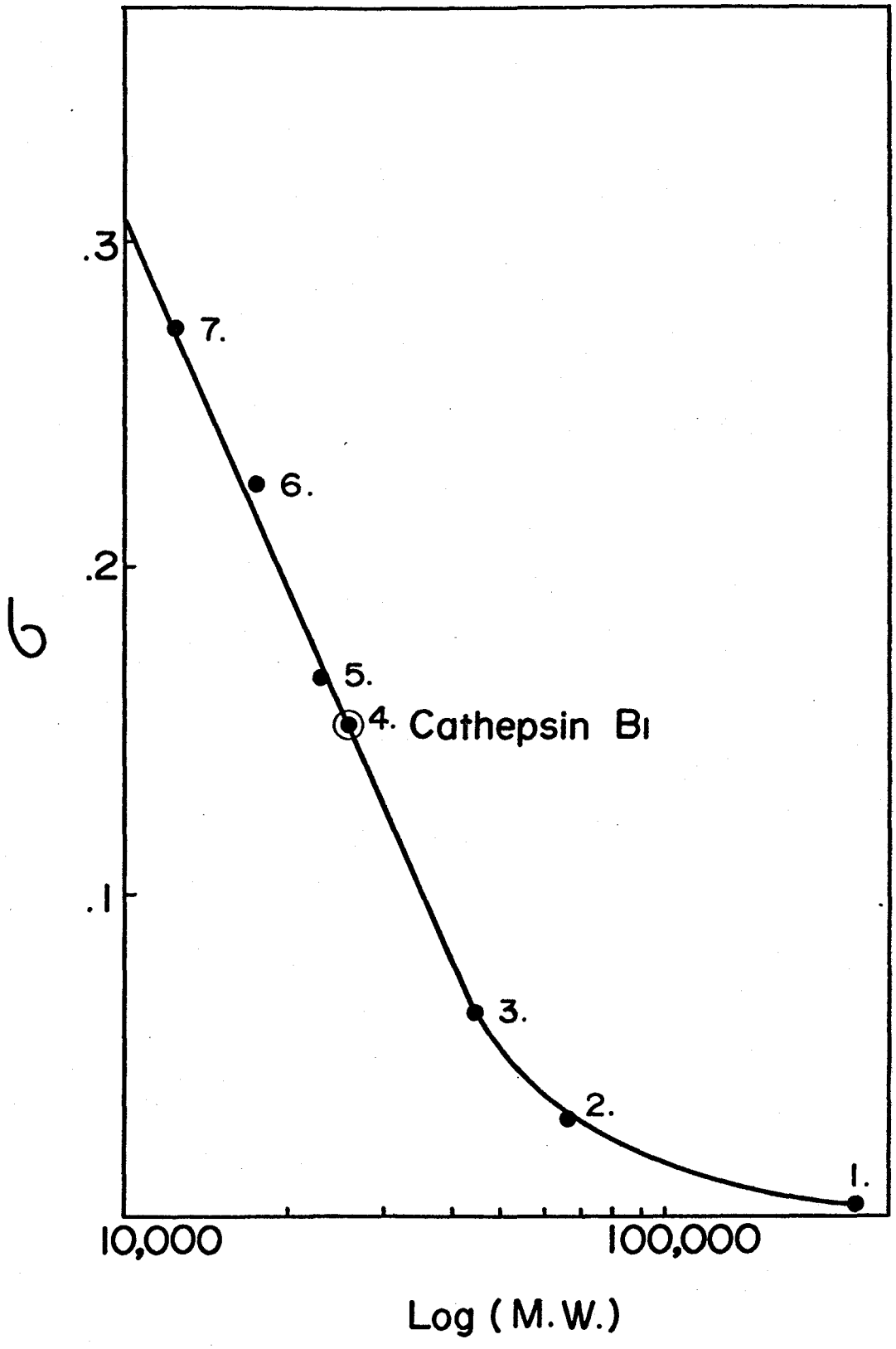
Protein	M.W.	Conc.[b]	V_e[c]	σ
Blue Dextran 2000	1,000,000	1.6	20.6	--
Catalase	230,000	0.5	20.8	0.0003
Bovine Serum Albumin	67,000	4.2	22.4	0.0286
Ovaalbumin	45,000	8.4	24.6	0.0635
Trypsinogen A	23,560	2.4	31.0	0.1651
Myoglobin	16,900	2.9	30.6	0.1587
Ribonuclease	12,600	1.5	37.8	0.2730
Cathepsin B1	X	3.1	30.0	0.1492

[a] The PM-30 column (1.6 x 33.3 cm) was thermostated at 4°C. The buffer was 0.025 M sodium acetate, pH 5.1, containing 1mM EDTA. The flow rate was 24 ml/hr.

[b] Expressed as mg/ml.

[c] Expressed as ml.

Figure 10. Plot of σ vs. log Molecular Weight. ⊙ cathepsin B1. The PM-30 column (1.6 X 33.3 cm) was thermostated at 4°C. The buffer was 0.025 M sodium acetate, pH 5.1, containing 1 mM EDTA. The flow rate was 24 ml/hr.
(1) Catalase; (2) Bovine Serum Albumin; (3) Ovalbumin; (4) Cathepsin B1; (5) Trypsinogen A; (6) Myoglobin; and (7) Ribonuclease.


.3
.2
.1
b
7.
6.
5.
4. Cathepsin B1
3.
2.
1.
10,000
100,000
Log (M.W.)

be resolved by chromatography on SP-Sephadex C-25 into at least three species, with cathepsin B1 (II) representing the major CLN hydrolase activity present. Polyacrylamide gel electrophoresis at pH 4.5 was able to separate cathepsin B1 (II) from cathepsin B1 (III) but it could not separate cathepsin B1 (II) from cathepsin B1 (I). Thus, when cathepsin B1 (I) and B1 (II) were present together they appeared as a single, broad band. In contrast, polyacrylamide gel electrophoresis at pH 7.5 could resolve all three species. Cathepsin B1 (I), B1 (II), and B1 (III) appeared as three distinct bands under these conditions. The possibility also exists that more than three species of cathepsin B1 were present after the Sephadex G-150 step but that they could not be fully resolved by chromatography on SP-Sephadex and electrophoresis at pH 4.5 and 7.5.

Electrophoresis was also performed in the presence of 10 mM 2-mercaptoethanol. Enzyme samples were activated with DTT and EDTA prior to their inclusion in the gels. The electrophoretic patterns from these experiments were exactly identical, within experimental error, to those patterns without 2-mercaptoethanol. These results suggest that the multiple forms of cathepsin B1 seen in electrophoresis and ion-exchange chromatography were not due to differences in the oxidation states of the enzymes.

The electrophoresis of cathepsin B1 afforded an opportunity to stain for glycoprotein and lipoprotein. For gly-

coprotein, gels were stained with either Alcian Blue (84) or the Periodic Schiff reagent (85). For lipoprotein, gels were stained with Sudan Black B (83). Cathepsin B1 did not stain for either carbohydrate or lipid. From the limits of detectability of the carbohydrate stains it is possible to calculate that cathepsin B1 contains less than 5 residues of hexose or hexosamine per molecule. Thus, cathepsin B1 is not a glycoprotein and its molecular multiplicity cannot be due to variations in the amount of bound carbohydrate. These results have been obtained by others (91).

5. Active Site Titration of Cathepsin B1

The enzymatic purity of cathepsin B1 can be determined by measuring the normality of active sites on the enzyme. Active site titrations are stoichiometric reactions between the active site of the enzyme and a substrate or a group-specific reagent. Two methods of titrating the active site sulfhydryl group of cathepsin B1 were investigated. These involved the reaction of the enzyme with DTNB and the reaction of the enzyme with PCMB.

a. Titration of Cathepsin B1 with DTNB

The purified enzyme, activated with DTT and EDTA, and separated from excess activator, was titrated with DTNB as described in the Experimental Section. The titration was initiated by mixing in a cuvette 1 ml of the effluent from

the anaerobic Sephadex G-25 column with 50 µl of a 1 mM solution of DTNB in 0.01 M phosphate buffer, pH 6.5. The cuvettes were sealed with Teflon plugs and placed in the cell compartment of a Cary 14 spectrophotometer which was flushed with a continuous stream of nitrogen. The absorbance, as measured at 412 nm, was corrected for a very slow spontaneous hydrolysis of DTNB at this pH. A second reaction mixture was simultaneously monitored for decreases in CLN hydrolase activity.

The reaction of cathepsin B1 (II) with DTNB is shown in Figure 11. The titration was biphasic with one species reacting moderately fast and a second species rather slowly. The moderately fast reacting species represented approximately 15% of the total expected absorbance change at 412 nm and was complete in 150 minutes. The slower reacting species accounted for 85% of the DTNB reactive material. The titration went to completion in approximately 36 hr and the color yield obtained was 98% of that expected based on the protein concentration as determined by the biuret procedure (75) and a molecular weight of 26,000.

The loss of CLN hydrolase activity during the course of the DTNB titration was also measured. These results are shown in Figure 12. Aliquots were taken from the reaction mixture at the time intervals shown and assayed for CLN hydrolase activity in the presence of 0.01 M potassium phosphate buffer, pH 6.5. The loss of CLN hydrolase activity

Figure 11. The Reaction of DTNB with Cathepsin B1 (II). The release of TNB was monitored at 412 nm. E_o = 3.46 X 10^{-6} M. The buffer was 0.01 M phosphate, pH 6.5.

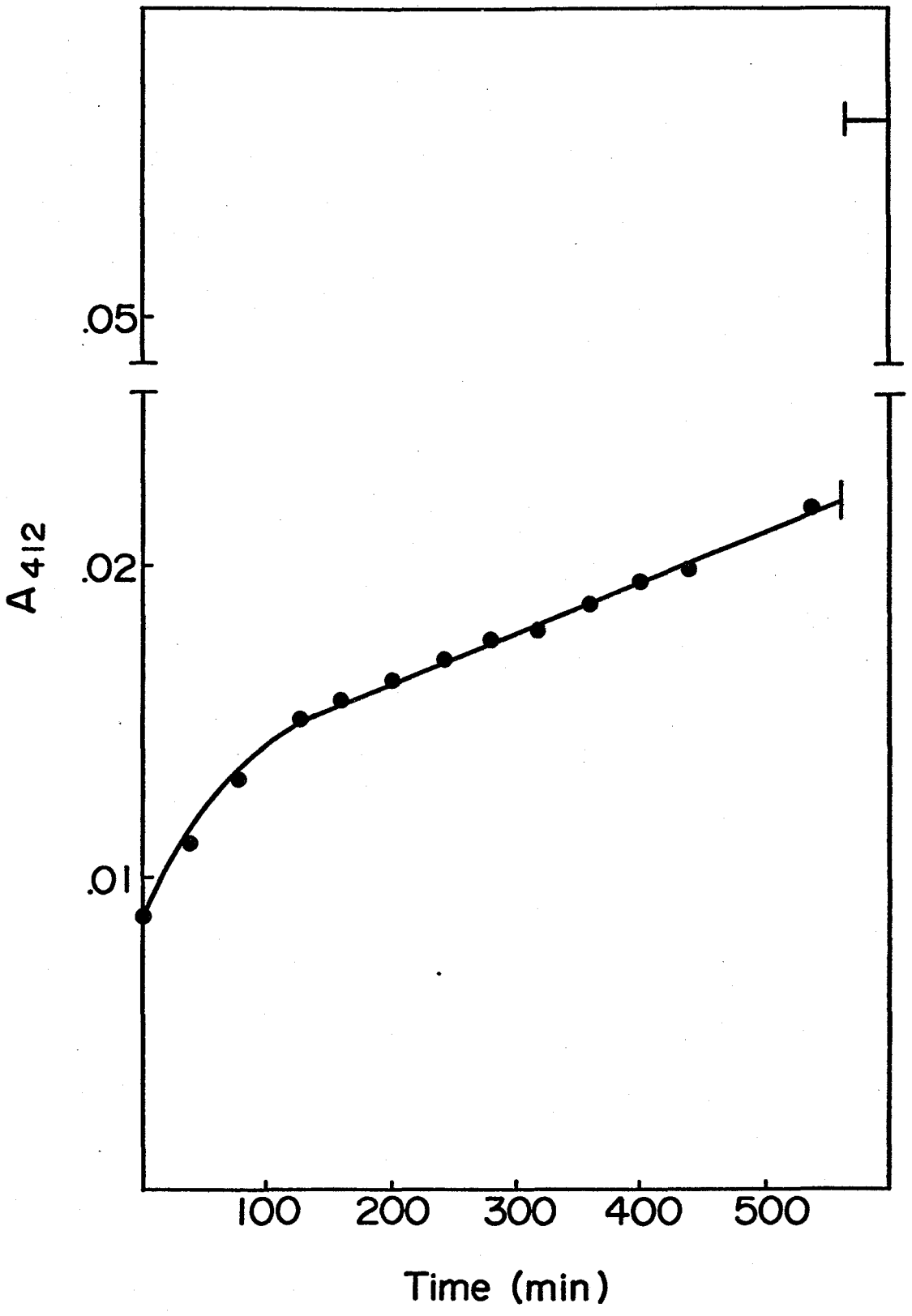
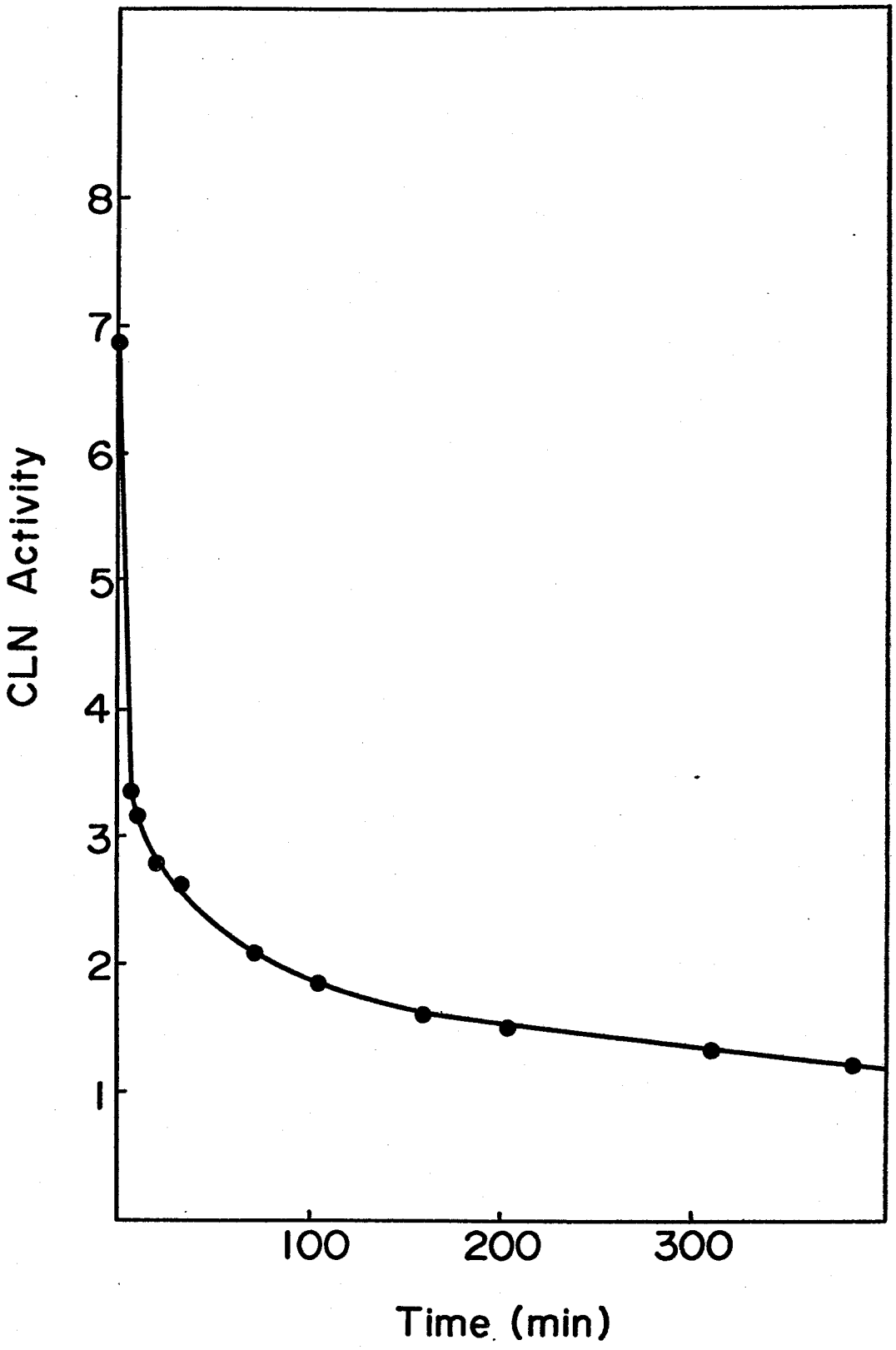

.05
.02
.01
A_{412}
100
200
300
400
500
Time (min)

Figure 12. Loss of CLN Hydrolase Activity During the DTNB Titration of Cathepsin B1 (II) CLN activity expressed in ΔA_{326}/ml/min. $E_o = 3.46 \times 10^{-6}$ M. The buffer was 0.01 M potassium phosphate, pH 6.5.


CLN Activity
8
7
6
5
4
3
2
1
100
200
300
Time (min)

appeared to be triphasic suggesting the existence of three species of cathepsin B1. One species very rapidly lost activity within the first 5 min of the titration. A second component lost CLN activity moderately fast and was totally inactive within 150 minutes. The third species lost its activity rather slowly. No activity remained after 36 hours.

Table 8 compares the results of the DTNB titration as measured by the increase in absorbance at 412 nm and by the decrease in CLN activity at pH 6.5. Presented in the table are the rate constants for each species identified and their relative contribution to each reaction.

It is quite evident from Table 8 that the titration of cathepsin B1 by DTNB when measured by the release of TNB at 412 nm and when measured by the loss of CLN hydrolase activity show distinct differences. These differences are reflected in the apparent number of components present in each reaction, the relative contribution of each component to the total reaction and the kinetic constants calculated for each component. Only in the case of the second component was there any correspondence between the rate constant and the proportion of that component in the reaction mixture obtained by the two methods. The poor agreement between the two titration experiments was clearly revealed by the fact that within the first 150 min of the reaction 75% of the CLN hydrolase activity was lost but only 15% of the expected color yield observed.

Table 8

Titration of Cathepsin B1:

Summary of Results

	A_{412}[a]		CLN Activity[b]	
Phase	$k/[DTNB]$[c]	%	$k/[DTNB]$[c]	%
I	--	--	86.76	56
II	6.68	15	6.09	17
III	0.14	85	0.36	27

[a] The increase in A_{412} represents the release of TNB in 0.01 M potassium phosphate buffer, pH 6.5.

[b] CLN activity was measured in the presence of 0.01 M potassium phosphate buffer, pH 6.5, containing 1.6% DMSO (v/v).

[c] $k/[DTNB]$ expressed as M^{-1} sec^{-1}.

This lack of correspondence between the release of TNB and the loss of CLN hydrolase activity by the enzyme was observed in all cathepsin B1 (II) preparations. Whether this result indicated the presence of two cathepsin B1 species in the reaction mixture or that the reaction proceeded by some unexplained mechanism was further investigated.

Since only 15% of the enzyme reacted with DTNB within 150 min as judged by A_{412} measurements, it should have been possible to separate this material from the slower reacting species by affinity chromatography on an Organomercurial Sepharose column. Based on the previous results it was expected that 15% of the protein would pass through the column and that 85% would be retained.

The reaction of cathepsin B1 with DTNB proceeded as outlined earlier. After 150 min the reaction was terminated by passing the reaction mixture through an anaerobic Sephadex G-25 column (1.6 X 19 cm). The column was equilibrated and eluted with 0.01 M potassium phosphate buffer, pH 6.5. The flow rate was 24 ml/hr and 4 ml fractions collected. The enzyme collected was free of TNB and unreacted DTNB.

The fractions containing cathepsin B1 were pooled, concentrated under nitrogen and applied to an anaerobic Organomercurial Sepharose column (0.9 X 27 cm). The elution profile for the Organomercurial Sepharose column is shown in Figure 13. Fractions were assayed in the presence and absence of activating solution. The results presented in Figure

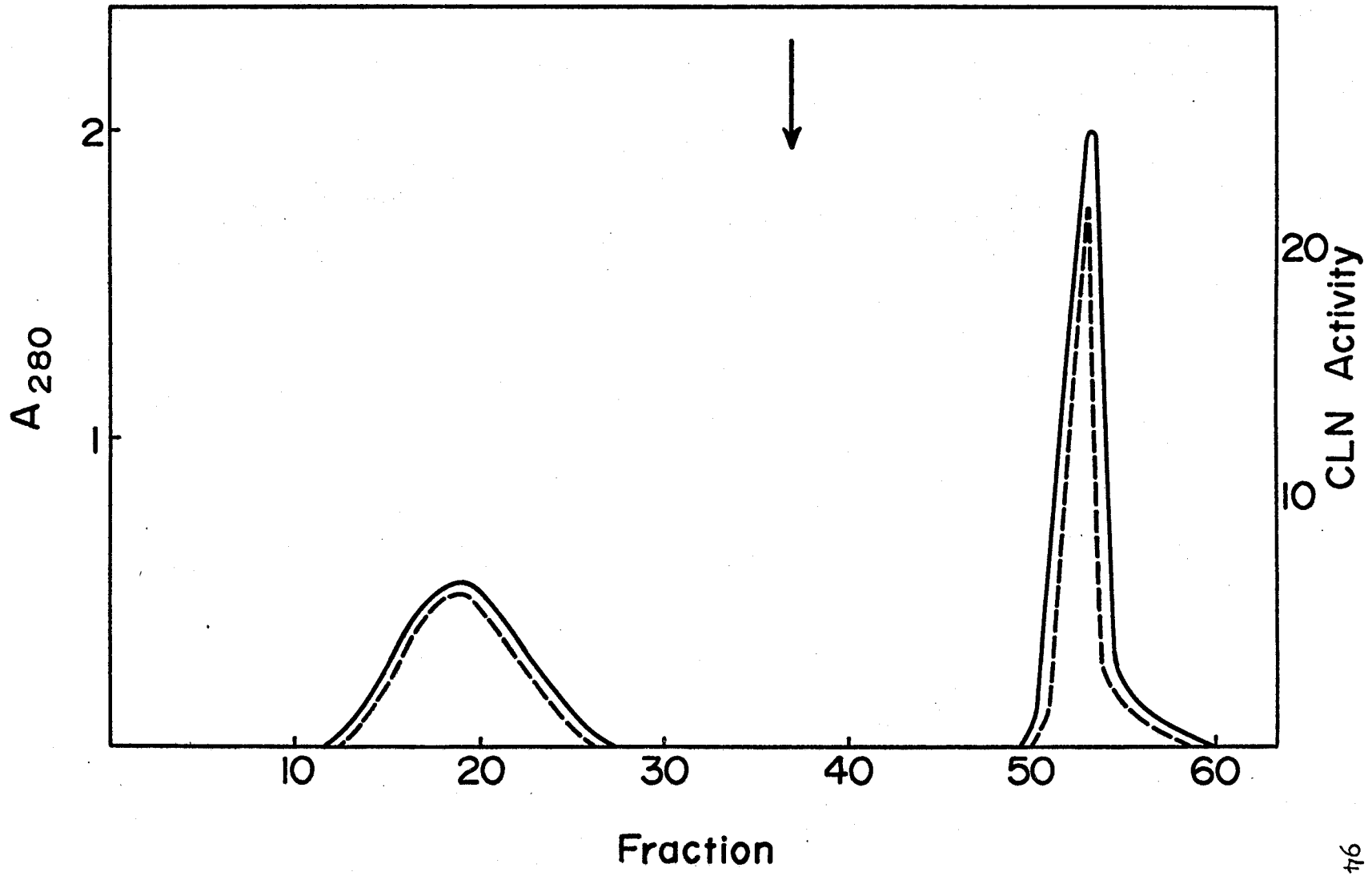
13 reflected assays with DTT and EDTA present.

The elution profiles reveal the presence of two components in the DTNB reaction mixture. One component, centered at fraction 19, did not bind to the Organomercurial Sepharose column and likely represented modified cathepsin B1. This material was active toward CLN when assayed in the presence of DTT and EDTA, but showed no activity in its absence. The second component which had bound to the column and which required 0.01 M L-cysteine for elution was active toward CLN. This material probably corresponded to unmodified cathepsin B1. A third minor peak, not shown in Figure 13 because of its small size, eluted from the column just prior to the non-binding enzyme. This peak possessed no CLN hydrolase activity and probably reflected denatured protein.

The non-binding cathepsin B1 fractions were pooled and assayed. The pool contained 5.6 mg of protein and had a total activity of 49 µmoles/ml/min. The cathepsin B1 fractions which had bound to the Organomercurial Sepharose column and were eluted with L-cysteine, were pooled and passed through a column of Sephadex G-25 to remove the cysteine. The enzyme pool contained 5.2 mg of protein and had a total activity of 51 µmoles/ml/min.

These results are in contrast to those predicted by the release of TNB. After 150 min it was observed that 15% of the enzyme had reacted with DTNB and that 85% had yet to

Figure 13. Chromatography of DTNB Reaction Products on Organomercurial Sepharose. The column (0.9 X 27 cm) was thermostated at 4°C. The buffer was 0.01 M potassium phosphate, pH 6.5. The flow rate was 12 ml/hr. Bound protein was eluted with 0.01 M L-cysteine in the phosphate buffer (arrow). (———) Absorbance at 280 nm; and (— — —) CLN hydrolase activity in μmoles/ml/min.


2
1
A_{280}
20
10
CLN Activity
10
20
30
40
50
60
Fraction

react. The results with the Organomercurial Sepharose column, however, indicated that approximately half of the sample had reacted with DTNB within 150 min, supporting the results of the activity measurements. The reasons for these differences will become apparent shortly.

In order to determine the number of species present in the non-binding enzyme pool, a sample was back-titrated with L-cysteine. The reaction was initiated by adding 10 µl of a solution containing 5 m$\underline{M}$ cysteine and 0.01 $\underline{M}$ potassium phosphate buffer, pH 6.5, to a 1 ml enzyme sample in a cuvette. The reaction was monitored at 412 nm. The results are shown in Figure 14. The back-titration of the non-binding enzyme pool was biphasic. One component, which accounted for about 24% of the total color yield, had a rate constant of 1.82×10^{-2} sec^{-1}. The other component had a rate constant for the back-titration of 3.15×10^{-3} sec^{-1} and represented 76% of the total amount of TNB released. The back-titration gave an absorbance change 4.8 times greater than that observed in the forward reaction and 1.6 times higher than expected assuming all the non-binding enzyme had one molecule of TNB associated with it.

Similar results were obtained from absorption spectra of the non-binding enzyme pool. A typical absorption spectrum is shown in Figure 15. The most notable feature of the spectrum was the presence of a large absorption peak at 325 nm. In the presence of L-cysteine this peak decreased and

Figure 14. The Back-Titration of the Non-Binding Enzyme Pool with L-Cysteine. Plot of log (A_{∞}-A) vs. time. The reaction was monitored at 412 nm. The concentration of L-cysteine in the cuvette was 5×10^{-5} M. The buffer was 0.01 M potassium phosphate, pH 6.5.

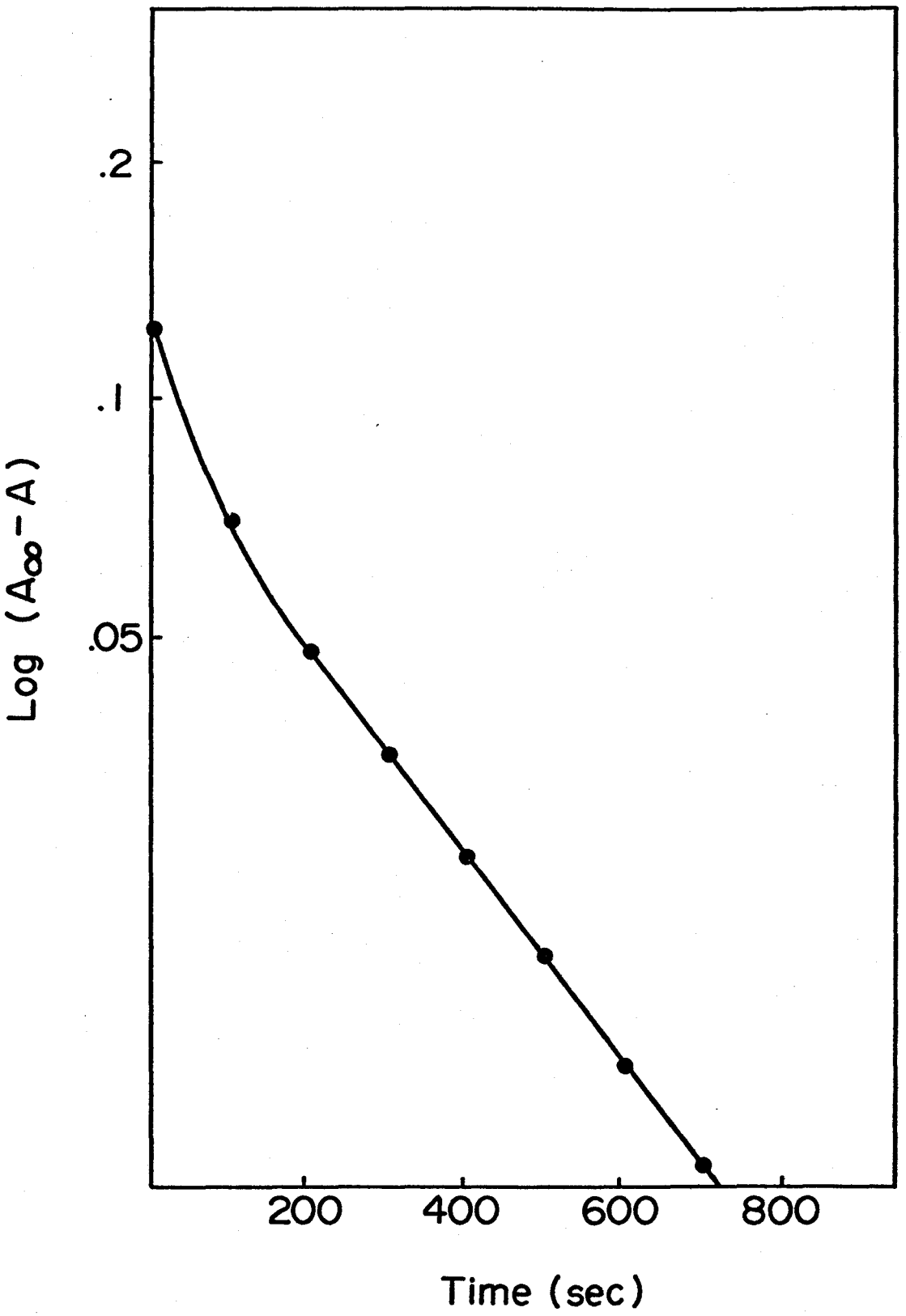
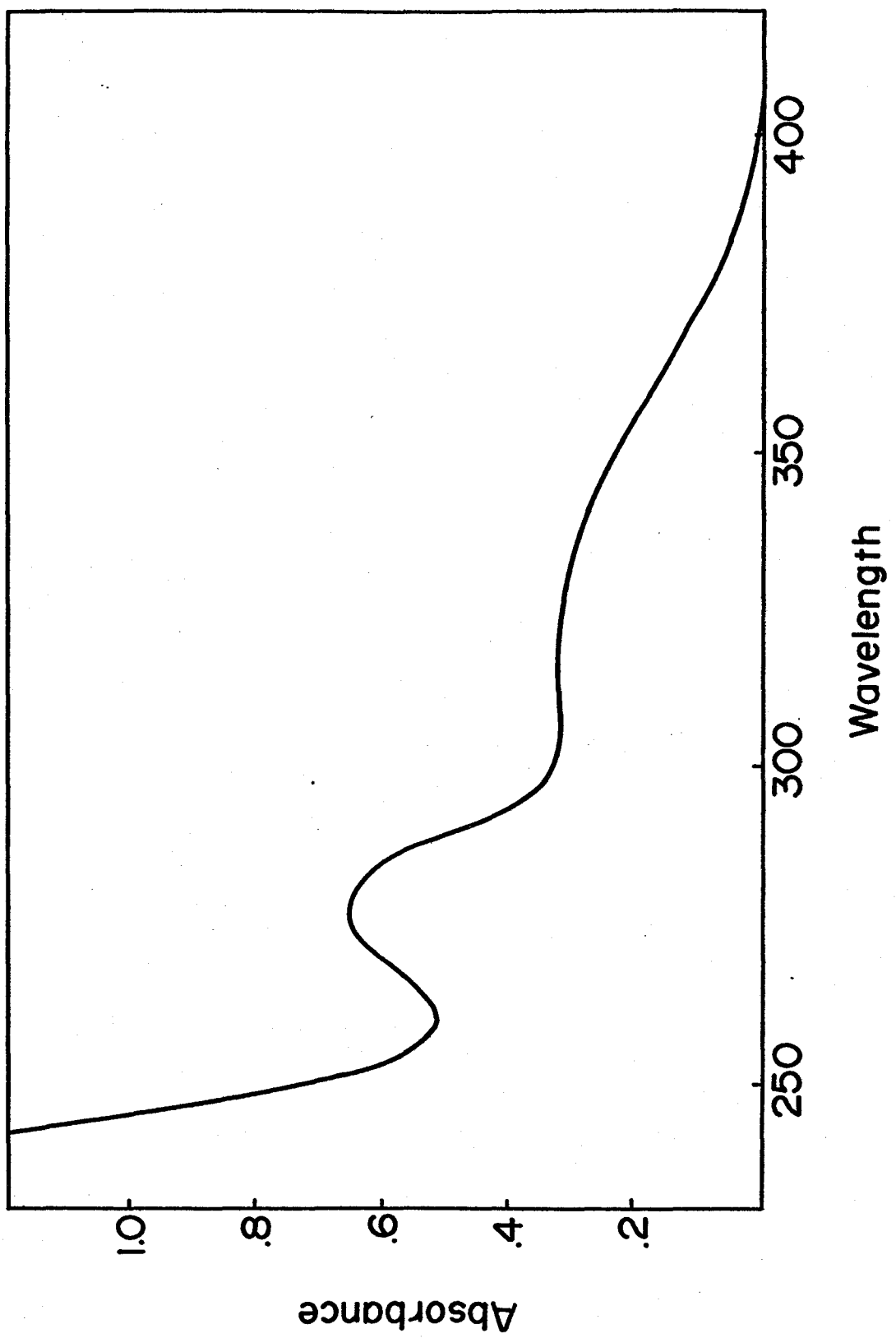

.2
.1
.05
Log (A∞ - A)
200
400
600
800
Time (sec)

Figure 15. Absorption Spectra of the Non-Binding Enzyme from the Organomercurial Sepharose Column. The enzyme concentration was 1.72×10^{-5} M in 0.01 M potassium phosphate buffer pH 6.5.


Absorbance
1.0
.8
.6
.4
.2
250
300
350
400
Wavelength

the characteristic peak of TNB appeared at 412 nm. These results suggested that the absorption peak at 325 nm was due to the formation of an enzyme-TNB mixed disulfide. An absorption coefficient for the mixed disulfide at 325 nm as determined in this experiment was 19,576. This absorption coefficient was 1.5 times higher than that expected by addition of the a_m^{325} of the enzyme and one-half of the a_m^{325} of DTNB. This later extinction coefficient was calculated to be 13,600.

Finally, it was observed that the non-binding species underwent a spontaneous reactivation. At the end of 100 hr 63% of the activity reappeared while 37% remained inactive. This residual inactive enzyme underwent no further activation even after two weeks. The inactive enzyme, however, could be reactivated with sulfhydryl compounds. The non-binding enzyme was clearly 2 species, one which could spontaneously reactivate in the absence of L-cysteine and one which required L-cysteine for reactivation.

The non-binding enzyme after spontaneous reactivation was then passed through a Sephadex G-25 column in order to remove any material released during the reactivation process. After gel filtration 1 ml of the enzyme was back-titrated with 10 µl of 5 m<u>M</u> L-cysteine in 0.01 <u>M</u> potassium phosphate buffer, pH 6.5. The observed absorbance change was now equal to that expected assuming all of the residual inac-

tive enzyme was TNB-enzyme. The kinetics of reactivation were first order with a rate constant of 1.78×10^{-2} sec^{-1}.

The results of all these experiments were entirely consistent with the following model. The model assumed that the original sample of cathepsin B1 (II) contained at least two species of cathepsin B1 which reacted with DTNB. The first species (E_1) reacted with DTNB to form the expected E_1-TNB mixed disulfide. The reaction corresponded to the fast phase of the A_{412} absorbance change and the intermediate phase of the activity loss (Figures 11 and 12).

The second species, (E_2), reacted with DTNB in two steps. In the first step, an unusually stable, non-covalent E_2-DTNB complex was formed. This species was enzymatically inactive and its formation was not accompanied by any change in absorbance at 412 nm. This step corresponded to the rapid loss of CLN activity in Figure 12. This complex could slowly dissociate to yield free enzyme, accounting for partial spontaneous reactivation of the DTNB inactivated enzyme, or in a second step of the reaction, slowly form the true covalent E_2-TNB adduct as evidenced by the slow increase in absorbance at 412 nm and by the slow phase of the activity loss curve.

The E_2-DTNB complex did not bind to the Organomercurial Sepharose column and co-eluted with the E_1-TNB species. This hypothesis explains the following observations: the ability

of the non-binding enzyme to undergo partial spontaneous reactivation; the higher than expected absorbance change at 412 nm in the back-titration of the non-binding enzyme; and the higher than expected absorbance of the non-binding enzyme at 325 nm. Furthermore, this model is in quantitative agreement with our observations. At the end of 150 min, about 15% of the enzyme reacted with DTNB to produce a covalent E-TNB adduct. Since about half of the titrated enzyme did not bind to the mercurial Sepharose column, this non-binding material was composed of 30% E_1-TNB and 70% E_2-DTNB. Spontaneous reactivation experiments indicated 36% TNB-enzyme and 64% DTNB enzyme. On the basis of a 30:70 mixture one would expect the back-titration with L-cysteine to yield 1.7 moles of free TNB per mole of enzyme. The observed value was 1.6 moles TNB per mole enzyme. Furthermore, the absorbance of the non-binding mixture at 325 nm would also be 1.7 times higher than expected for pure TNB-enzyme. The observed absorbance at 325 nm was 1.5 times higher than expected. Finally, the color yield at 412 nm in the back reaction of the non-binding enzyme would be 5.5 times higher than that of the forward reaction. The observed value was 4.8 times higher than that of the forward reaction.

b. Titration of Cathepsin B1 with PCMB

Titration of the active site sulfhydryl group of cathepsin B1 was also performed with PCMB. PCMB aliquots of

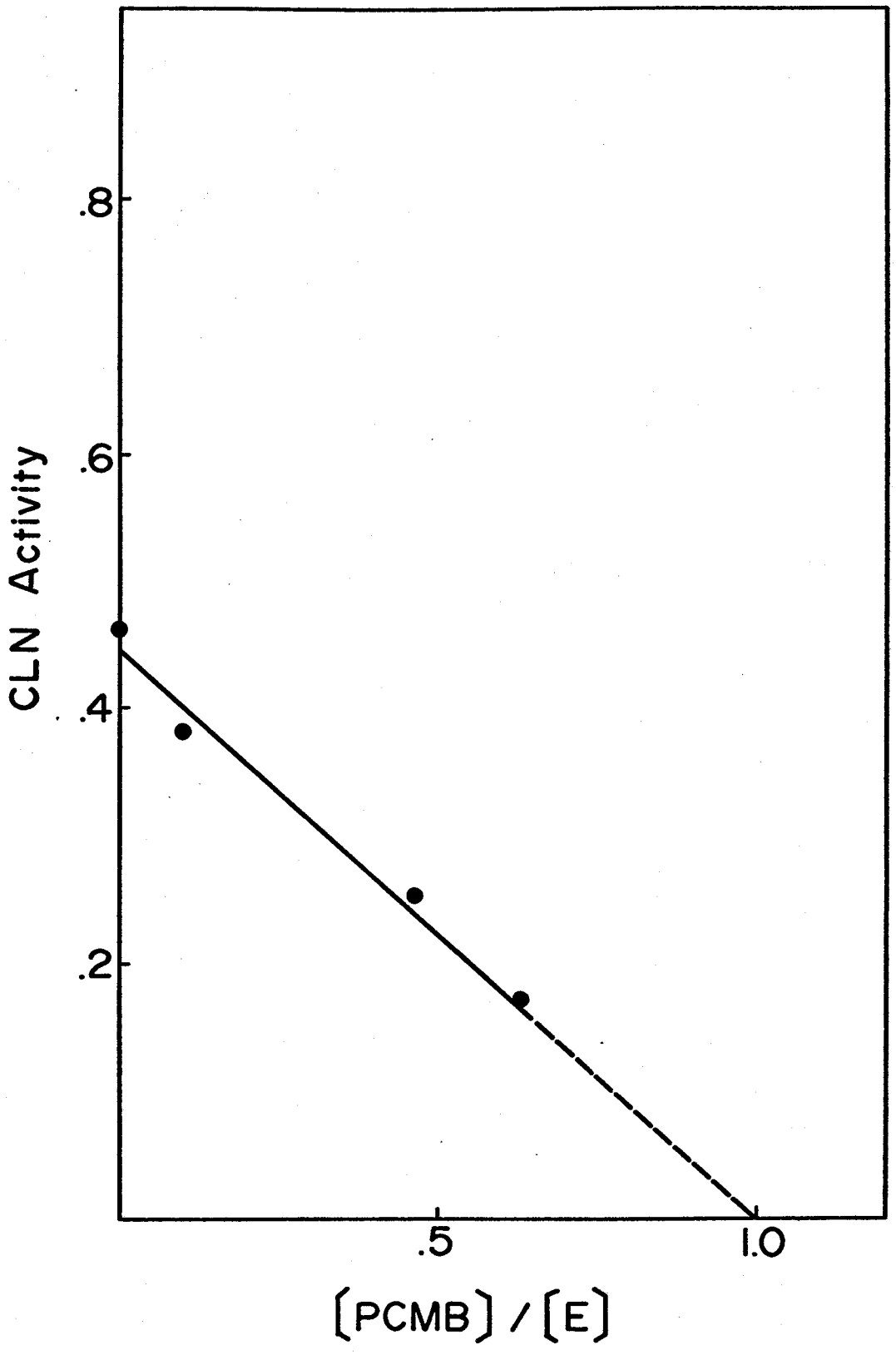
known concentration were added to an enzyme sample and the amount of activity remaining measured.

The enzyme sample used in the PCMB titration was the material that had bound to the Organomercurial Sepharose column. A portion of the pool was activated with DTT and EDTA, and passed through an anaerobic column of Sephadex G-25 to remove excess activator as described in the Methods Section. Samples of the active enzyme (500 µl) were placed in test tubes to which were added 50 µl of various PCMB solutions of different known concentrations. Aliquots of 50 µl were removed from the reaction mixtures over time and assayed for CLN hydrolase activity at pH 6.5. The reactions with PCMB were complete within the first 1.5 min of the reaction.

A plot of final activity versus [PCMB]/[E] is shown in Figure 16. An extrapolation of the line gave a sulfhydryl content for cathepsin B1 of approximately 1 mole of sulfhydryl group per mole of enzyme. Whereas the reaction of cathepsin B1 with DTNB produced very complex results, the titration of cathepsin B1 with PCMB was rather straightforward; both the PCMB and DTNB titrations indicated that cathepsin B1 preparations contained 1 mole of SH group per mole of protein and both could be used to determine the normality of active enzyme.

By using the results of PCMB titrations, it became

Figure 16. Titration of Cathepsin B1 with PCMB. Plot of Activity versus [PCMB]/[E]. CLN activity in umoles/ml/min. E_o was 1.70×10^{-6} M. The buffer was 0.01 M potassium phosphate, pH 6.5.


.8
.6
.4
.2
CLN Activity
.5
1.0
[PCMB] / [E]

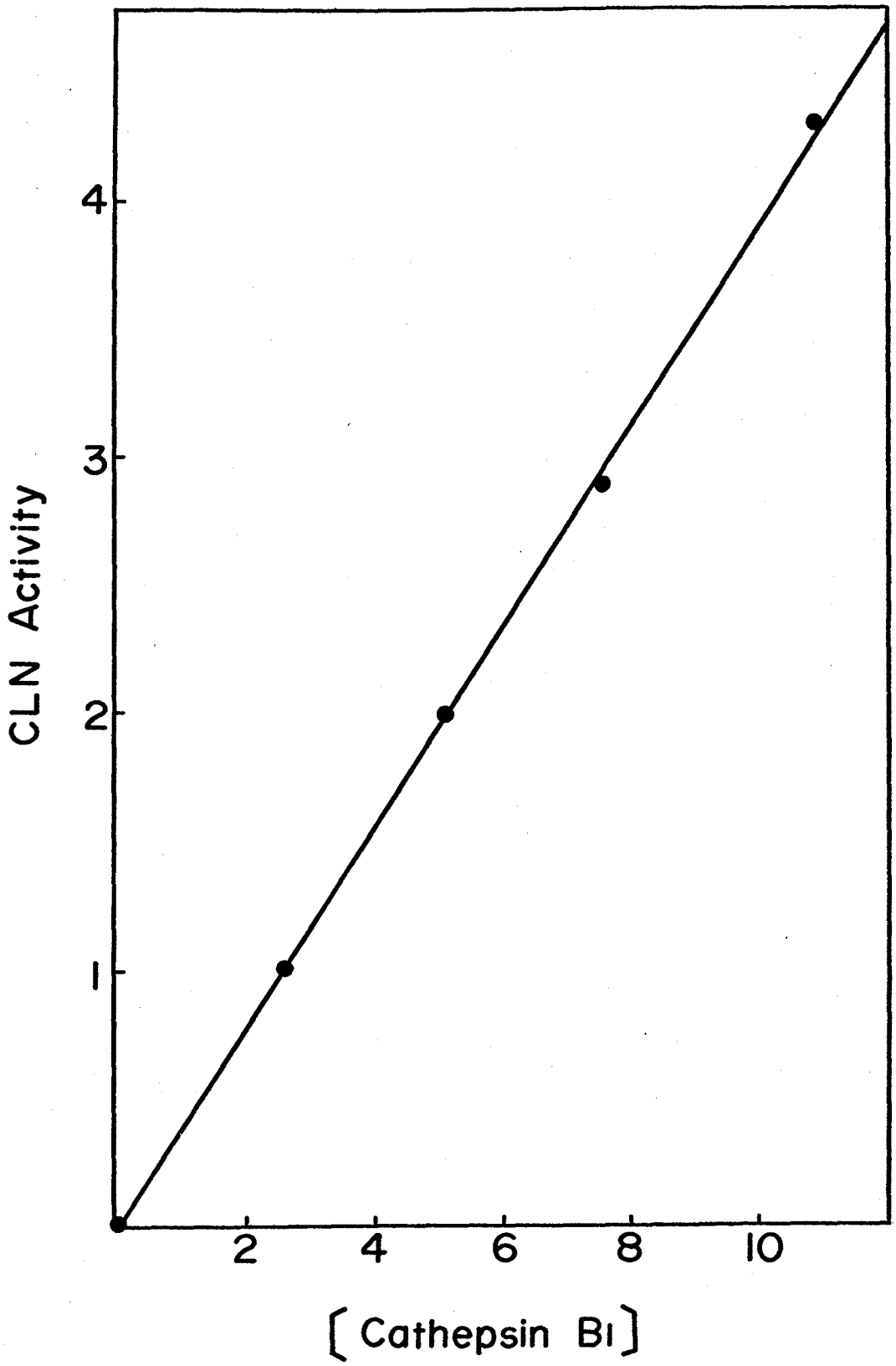
possible to relate the initial velocity of the cathepsin B1 catalyzed hydrolysis of CLN to the concentration of cathepsin B1. Figure 17 is a plot of the initial rate of hydrolysis of CLN as a function of cathepsin B1 concentration as determined by PCMB titration. The rate of hydrolysis is linear with enzyme concentration over at least a ten-fold range. From the slope of the line it could be determined that cathepsin B1, at a final concentration of 1 μM in the spectrophotometer cell, catalalyzed the release of 0.382 μmoles/ml/min of p-nitrophenol from CLN under the standard assay conditions. Since the CLN hydrolase assay is performed under conditions where $S \gg K_m$, the initial velocity of the reaction is essentially equal to V_{max}. Thus, it is possible to calculate that the turnover number, k_{cat}, for this substrate under the conditions of the assay is 6.36 sec^{-1}.

D. Mechanism of Catalysis

1. The Catalysis of alpha-N-Benzyloxy Carbonyl-L-lysine Esters by Cathepsin B1

Indirect evidence for the formation of an acyl-enzyme intermediate in reactions catalyzed by proteolytic enzymes can sometimes be inferred from the observation that substrates derived from the same amino acid and differing only in the identity of the leaving group are hydrolyzed with identical rates. This suggests that for such substrates, a common acyl-enzyme intermediate is formed whose hydrolysis

Figure 17. The Dependence of CLN Activity on Cathepsin B1 Concentration. CLN activity in μmoles/ml/min, [cathepsin B1] in μM as determined by PCMB titration. The enzyme was assayed in 0.01 M potassium phosphate, pH 6.5.


CLN Activity
1
2
3
4
2
4
6
8
10
[Cathepsin B1]

is in each case rate limiting (67). Values of K_m and k_{cat} were determined for the cathepsin B1 catalyzed hydrolyses of p-nitrophenyl, methyl, and benzyl esters of alpha-N-benzyloxycarbonyl-L-lysine. The kinetic constants obtained with these various esters are collected in Table 9.

The most striking characteristic of cathepsin B1 is its inability to hydrolyze CLBE, a substrate hydrolyzed by papain (58). In order to check the commercial preparation of CLBE, its hydrolysis by papain was studied. Based on the absorbance change at 236 nm, CLBE was found to be at least 97% pure. The failure of CLBE to react with cathepsin B1 was not due to the presence of a contaminating inhibitor since CLBE did not affect the hydrolysis of CLN by cathepsin B1.

Similar values of k_{cat} were obtained for both CLN and CLME, suggesting an acyl-enzyme intermediate. Differences between the two substrates are reflected in their values of K_m and, consequently, in the ratios k_{cat}/K_m. CLN binds 400 times more strongly to cathepsin B1 and has a value of k_{cat}/K_m 400 times greater than CLME. Similar results were observed with papain (58).

2. The pH Dependency of CLN Hydrolysis

If cathepsin B1 does indeed hydrolyze small synthetic substrates through the formation of an acyl-enzyme intermediate as depicted by Equation 1, then, the steady state rate equation for substrate hydrolysis is given by Equation 11

Table 9

Kinetic Constants for the Cathepsin B1 Catalyzed Hydrolysis of Alpha-N-Benzyloxycarbonyl-Lysine Esters[a]

Substrate	k_{cat}[b] (sec^{-1})	K_m ($M \times 10^6$)	k_{cat}/K_m[b] ($M^{-1} sec^{-1} \times 10^{-6}$)
CLN[c]	6.52 ± .31	5.57 ± .31	1.17 ± .09
CLME[c]	6.06 ± .27	2080 ± 22	0.003 ± .0001
CLBE	0	0	--

a The buffer was 0.01 M potassium phosphate, pH 6.52, containing 1.1 mM EDTA, 0.9 mM DTT and 1.6% (v/v) DMSO at 25°C.

b The concentration of Cathepsin B1 was 7.90×10^{-8} M as determined by titration with PCMB.

c Average of three determinations ± s.d.

$$v = \frac{\frac{k_2 k_3}{k_2 + k_3}(E)_o}{\left[\frac{k_3}{k_2 + k_3}\right]\frac{K_s}{S}} \qquad (11)$$

It can be seen that $k_{cat} = k_2k_3/(k_2 + k_3)$, $K_m = k_3K_s/(k_2 + k_3)$, and $k_{cat}/K_m = k_2/K_s$.

Under certain conditions, it may be possible to calculate k_3, K_s and k_2 by studying the pH dependency of k_{cat} and K_m. Accordingly, the pH dependencies of the steady state kinetic constants for the cathepsin B1 catalyzed hydrolysis of CLN were determined. The experimental values of k_{cat}, K_m and k_{cat}/K_m at each pH are collected in Table 10. The value of k_{cat} appears to be relatively insensitive to pH over the range studied. In contrast, the ratio k_{cat}/K_m appears to be pH dependent. Figure 18 is a plot of log (k_{cat}/K_m) vs. pH. The open circles are experimental points and these fall on a bell-shaped curve.

The pK_a's of the two amino acids whose ionization control k_{cat}/K_m can be obtained with the help of the following relationship (92).

$$K_m/k_{cat} = (K_m/k_{cat})_{lim} + \frac{1}{K_{a1}}(K_m/k_{cat})_{lim}\left[\frac{H^2 + H_{max}^2}{H}\right] \qquad (12)$$

Table 10

Kinetic Constants for the Cathepsin B1 Catalyzed Hydrolysis of CLN[a]

Buffer	pH	k_{cat}[b,c] (sec^{-1})	K_m[b] ($M \times 10^6$)	k_{cat}/K_m ($M^{-1}sec^{-1} \times 10^{-6}$)
13 mM formate	3.12	3.58 ± .03	25.21 ± .53	0.14 ± .01
	3.52	4.16 ± .04	12.78 ± .32	0.33 ± .01
	3.88	4.53 ± .04	5.21 ± .21	0.87 ± .04
25 mM acetate	4.35	4.64 ± .09	2.91 ± .18	1.60 ± .10
	4.85	5.22 ± .04	2.69 ± .10	1.94 ± .07
	5.39	6.02 ± .09	1.92 ± .12	3.14 ± .20
10 mM phosphate	6.05	6.38 ± .01	1.60 ± .11	4.00 ± .27
	6.62	6.55 ± .07	1.64 ± .11	3.98 ± .27
	7.12	6.62 ± .17	1.84 ± .41	3.60 ± .81
	7.58	7.69[d]	3.43	2.27

Table 10 (Cont'd)

Kinetic Constants for the Cathepsin B1

Catalyzed Hydrolysis of CLN

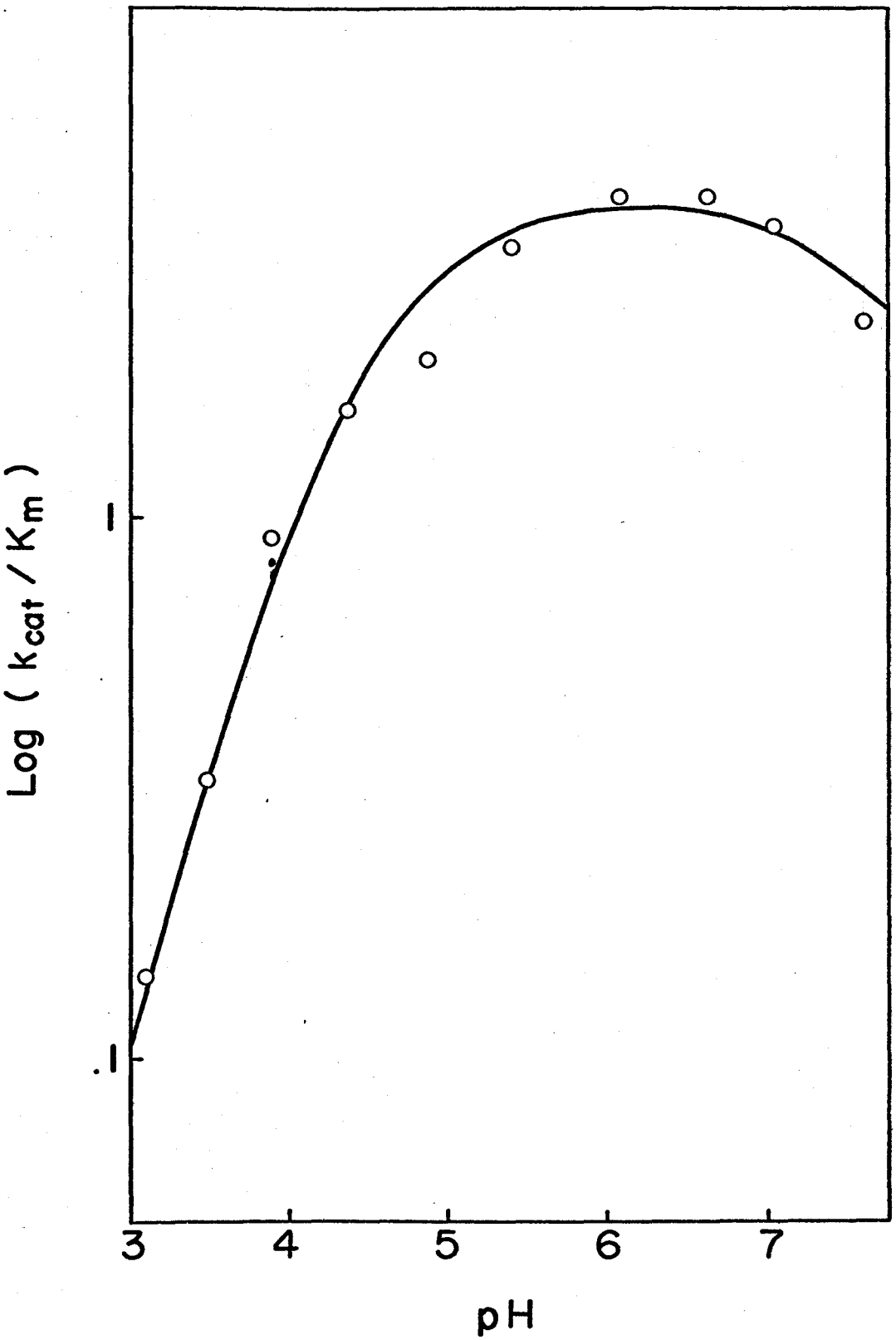
[a] 1.6% DMSO (v/v); 0.2 M NaCl, 1.1 mM EDTA; 0.9 mM DTT; 25°C.
$S_0 = 8.86 \times 10^{-5}$ M.

[b] Average of three determinations ± s.d.

[c] $E_0 = 6.03 \times 10^{-8}$ M.

[d] Represents a single determination.

Figure 18. Plot of log (k_{cat}/K_m) vs. pH. Data from Table 10. The solid line was calculated according to Equation 13. Values of ka_1, ka_2 and $(k_{cat}/K_m)_{lim}$ equaled 2.88×10^{-5}, 1.10×10^{-8} and 3.88×10^{6} $\underline{M}^{-1}sec^{-1}$, respectively.


Log (kcat / Km)
1
.1
3
4
5
6
7
pH

In this equation, H_{max} corresponds to the hydrogen ion concentration at the maximum height of the curve. Thus, a plot of K_m/k_{cat} vs. $(H^2 + H_{max}^2)/H$ at each pH should yield a straight line whose slope is $(1/K_{a1})(K_m/k_{cat})_{lim}$ and whose intercept is $(K_m/k_{cat})_{lim}$. The value of K_{a1} can be calculated from the slope and intercept, and the value for K_{a2} can be determined from the relationship

$$K_{a2} = (H_{max})^2/K_{a1} \qquad (13).$$

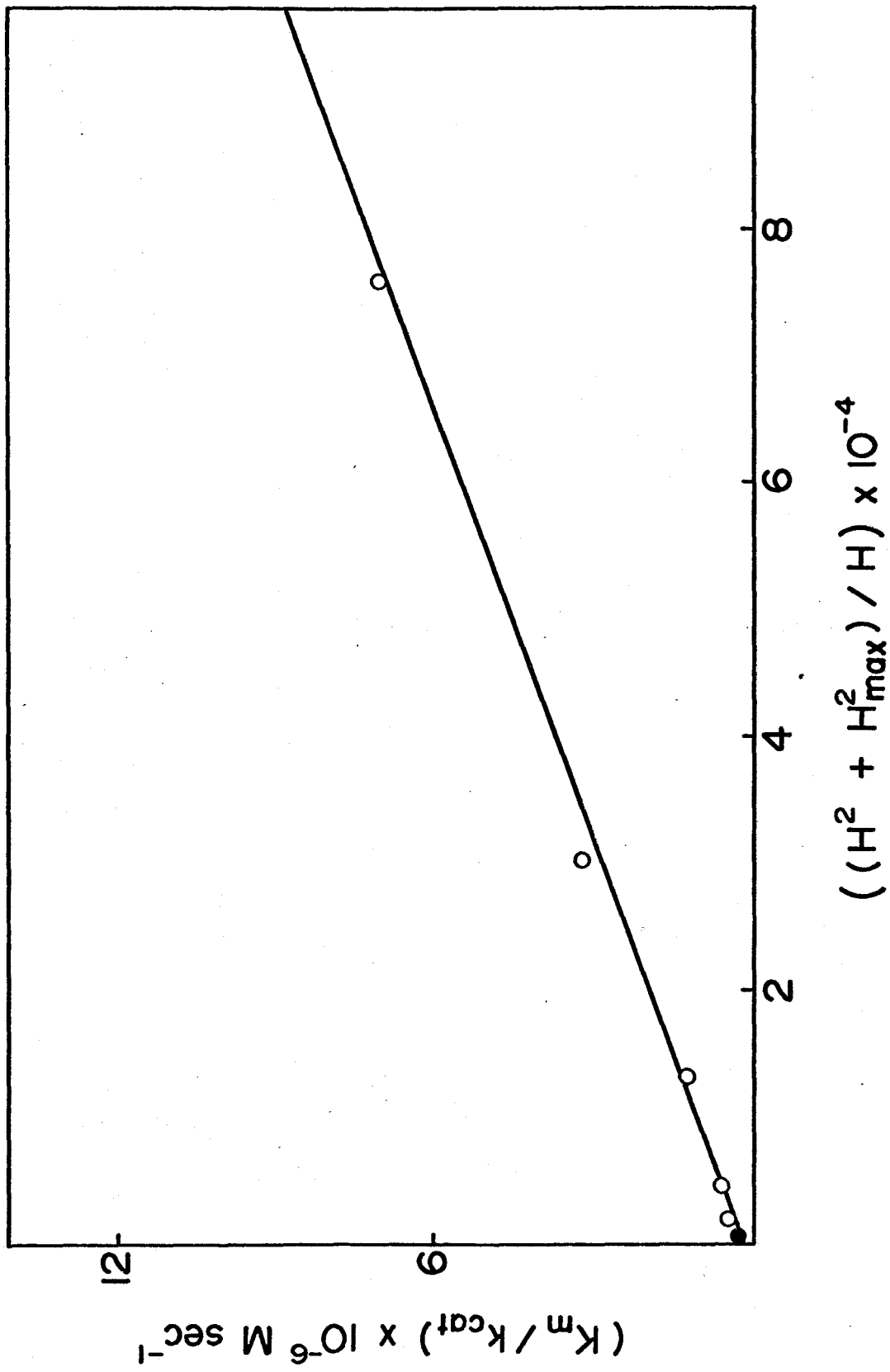
A plot of K_m/k_{cat} vs. $(H^2 + H_{max}^2)/H$ for cathepsin B1 and CLN is presented in Figure 19. Values of K_{a1} and K_{a2} were found to be 2.88×10^{-5} and 1.10×10^{-8}, respectively. These values correspond to apparent pK_a's of 4.54 and 7.95. The value of $(k_{cat}/K_m)_{lim}$ equaled $3.88 \pm .70 \times 10^6$ $\underline{M}^{-1}$ sec.

Having determined the pK_a's of the ionizable groups which control k_{cat}/K_m, a theoretical bell-shaped curve can be calculated according to Equation 14.

$$(k_{cat}/K_m)_{calc.} = (k_{cat}/K_m)_{lim}/(1 + H/K_{a1} + K_{a2}/H) \qquad (14)$$

Having the values of K_{a1}, K_{a2}, and $(k_{cat}/K_m)_{lim}$ from the plot in Figure 19, the solid line in Figure 18 was calculated. Since k_{cat}/K_m can be shown to be equal to k_2/K_S the acylation of cathepsin B1 by CLN appears to be dependent upon an ionizable acidic group of pK_a = 4.54 and upon an ionizable basic group of pK_a 7.95. On the basis of the

Figure 19. Plot of K_m/k_{cat} vs. $(H^2 + H_{max})/H$. Data from Table 10 was used. The closed circle represents values from pH 5.39 to pH 7.58.


12
9
2
4
6
8
$((K_m / k_{cat}) \times 10^{-6}\ M\ sec^{-1}$
$((H^2 + H^2_{max}) / H) \times 10^{-4}$

observations that the k_{cat} values for the hydrolysis of CLN and CLME were identical, it was inferred that the deacylation step is rate determining for these substrates. Consequently, k_{cat} for CLN and CLME hydrolysis is approximately equal to k_3. Since k_{cat} is essentially pH independent over the pH range studied, we may, as a first approximation, assume that k_3 is similarly pH independent. If K_s is also pH independent, as is the case for papain, then it is possible to calculate k_3, K_s and k_2 as described by Whitaker and Bender for papain (57). Equation 15 describes the relationship between K_m and k_{cat}/K_m(57).

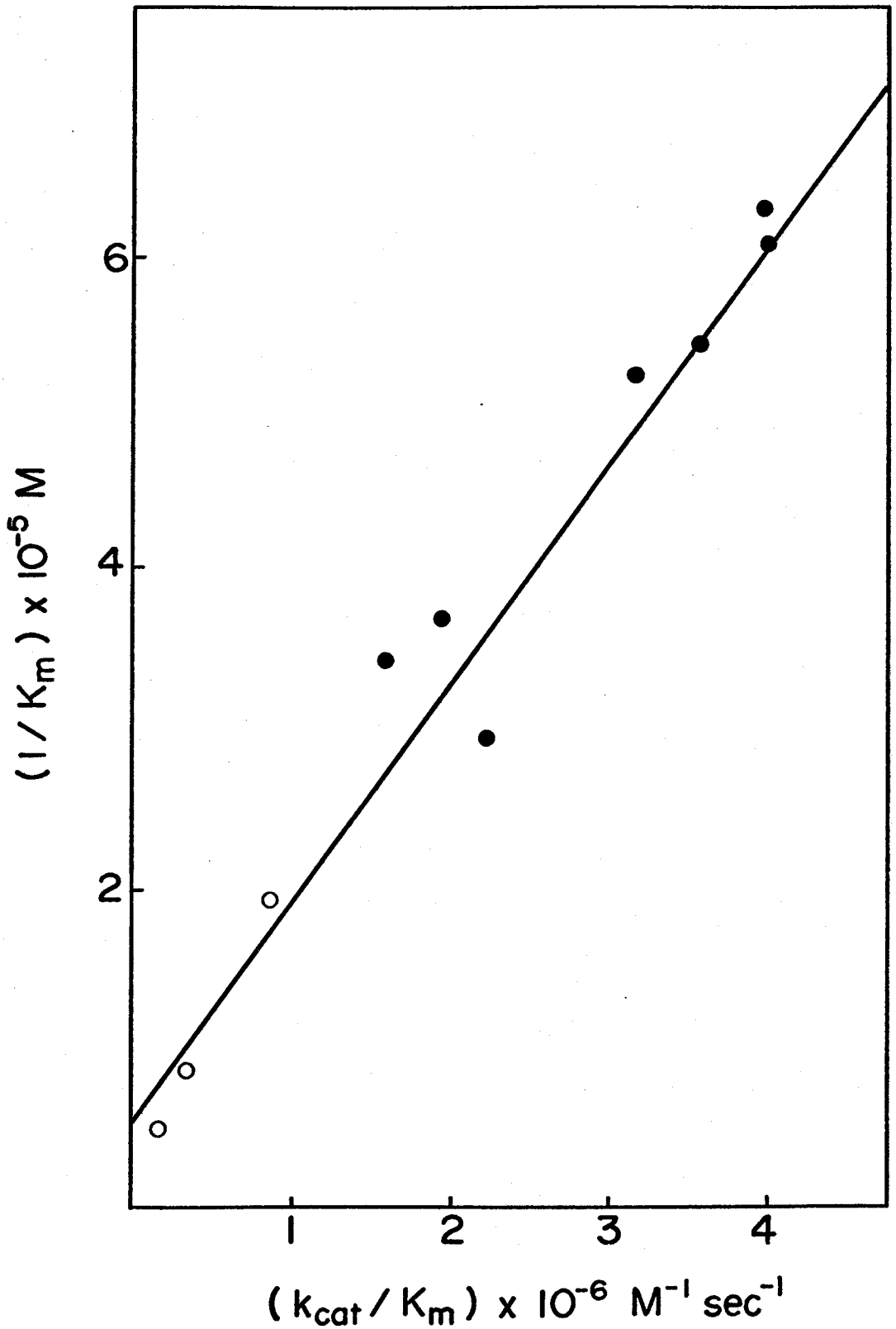
$$(1/K_m)_{observed} = 1/K_s + (1/k_3)(k_{cat}/K_m)_{observed} \quad (15)$$

Thus, a plot of $1/K_m$ vs. k_{cat}/K_m should yield a straight line with an intercept of $1/K_s$ and a slope of $1/k_3$. Such a plot was constructed with the data in Table 10 and is shown in Figure 20. A value of $7.00 \pm .50$ sec^{-1} was calculated for the deacylation rate constant, k_3, and a value of $2.04 \pm 1.08 \times 10^{-5}$ M for the dissociation constant K_s. Having both knowledge of $(k_{cat}/K_m)_{lim}$ and K_s, the value for $(k_2)_{lim}$ can be calculated using Equation 16.

$$(k_2)_{lim} = (k_{cat}/K_m)_{lim} (K_s) \quad (16)$$

The value for $(k_2)_{lim}$ was determined to be 79.15 ± 42 sec^{-1}.

Figure 20. Plot of $1/K_m$ vs. k_{cat}/K_m. Data from Table 10 was used. Open circles represent low pH values. Closed circles represent high pH values.


6
4
2
1
2
3
4
$(1/K_m) \times 10^{-5}$ M
$(k_{cat}/K_m) \times 10^{-6}$ M^{-1} sec^{-1}

3. The Effect of Nucleophiles on the Cathepsin B1 Catalyzed Hydrolysis of Ester and Amide Substrates

a. The Cathepsin B1 Catalyzed Hydrolysis of CLN and CGN in the Presence of MeOH

The effect of methanol on the cathepsin B1 catalyzed hydrolysis of CLN and CGN was investigated. Buffers were prepared with known concentrations of MeOH. If nucleophiles other than water can facilitate the deacylation of an acyl-cathepsin B1 intermediate, then, in the presence of an added nucleophile, Equation 2 will describe substrate hydrolysis.

$$E + S \underset{}{\overset{K_s}{\rightleftharpoons}} ES \xrightarrow{k_2} E' \begin{cases} \xrightarrow{k_3'(H_2O)} E + P_2 \\ \xrightarrow{k_4(N)} E + P_3 \end{cases} \qquad (2)$$

With CLN as substrate, P_1 is p-nitrophenol, P_2 is CBZ-lysine, and P_3 is the CBZ-lysine nucleophile adduct. The steady state rate equation describing the rate of release of p-nitrophenol in the presence of an added nucleophile is then given by Equation 3.

$$\frac{dP_1}{dt} = v = \frac{\dfrac{k_2(k_3(H_2O) + k_4(N))}{k_2 + k_3(H_2O) + k_4(N)}(E)_o}{\dfrac{k_3 + k_4(N)}{k_2 + k_3 + k_4(N)}\dfrac{K_s}{(S)} + 1} \qquad (3)$$

From this equation it can be seen that in the presence of an added nucleophile V_{max} is given by Equation 4.

$$(V_{max})_{ob} = \frac{k_2(k'_3(H_2O) + k_4(N))(E_o)}{k_2 + k_3(H_2O) + k_4(N)} \qquad (4)$$

Under conditions where $k_2 \gg (k'_3(H_2O) + k_4(N))$,

$$(V_{max})_{ob} = k'_3(E_o)(H_2O) + k_4(E_o)(N) \qquad (7)$$

Transformation of Equation 7 yields

$$\frac{(V_{max})_{ob}}{H_2O} = k'_3(E_o) + \frac{k_4(E_o)(N)}{H_2O} \qquad (17)$$

Such a plot of $(V_{max})_{obs}/[H_2O]$ vs. $[MeOH]/[H_2O]$ for CLN is shown in Figure 21. A similar plot for CGN is shown in Figure 22. Both plots yield a straight line and, in each case, indicates that MeOH acts as a nucleophile in the cathepsin B1 catalyzed hydrolysis of these substrates. From the ratios of k_{H_2O}/k_N, methanol is a 4.5 times better nucleophile than water in the reaction of cathepsin B1 with CLN and a 31.0 times better nucleophile in the reaction of cathepsin B1 with CGN.

b. <u>The Cathepsin B1 Catalyzed Hydrolysis of CLN in the Presence of Amino Acids and Simple Amino Acid Derivatives</u>

The ability of amino acids to stimulate the release of p-nitrophenol in the reaction of cathepsin B1 with CLN was studied. The amino acids tested included glycine, L-

Figure 21. Plot of $(V_{max})_{obs}/[H_2O]$ vs. $[MeOH]/[H_2O]$ for CLN. The buffer was 0.025 M sodium acetate, pH 5.1, containing 1.1 mM EDTA, 0.9 mM DTT and 1.6% (v/v) DMSO at 25°C.

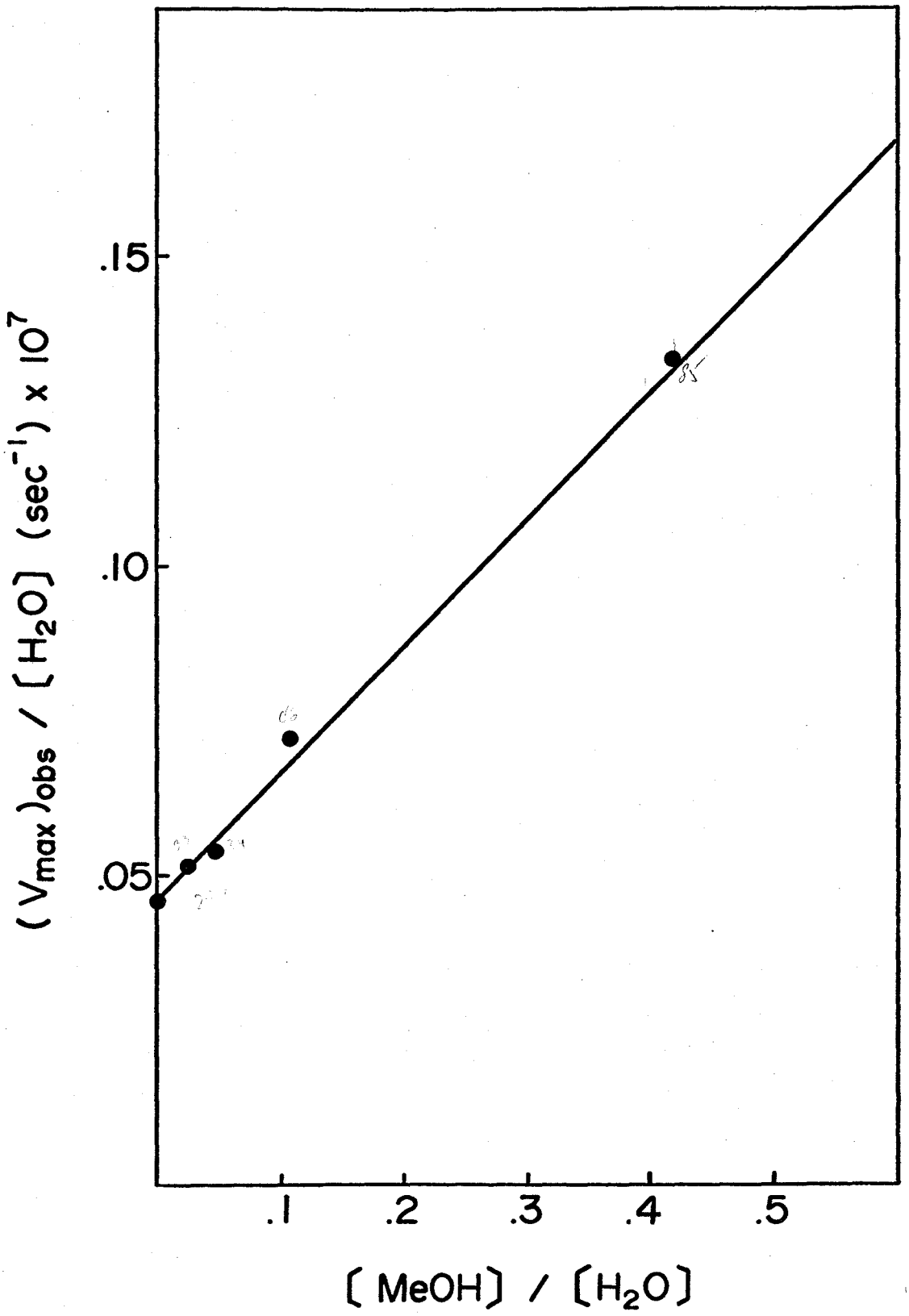
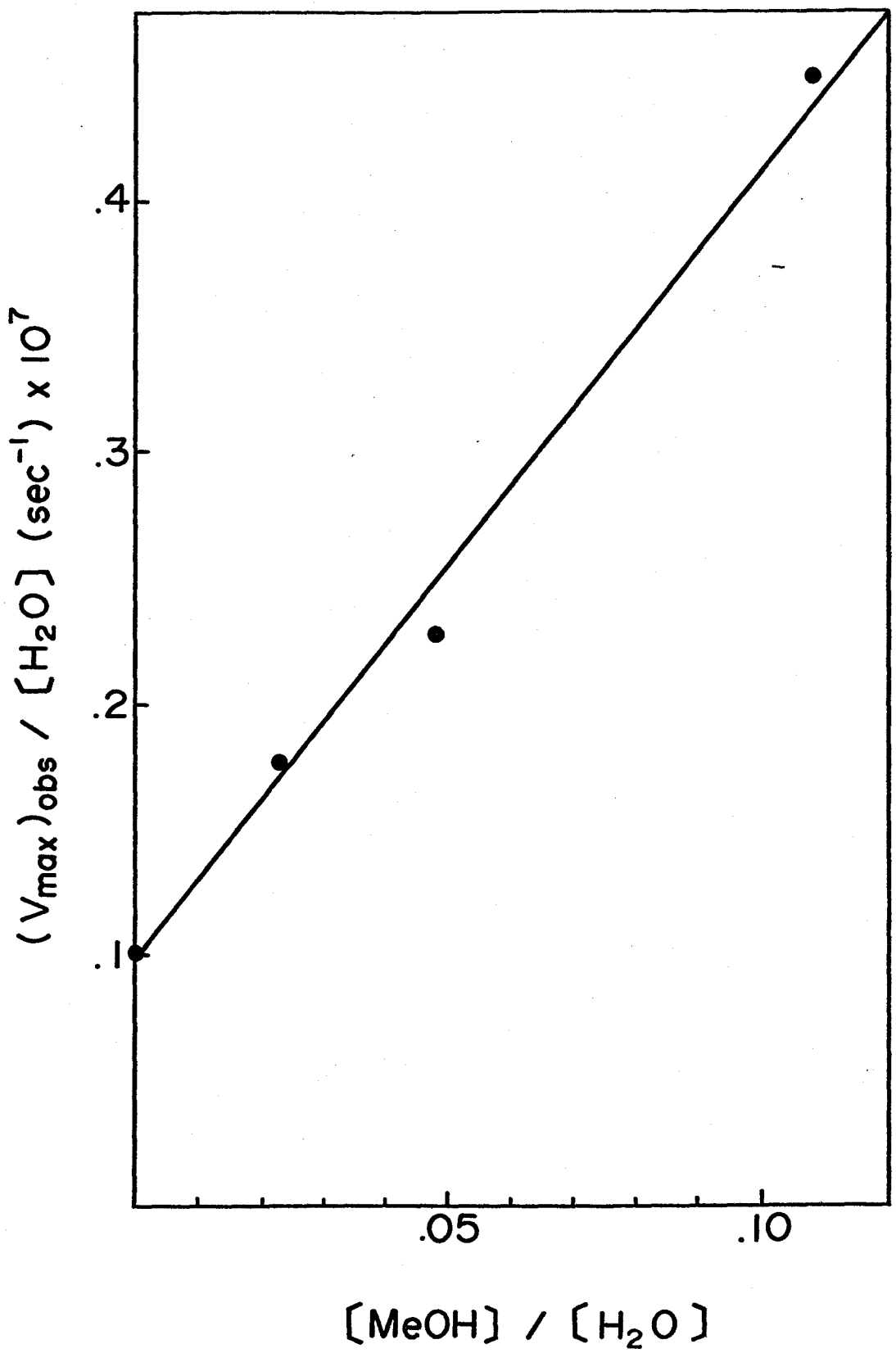

.15
.10
.05
.1
.2
.3
.4
.5
$(V_{max})_{obs}$ / $[H_2O]$ (sec^{-1}) x 10^7
[MeOH] / $[H_2O]$

Figure 22. Plot of $(V_{max})_{obs}/[H_2O]$ vs. $[MeOH]/[H_2O]$ for CGN. The buffer was 0.025 M sodium acetate, pH 5.1, containing 1.1 mM EDTA, 0.9 mM DTT, and 1.6% (v/v) DMSO at 25°C.


.4
.3
.2
.1
.05
.10
$(V_{max})_{obs}$ / $[H_2O]$ (sec^{-1}) x 10^7
[MeOH] / $[H_2O]$

alanine, L-leucine, L-isoleucine, L-serine, L-methionine, L-phenylalanine, L-tryptophan, L-asparagine, L-glutamine, L-lysine, L-arginine, and L-histidine. These amino acids had no effect on the rates of reaction of cathepsin B1 with CLN.

A number of simple amino acid derivatives were also studied to determine their effect on the rate of release of p-nitrophenol from CLN by cathepsin B1. Glycine ethyl ester (.092 M) was without effect as was 2-phenylethylamine (.092 M), a compound which is a good acyl acceptor in papain-catalyzed reactions (4). A number of tryptophan derivatives produced contrastingly different results. Table 11 summarizes the effect of these compounds on the cathepsin B1 catalyzed hydrolysis of CLN. All of the tryptophan derivatives acted as competitive inhibitors and their K_I's were calculated. The greatest degree of inhibition was seen with tryptamine which had a K_I of 2.14×10^{-3} M. Tryptophan methyl ester and amide were also nucleophiles, stimulating the release of p-nitrophenol by factors of 5 and 8, respectively at the indicated concentrations.

c. The Cathepsin B1 Catalyzed Hydrolysis of Substrates in the Presence of Glycylglycine

Glycylglycine was also found to markedly stimulate the cleavage of CLN by cathepsin B1. Figure 23 shows a reciprocal plot of rate data obtained in the presence of

Table 11

The Cathepsin B1 Catalyzed Hydrolysis of CLN

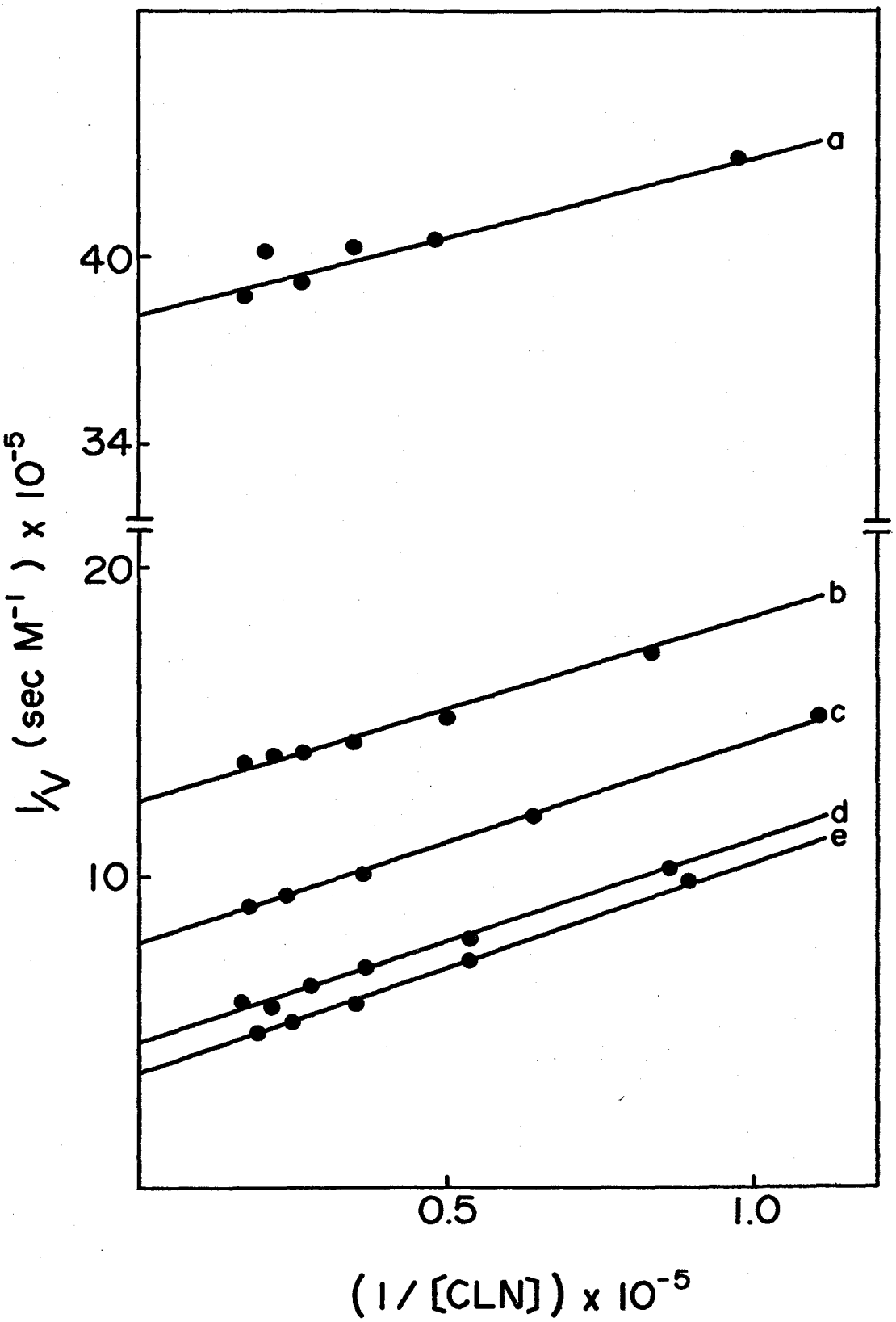
in the Presence of Tryptophan Derivatives

Analog	Conc.[a] (M)	k_{cat}[b] (sec^{-1})	K_m (M x 10^6)	k_{cat}/K_m[b] ($M^{-1}sec^{-1}$ x 10^{-6})	K_I (M)
None	--	5.78	1.45	3.96	--
Try	.092	7.21	3.10	2.33	.14
Try methyl ester	.092	28.00	38.3	0.73	2.2×10^{-2}
Try amide	.092	46.19	100.0	0.46	1.31×10^{-2}
Tryptamine	.0092	5.85	8.32	0.70	2.14×10^{-3}

[a] The buffer was 0.01 M potassium phosphate, pH 6.5, containing 1.1 mM EDTA, 0.9 mM DTT, 0.2 M NaCl and 1.6% (v/v) DMSO at 25°C.

[b] $E_0 = 9.57 \times 10^{-9}$ M.

Figure 23. Reciprocal Plots of Velocity vs. CLN Concentration at Increasing Concentrations of Glycylglycine. The buffer was 0.01 M potassium phosphate buffer, pH 6.5, containing 1.1 mM EDTA, 0.9 mM DTT, 0.2 M NaCl and 1.6% (v/v) DMSO at 25°C. Glycylglycine concentrations: (a) 0; (b) 0.092 M; (c) 0.18 M; (d) 0.37 M; and (e) 0.55 M.


40
34
20
10
0.5
1.0
a
b
c
d
e
1/V (sec M⁻¹) x 10⁻⁵
(1/[CLN]) x 10⁻⁵

different concentrations of gly-gly. A series of essentially parallel lines are obtained with V_{max} increasing with increasing nucleophile concentration.

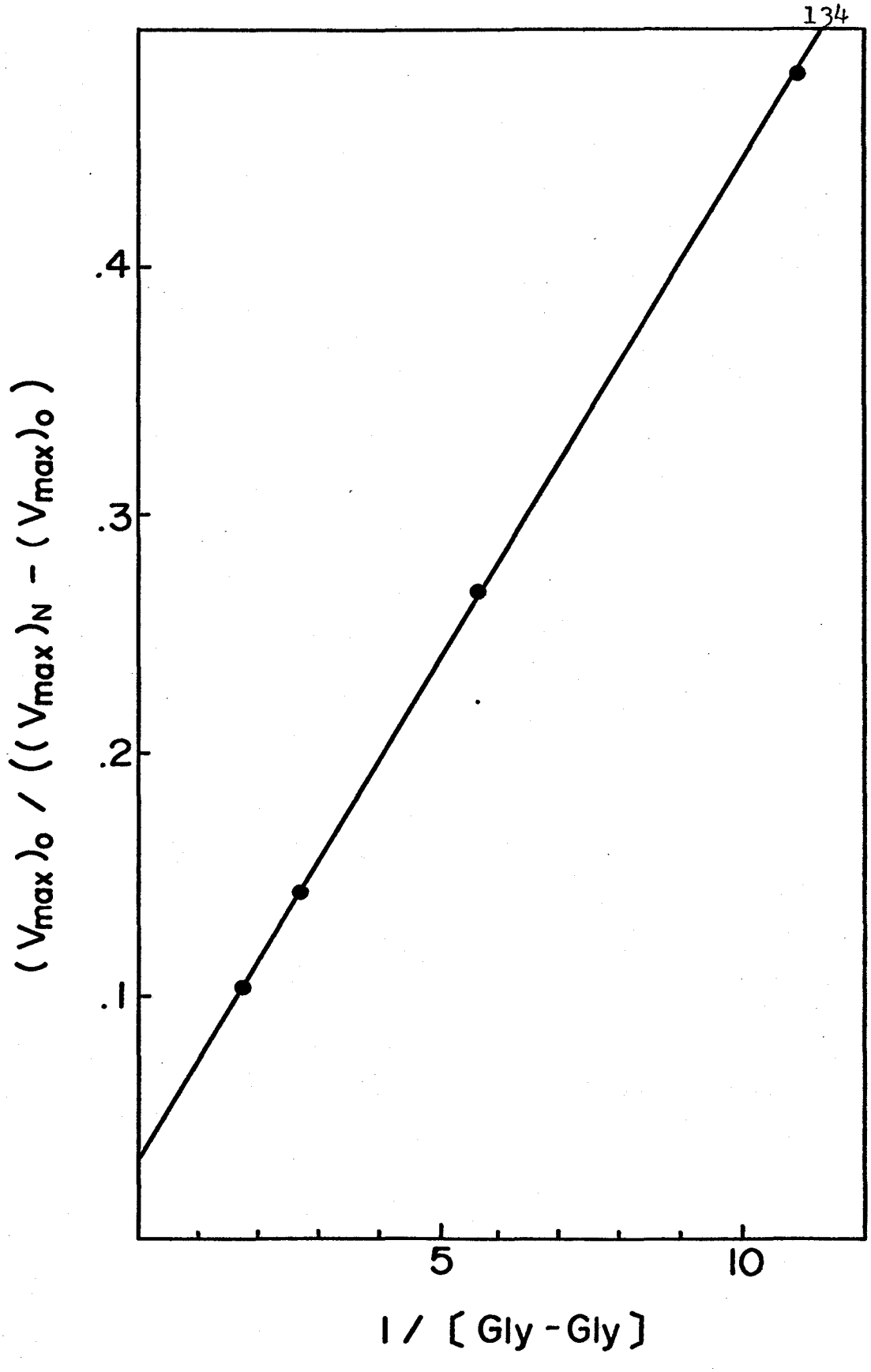
With good nucleophiles such as glycylglycine and other dipeptides, k_2 may no longer be much greater than $k_3'(H_2O) + k_4(N)$. With strong nucleophiles, Equation 18 describes the exact relationship between nucleophile concentration and maximum velocity. (Appendix A).

$$\frac{(V_{max})_o}{(V_{max})_N(V_{max})_o} = \frac{k_3(k_2 + k_3)}{k_2k_4(N)} + \frac{k_3}{k_2} \qquad (18)$$

A plot of $(V_{max})_o/((V_{max})_N - (V_{max})_o)$ vs. $1/N$ should yield a straight line whose intercept is k_3/k_2, the ratio of rate constants for deacylation and acylation, and whose slope is $k_3(k_2 + k_3)/k_2k_4$. Such a plot for gly-gly is shown in Figure 24. As predicted by Equation 18, a straight line is observed. From the intercept the ratio of acylation to deacylation, k_2/k_3, was found to be about 31.

In order to verify the acyl-enzyme mechanism for cathepsin B1 it is necessary to demonstrate that cathepsin B1 catalyzes the transfer of the CBZ-L-lysyl residue from p-nitrophenol to the nucleophile. To this end, aliquots were removed from a reaction mixture containing glyclyglycine and CLN, and chromatographed on silica gel thin-layer plates. The developing solvent was butanol:acetic acid: water, 4:1:1. A new spot appeared having a mobility inter-

Figure 24. Plot of $(V_{max})_o/((V_{max})_N - (V_{max})_o)$ vs. $1/[\text{Gly-Gly}]$. Data was obtained from Figure 23.


.4
.3
.2
.1
5
10
$(V_{max})_o / ((V_{max})_N - (V_{max})_o)$
1 / [Gly - Gly]

mediate between CBZ-L-lysine and glycylglycine. A drawing of the plate is shown in Figure 25. This component did not appear when either glycylglycine or cathepsin B1 was omitted from the reaction mixture.

The new product was isolated by preparative thin layer chromatography, hydrolyzed for 24 hr in 6 N HCl at 110°C in vacuo, and rechromatographed on silica gel with a solvent of 95% ethanol:water, 7:3. The spot corresponding to the unknown compound disappeared and was replaced by two spots having mobilities identical to those of glycine and lysine. The amino acid analysis of the 6 N HCl hydrolysate indicated the ratio of lysine to glycine for the new peptide product was 1 to 2, suggesting that the new product is CBZ-L-lysylglycylglycine.

In contrast to esters, the catalysis of amides by cathepsin B1 is expected to be acylation rate limiting (57). In the presence of nucleophiles no effect on the maximum velocity of the reaction should be observed since nucleophiles participate in the deacylation step. Lineweaver and Burk plots of rate data for the hydrolysis of BAPA by cathepsin B1 in the presence and absence of glycylglycine, as shown in Figure 26, confirm this prediction. In addition, there is no indication that glycylglycine is a competetive inhibitor of BAPA hydrolysis by cathepsin B1.

Figure 25. Silica Gel Thin Layer Chromatogram of the Reaction Mixture Obtained from the Cathepsin B1 Catalyzed Cleavage of CLN in the Presence and Absence of Glycylglycine. Developing solvent was n-butanol:acetic acid: water, 4:1:1. Cross hatched spots represent the new peptide.

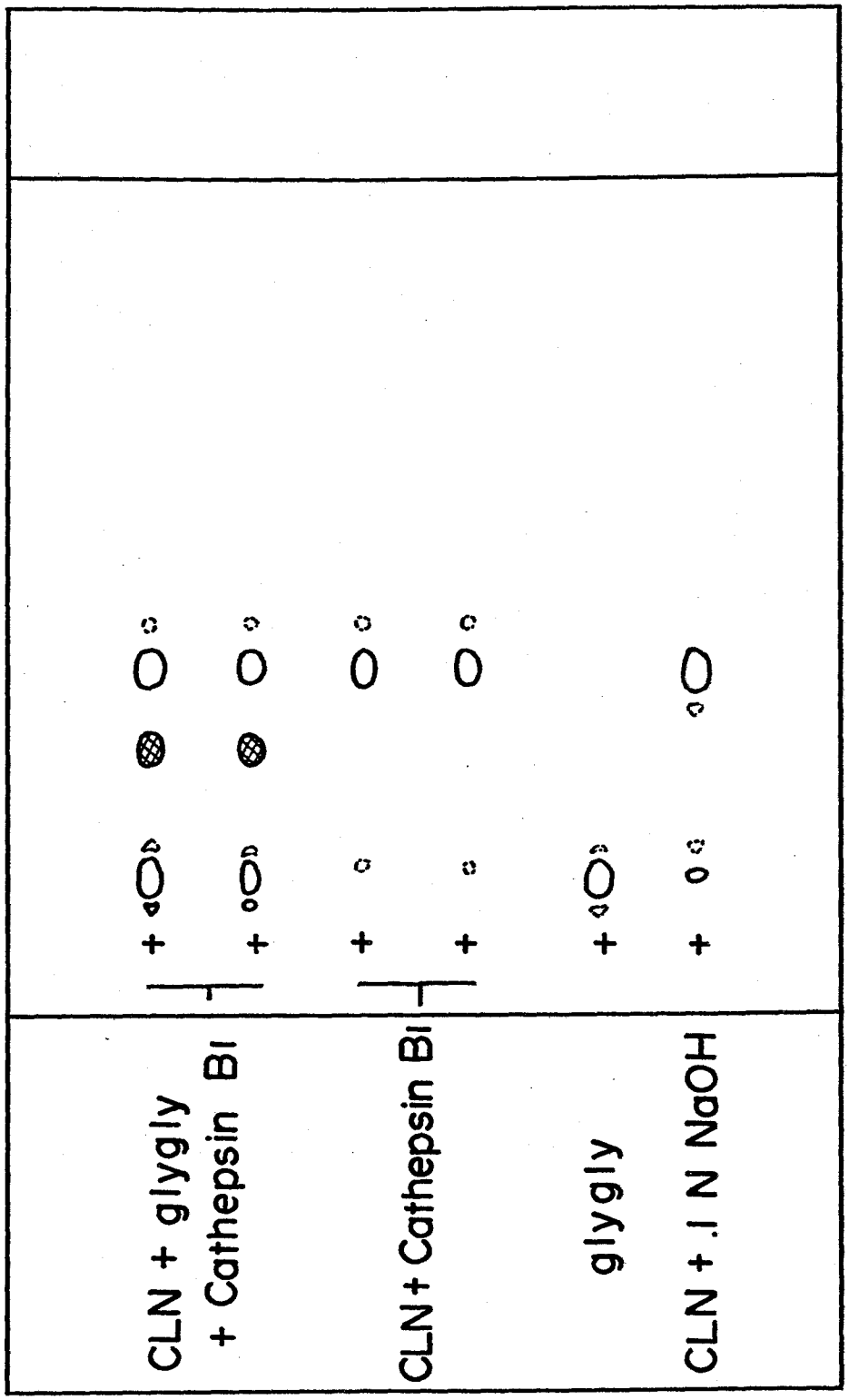
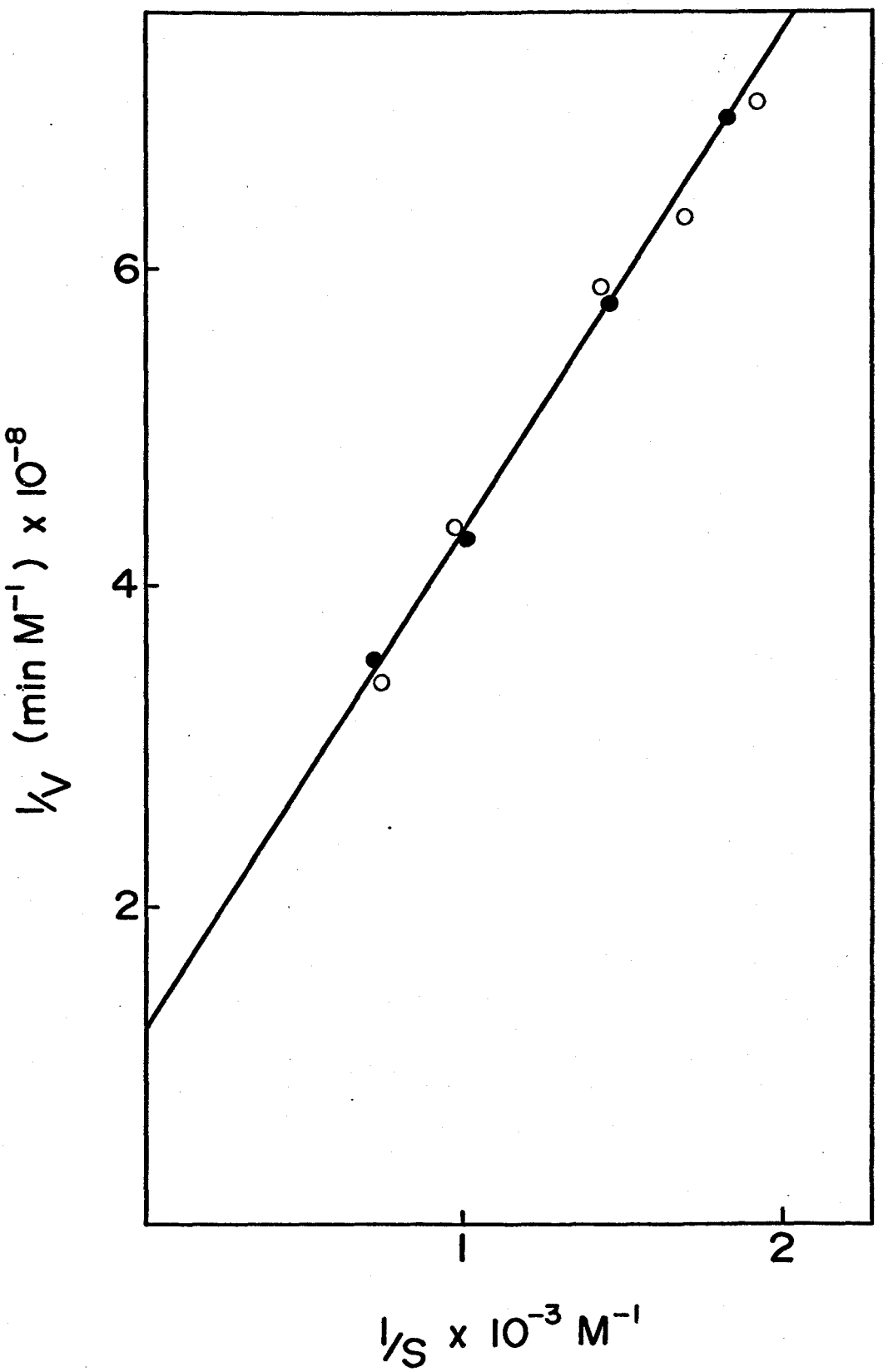

CLN + glygly
+ Cathepsin BI
CLN + Cathepsin BI
glygly
CLN + .1 N NaOH

Figure 26. Lineweaver and Burk Plots of Rate Data for the Hydrolysis of BAPA in the Presence and Absence of Glycylglycine. The buffer was 0.01 M potassium phosphate, pH 6.5, containing 1.1 mM EDTA, 0.9 mM DTT, and 1.6% (v/v) DMSO at 25°C. Glycylglycine concentrations: (a) 0 (closed circles); and (b) 0.37 M (open circles).


6
4
2
1
2
1/V (min M⁻¹) x 10⁻⁸
1/S x 10⁻³ M⁻¹

d. The Effects of Other Dipeptide Nucleophiles on Substrate Hydrolysis

i. The Effect of Dipeptides on the Kinetic Parameters of CLN Hydrolysis

The dipeptides, gly-phe, leu-gly and leu-phe, were studied to determine the extent to which they stimulated the cathepsin B1 catalyzed hydrolysis of CLN. From these studies the rate constant, k_4, was calculated for each dipeptide. In addition, these results could also be used to calculate the rate constants, k_2 and k_3, independently of the method using pH dependency data.

As was mentioned previously for gly-gly, the assumption that $k_2 >> [k_3 + k_4(N)]$ is not valid with good nucleophiles at higher concentrations. Indeed, with higher concentrations of dipeptides, $[k_3 + k_4N]$ approaches k_2, and $(V_{max})_N$ approaches k_2E_o. Under these conditions a plot of V_N with increasing concentrations of N should show apparent satuation with N. This was observed with gly-phe as shown in Figure 27. Since Equation 18 describes an exact relationship between V_{max} and [N], a plot of $(V_{max})_o/((V_{max})_N - (V_{max})_o)$ vs. $1/[N]$ should be linear. These predictions were confirmed for gly-phe as shown in Figure 28. The intercept yields k_3/k_2. Alternatively, one can also plot $((V_{max})_N(V_{max})_o)/((V_{max})_N - (V_{max})_o)(E_o)$ vs. $1/N$ (Equation 12, Appendix A). Such a plot should yield a straight line with an intercept equal to k_3 and a slope equal to k_4/k_3. Typical plots of $(V_{max})_N(V_{max})_o/((V_{max})_N - (V_{max})_o)(E_o)$

Figure 27. Plot of V_{max} vs. Glycylphenylalanine Concentration. The buffer was 0.01 M potassium phosphate, pH 6.5, containing 1.1 mM EDTA, 0.9 mM DTT, 0.2 M NaCl, and 1.6% (v/v) DMSO at 25°C. The substrate was CLN.

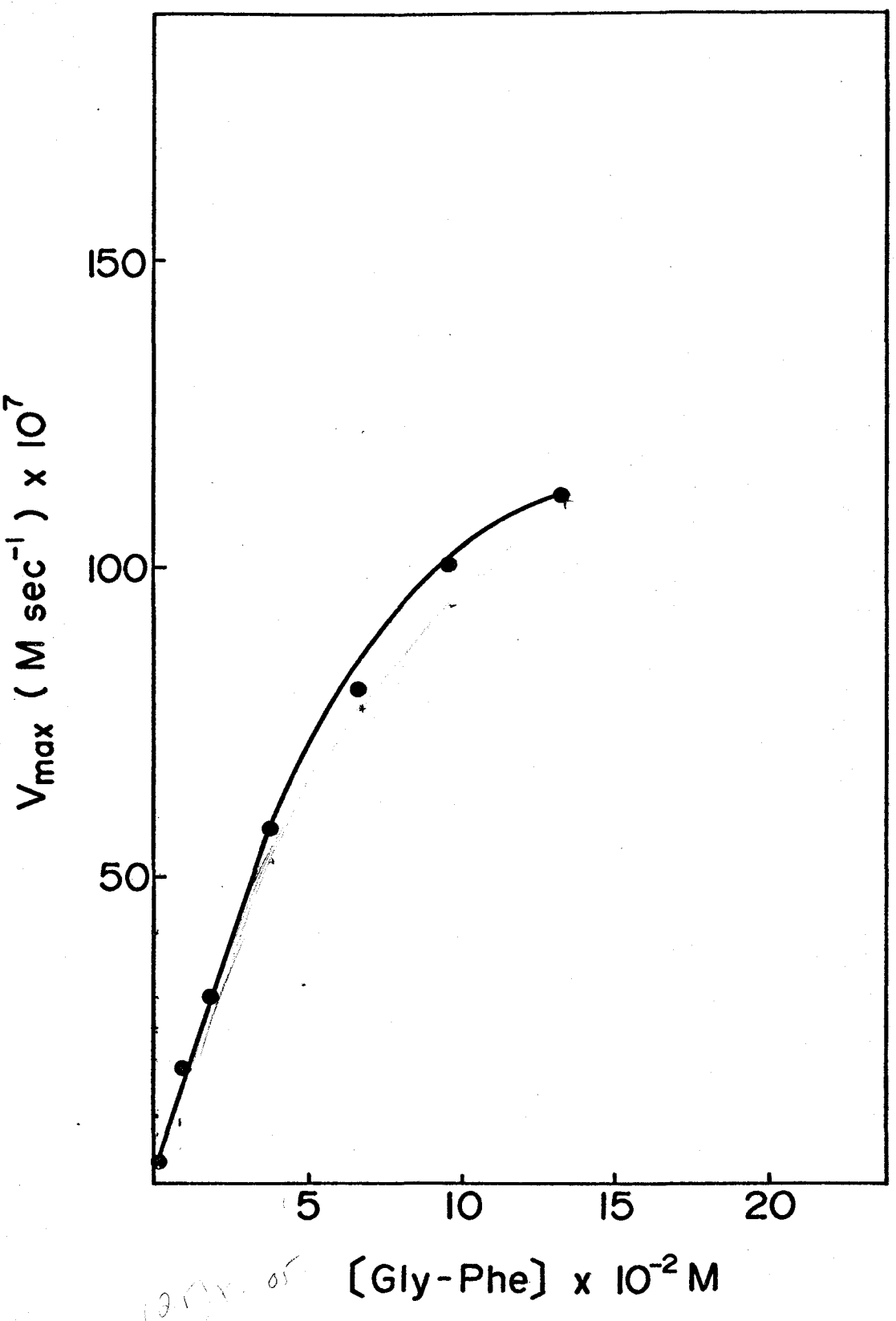
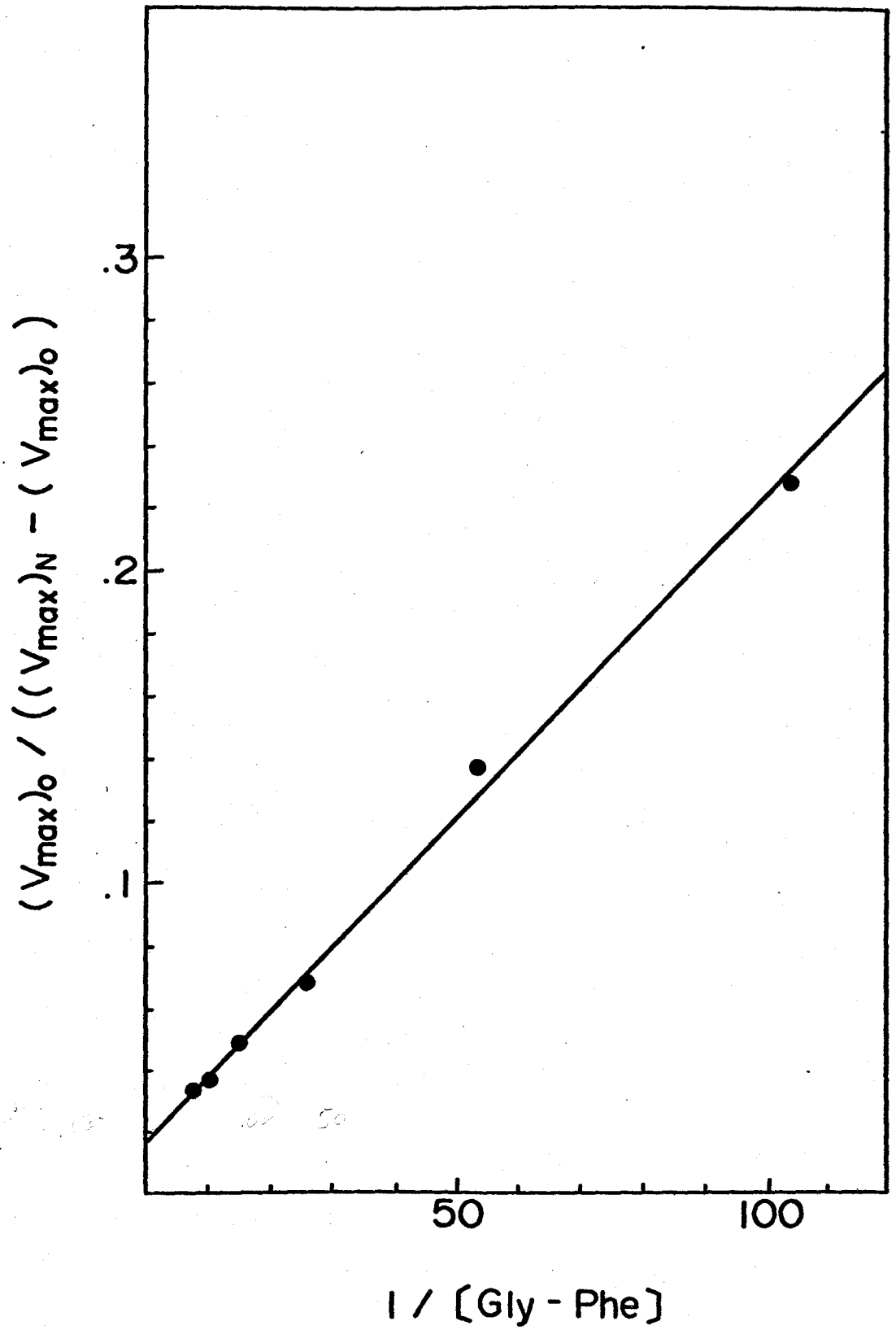

150
100
50
V_{max} (M sec^{-1}) x 10^7
5
10
15
20
[Gly-Phe] x 10^{-2} M

Figure 28. Plot of $(V_{max})_o/((V_{max})_N - (V_{max})_o)$ vs. 1/Gly-Phe. The buffer was 0.01 M potassium phosphate, pH 6.5, containing 1.1 mM EDTA, 0.9 mM DTT, 0.2 M NaCl and 1.6% (v/v) DMSO at 25°C. The substrate was CLN.


$(V_{max})_o / ((V_{max})_N - (V_{max})_o)$
.3
.2
.1
50
100
1 / [Gly - Phe]

vs. 1/[N] for leu-phe and leu-gly are shown in Figures 29 and 30, respectively.

The values of k_4/k_3, k_3/k_2, k_3, k_4 and k_2 were calculated for gly-gly, gly-phe, leu-gly and leu-phe and are collected in Table 12. The values for k_2 and k_3 were found to be approximately 390 sec^{-1} and 6.58 sec^{-1}, respectively. The value for k_3 is in good agreement with that calculated from the pH dependency of CLN hydrolysis. The value of k_2, however, appears to be 4.9 times higher than was calculated from the pH studies. The reasons for this discrepency is unknown, but probably reflects the large uncertainties in both the experimentally determined values of K_S and the ratio (k_3/k_2). Based on the value for k_4, the order of decreasing nucleophilicity for the dipeptides appears to be leu-gly > gly-phe > leu-phe > gly-gly.

The ability of leu-gly to stimulate the cathepsin B1 catalyzed hydrolysis of CLN is dependent upon the dipeptide's enantiomeric form. Table 13 compares the hydrolysis of CLN in the presence of a racemic mixture of D,L-leu-D, L-phe with optically pure L-leu-L-phe. In the presence of the racemic mixture the observed value for k_4/k_3 was one-fourth that of the optically active dipeptide. Thus, it appears that cathepsin B1 displays stereo-specifity for both amino acids in dipeptide nucleophiles during deacylation.

Figure 29. Plot of $((V_{max})_N(V_{max})_o)/((V_{max})_N - (V_{max})_o)(E_o)$ vs. $1/[\text{Leu-Phe}]$. The buffer was 0.01 M potassium phosphate, pH 6.5, containing 1.1 mM EDTA, 0.9 mM DTT, 0.2 M NaCl and 1.6% (v/v) DMSO at 25°C. The substrate was CLN.

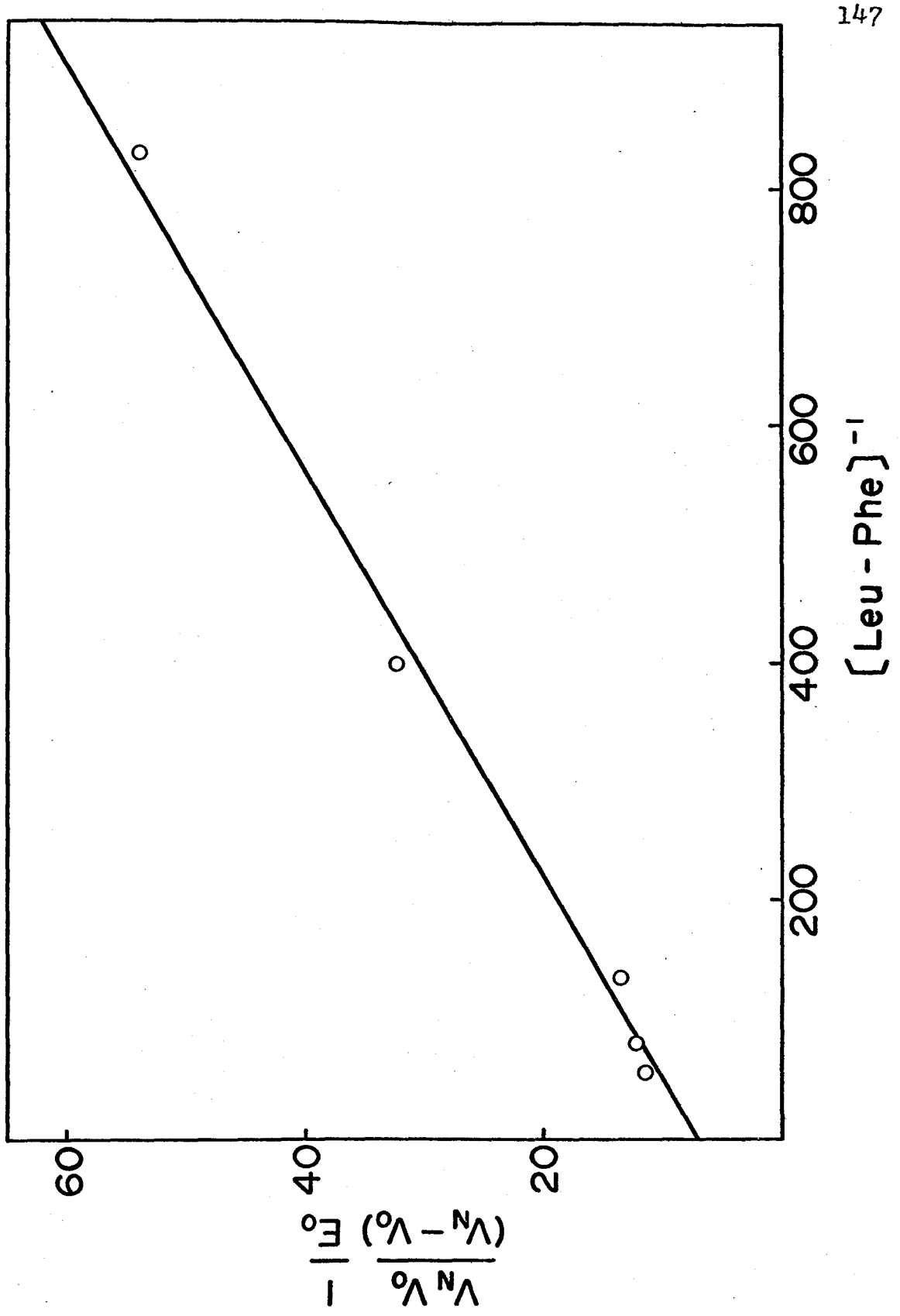

60
40
20
$\frac{V_N V_o}{(V_N - V_o)} \frac{1}{E_o}$
200
400
600
800
$[Leu-Phe]^{-1}$

Figure 30. Plot of $((V_{max})_N(V_{max})_o)/((V_{max})_N - (V_{max})_o)$ (E_o) vs. $1/[\text{Leu-Gly}]$. The buffer was 0.01 M potassium phosphate, pH 6.5, containing 1.1 mM EDTA, 0.9 mM DTT, 0.2 M NaCl and 1.6% (v/v) DMSO at 25°C. The substrate was CLN.

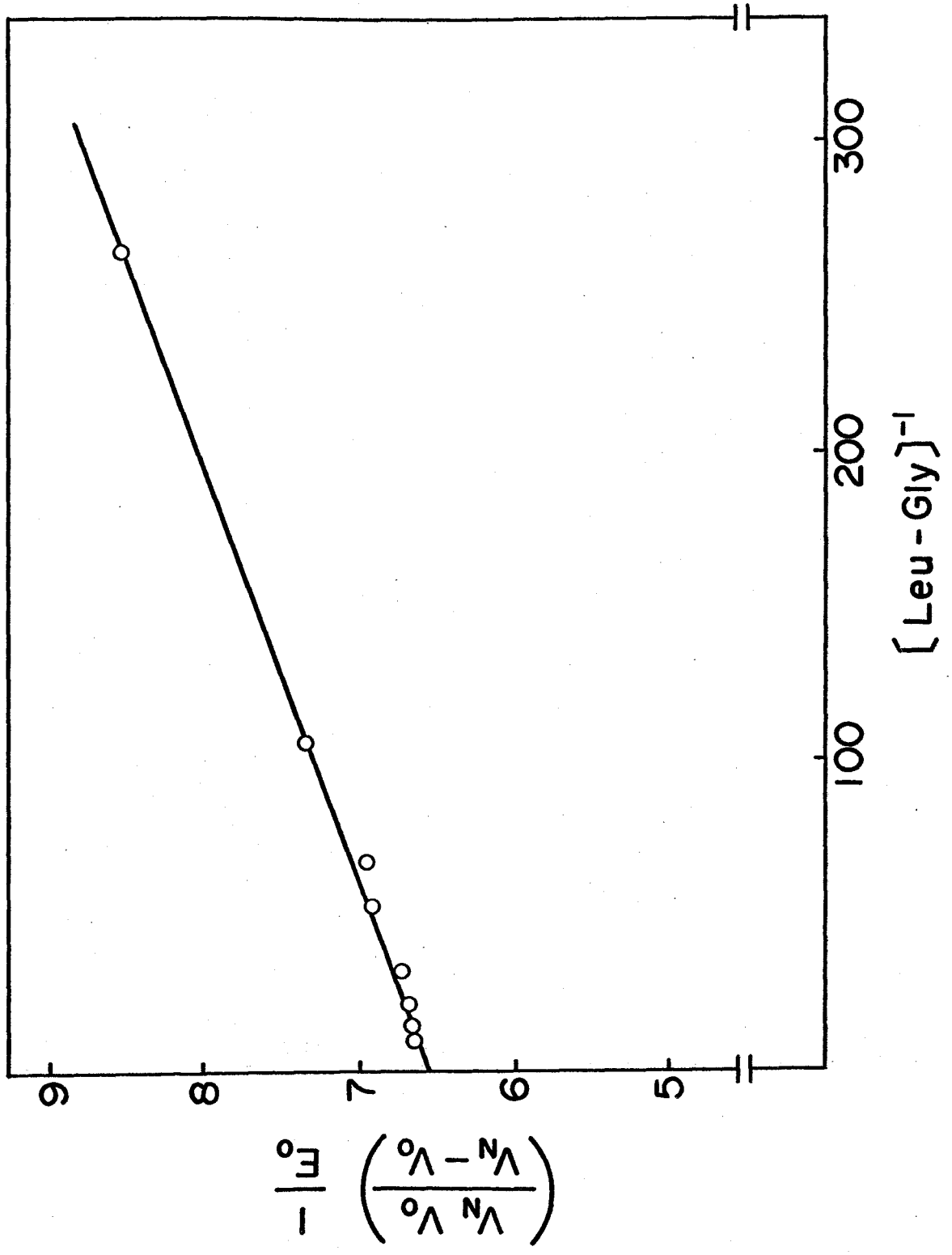

9
8
7
6
5
100
200
300
[Leu-Gly]⁻¹
1/E₀ (V_N V₀ / V_N − V₀)

Table 12

Determination of Kinetic Constants for the Cathepsin B1 Catalyzed Hydrolysis of CLN in the Presence of Dipeptides

Dipeptide	k_4/k_3 (M^{-1})	k_4 ($\times 10^3$ $M^{-1}sec^{-1}$)	k_3 (sec^{-1})	k_3/k_2	k_2 (sec^{-1})
Gly-Gly	21.7 ± 1.2	0.14 ± .01	6.64 ± .07	0.0172 ± .0107	386 ± 151
Leu-Phe	113 ± 4	0.71 ± .41	6.50 ± 1.80	--	--
Gly-Phe	496 ± 13	3.29 ± .08	6.64 ± .02	0.0169 ± .0034	393 ± 85
Leu-Gly	854 ± 29	5.61 ± .23	6.54 ± .03	0.0153 -- .0011	-- 368

a The buffer was 0.01 M potassium phosphate, pH 6.5, containing 1.1 mM EDTA, 0.9 mM DTT, 0.2 M NaCl, and 1.6% (v/v) DMSO at 25°C. $E_0 = 6.10 \times 10^{-8}$ M.

Table 13

The Effect of a Racemic Mixture of Leucylphenylalanine on the Cathepsin B1 Catalyzed Hydrolysis of CLN[a]

Dipeptide	Conc. ($\underline{M} \times 10^{-3}$)	k_{cat}[b] (sec^{-1})	K_m ($\underline{M} \times 10^{6}$)	k_{cat}/K_m[b] ($\underline{M}^{-1}sec^{-1} \times 10^{-6}$)	k_4/k_3
None[c]	--	6.52	1.85	3.52	--
D,L-Leu-D,L-Phe[c]	2.60	15.72	6.66	2.36	542
L-Leu-L-Phe[c]	2.00	34.47	32.99	1.04	2144

[a] The buffer was 0.01 $\underline{M}$ potassium phosphate, pH 6.5, containing 1.1 m$\underline{M}$ EDTA, 0.9 m$\underline{M}$ DTT, 0.2 $\underline{M}$ NaCl and 1.6% (v/v) DMSO at 25°C.

[b] $E_0 = 4.85 \times 10^{-8}$ $\underline{M}$.

[c] Average of two determinations.

ii. Comparison of CLN and CLME Hydrolysis in the Presence of Leu-Gly

It was shown previously that substrates derived from the same amino acid and differing only in the identity of the leaving group were hydrolyzed with identical rates. This suggested that a common acyl-enzyme intermediate was formed whose hydrolysis was in each case rate limiting. Further support for this hypothesis comes from a study of the dipeptide, leu-gly, and its ability to stimulate the hydrolysis of these substrates. The results are presented in Table 14.

L-Leucyl-L-glycine not only stimulates the hydrolysis of both CLN and CLME, but does so to the same extent. The ratio k_4/k_3 for the two substrates are essentially identical, providing further support for the hypothesis that the hydrolysis of CLN and CLME by cathepsin B1 proceeds through a common acyl-enzyme intermediate. One difference observed in this study was that leu-gly decreased the value of k_{cat}/K_m for CLME and not for CLN, suggesting that leu-gly is a competitive inhibitor of CLME but not CLN. The K_I for leu-gly in the reaction of cathepsin B1 with CLME was found to be 3.3×10^{-3} M.

iii. The Effect of Phe-Gly on CLN Hydrolysis

In contrast to the results obtained with other dipeptides, phe-gly did not stimulate the cathepsin B1 catalyzed

Table 14

Kinetic Constants for the Cathepsin B1 Catalyzed Hydrolysis of Alpha-N-Benzyloxycarbonyl-L-Lysine Esters in the Presence of 3.26 mM Leucyl-L-Glycine[a]

Substrate	k_{cat}[b] (sec^{-1})	K_m ($M \times 10^6$)	k_{cat}/K_m[b] ($M^{-1}sec^{-1} \times 10^{-3}$)	k_4/k_3
CLN[c]	6.52 ± .31	5.57 ± .31	1171 ± 86	--
CLN + Leu-gly[c]	28.13 ± .97	20.00 ± 2	1406 ± 295	1016 ± 48
CLME[c]	6.06 ± .27	2080 ± 22	3.0 ± 0.1	--
CLME + Leu-gly[c]	28.10 ± .92	18,300 ± 470	1.5 ± 0.1	1123 ± 50

[a] The buffer was 0.01 M potassium phosphate, pH 6.52, containing 1.1 mM EDTA, 0.9 mM DTT, and 1.6% (v/v) DMSO at 25°C.

[b] The concentration of Cathepsin B1 was 7.90×10^{-8} M.

[c] Average of three determinations ± s.d.

hydrolysis of CLN. Lineweaver and Burk plots of rate data for the hydrolysis of CLN in the presence and absence of phe-gly are shown in Figure 31. The dipeptide acted not as a nucleophile but as a competitor inhibitor. The K_I for phe-gly was found to be 1.32×10^{-2} M.

iv. The Effect of Glycylglycyl Amide on CLN Hydrolysis

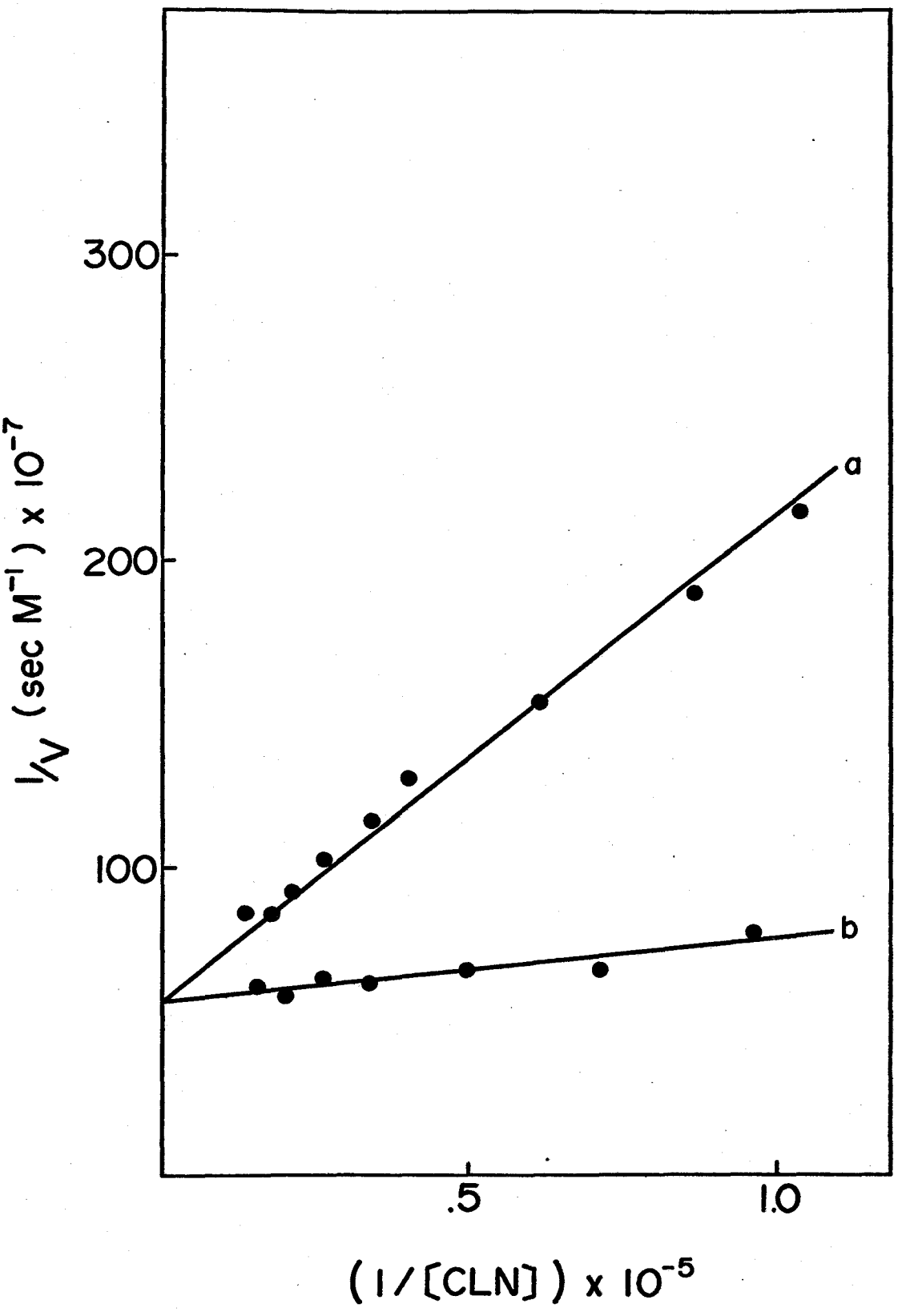
Although gly-gly was shown to act as a good nucleophile in the cathepsin B1 catalyzed hydrolysis of CLN, .092 M glycylglycine amide did not stimulate V_{max}. Instead, gly-gly amide was a competitive inhibitor of CLN hydrolysis, the K_I being 0.16 M. Such results suggest that the free carboxyl group of gly-gly is necessary for the dipeptide to act as a nucleophile.

e. The pH Dependency of CLN Hydrolysis in the Presence of Dipeptides

In a previous section it was shown that the rate constants for the individual steps in the cathepsin B1 catalyzed hydrolysis of CLN could be calculated by studying the rate of p-nitrophenol release in the presence of an added nucleophile. It was of some interest, therefore, to repeat those studies at various pH values in order to determine the pH dependency of the individual steps in the reaction.

From Equations 9 and 12 (Appendix A), the following relationships can be derived

Figure 31. Lineweaver and Burk Plots of Rate Data for the Hydrolysis of CLN in the Presence and Absence of Phenylalanylglycine. The buffer was 0.01 M potassium phosphate, pH 6.5, containing 1.1 mM EDTA, 0.9 mM DTT, 0.2 M phenylalanylglycine concentrations: (a).092 M; and (b) 0.


300
200
100
1/V (sec M⁻¹) x 10⁻⁷
a
b
.5
1.0
(1/[CLN]) x 10⁻⁵

$$\frac{\left[\frac{V_o(N_2)}{V_{N2} - V_o} - \frac{V_o(N_1)}{V_{N1} - V_o}\right]}{(N_2) - (N_1)} = k_3/k_2 \qquad (19)$$

$$\left[\frac{V_o}{V_{N1} - V_o} - \frac{V_o}{V_{N2} - V_o}\right] \frac{(N_1)(N_2)}{(N_2) - (N_1)} = \frac{k_3}{k_2}\left[\frac{k_2 + k_3}{k_4}\right] \qquad (20)$$

$$\frac{1}{E_o}\frac{\left[\frac{V_{N2}V_o(N_2)}{V_{N2} - V_{N1}} - \frac{V_{N1}V_o(N_1)}{V_{N1} - V_o}\right]}{(N_2) - (N_1)} = \frac{k_3^2}{k_4} \qquad (21)$$

$$\frac{1}{E_o}\left[\frac{V_{N1}V_o}{V_{N1} - V_o} - \frac{V_{N2}V_o}{V_{N2} - V_o}\right] \frac{(N_1)(N_2)}{(N_2) - (N_1)} = k_3 \qquad (22)$$

In these equations $(E)_o$ is the total enzyme concentration; V_o, V_{N1} and V_{N2} are V_{max} values in the absence and in the presence of two different nucleophile concentrations; and (N_1) and (N_2) are the respective nucleophile concentrations.

Figure 32 shows a plot of log (V_{max}) vs. pH in the absence and in the presence of 0.092 M and 0.37 M glycylglycine. The points represent the experimental data and the solid lines were drawn by hand to represent reasonable fits to the data points. At intervals of 0.5 pH units, values of V_{max} were estimated from the curves. These are collected in Table 15. The individual rate constants k_2, k_3, and k_4

Figure 32. The Cathepsin B1 Catalyzed Hydrolysis of CLN in the Absence and Presence of 0.092 M and 0.37 M Glycylglycine. Plot of log (V_{max}) vs. pH. (———) 0 M gly-gly; (● ● ●) 0.092 M gly-gly; and (▲▲▲▲) 0.37 M gly-gly. The following buffers were used: pH 4.0, 13 mM formate; pH 4.0 - pH 6.0, 25 mM sodium acetate; and pH 6.0, 10 mM phosphate. All buffers contained 1.1 mM EDTA, 0.9mM DTT, 0.2 M NaCl and 1.6% (v/v) DMSO at 25°C. (I) range of values. Data points represent 3 or 4 determinations.

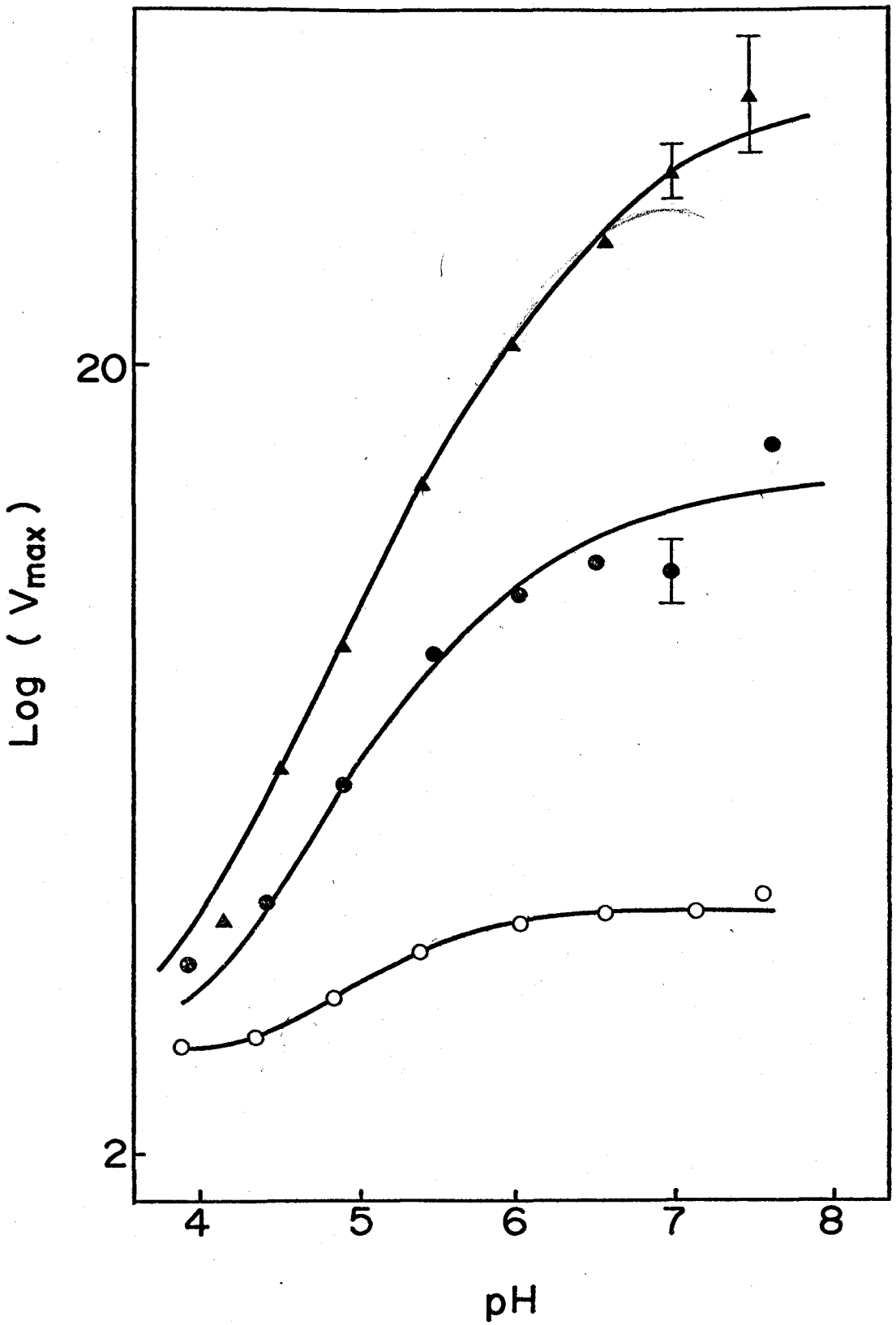

Log (Vmax)
20
2
4
5
6
7
8
pH

Table 15

The Cathepsin B1 Catalyzed Hydrolysis of CLN: V_{max} vs. pH in the Presence and Absence of Glycylglycine[a]

pH	V_0 ($\underline{M}sec^{-1} \times 10^7$)	$V_{0.092}$ ($\underline{M}sec^{-1} \times 10^7$)	$V_{0.37}$ ($\underline{M}sec^{-1} \times 10^7$)
4.0	2.73	3.20	3.95
4.5	2.86	4.25	6.10
5.0	3.25	6.15	9.70
5.5	3.69	8.30	14.90
6.0	3.88	10.40	21.30
6.5	3.99	11.80	28.00
7.0	4.03	13.00	34.50
7.5	4.07	13.60	39.00

[a] Values of V_{max} at each pH were extrapolated from the line drawn in Figure 32. Experimental conditions are given in Figure 32.

were then calculated with the help of Equations 19 through 22. These are collected in Table 16.

Values for k_2 can also be calculated at each pH from V_o and k_3 according to the relationship

$$k_2 = \frac{k_3 k_{cat}}{k_{cat} - k_3} \qquad (23)$$

In these calculations, k_3 was assumed to be equal to 7.00 sec^{-1}, the value obtained from the earlier plot of $1/K_m$ vs. k_{cat}/K_m in Figure 20. Values for k_{cat} in this calculation were obtained from V_{max} in the absence of nucleophile according to the relationship $k_{cat} = V_o/(E)_o$. These calculations of k_2 are also collected in Table 16 for comparison.

One interesting result of this study is that k_4 shows only a relatively small pH dependency between pH 5 and 7.5. Over this pH range, the concentration of the fully unprotonated form of glycylglycine, $H_2N\text{-}R\text{-}COO^{(-)}$, varies by about 3 to 4 orders of magnitude. Thus, this form of the nucleophile presumably does not participate significantly in deacylation. In contrast, the concentration of the zwitterionic form of glycylglycine varies only slightly over this pH range, suggesting that it is the active form of the nucleophile in the deacylation reaction.

4. Attempted Direct Demonstration of an Acyl-Enzyme Intermediate

Table 16

Calculated Values of the Rate Constants for the Cathepsin B1 Catalyzed Hydrolysis of CLN[a]

pH	k_{cat}[a] (sec^{-1})		k_3 (sec^{-1})	k_4 ($M^{-1}sec^{-1}$)	k_2 (sec^{-1})	$(k_2)_{calc}$[b] (sec^{-1})
4.0	4.53	4.18	9.31	43.8	8.8	12.8
4.5	4.74	4.92	7.06	74.3	14.3	14.7
5.0	5.39	5.42	7.01	120.5	23.4	23.4
5.5	6.12	5.99	7.18	146.0	41.4	48.7
6.0	6.43	6.42	7.09	170.3	71.2	79.0
6.5	6.61	6.61	6.98	173.2	134.0	118.6
7.0	6.67	6.67	6.88	181.4	252.0	141.5
7.5	6.70		6.84	182.4	497.8	156.3

[a] Values of k_{cat} at each pH were calculated from V_{max} data presented in Table 15. $E_0 = 5.72 \times 10^{-8}$ M. Experimental conditions are given in Figure 32.

[b] The value k_2 was calculated from the equation $(k_2)_{calc} = k_3 k_{cat}/(k_3 - k_{cat})$. The value of k_3 used equalled 7.00 sec^{-1} as determined from the plot of $1/K_m$ vs. k_{cat}/K_m in Figure 20.

a. Reaction of Cathepsin B1 with Trans-Cinnamoylimidazole

Trans-cinnamoylimidazole has been shown to react with papain to produce an acyl-enzyme intermediate (64). This acyl-enzyme intermediate is particularly stable and can be separated on a column of Sephadex G-25 from unreacted trans-cinnamoylimidazole. Once separated, the disappearance of the acyl-enzyme can be measured spectrophotometrically at 330 nm.

Similar experiments were performed with cathepsin B1. Cathepsin B1 was activated with DTT and EDTA and after 30 min passed through an anaerobic column of Sephadex G-25 (0.9 X 18 cm) at 25°C to free the enzyme from excess activator as described in the Methods Section. To cathepsin B1 was added a 50 µl aliquot of a solution containing 0.01 M trans-cinnamoylimidazole in 0.01 M formate buffer, pH 3.45. The reaction mixture was monitored at 296 nm. Under these conditions cathepsin B1 did not react with trans-cinnamoylimidazole. Thus, it appears that trans-cinnamoylimidazole is not a substrate for cathepsin B1.

b. Reaction of Cathepsin B1 with p-Nitrophenyl-p'-Guanidinobenzoate

p-Nitrophenyl-p'-guanidinobenzoate has been shown to be an active site titrant for trypsin (66). We therefore investigated the reaction of p'-nitrophenyl-p'-guanidino-

benzoate with cathepsin B1. To a 1 ml sample of activated cathepsin B1 (3.61×10^{-5} M) in 0.1 M Veronal buffer, pH 8.3, was added 10 µl of a 0.01 M solution of p-nitrophenyl-p'-guanidinobenzoate in N,N'-dimethylformamide and the release of p-nitrophenol measured at 410 nm. The expected burst of p-nitrophenol was not observed and, like trans-cinnamoylimidazole, it appeared that p-nitrophenyl-p'-guanidinobenzoate was not a substrate for cathepsin B1.

E. Specificity of Cathepsin B1

1. The Effect of Dipeptides on the Cathepsin B1 Catalyzed Hydrolysis of CLN

In order to obtain information about the specificity of cathepsin B1, the effects of a variety of dipeptides on the cathepsin B1 catalyzed release of p-nitrophenol from CLN was studied. Values for k_{cat}, K_m/k_{cat}, k_4 and K_I are collected in Table 17. It is apparent that there is a large variation in the ability of these dipeptides to stimulate the cleavage of CLN by cathepsin B1. Thus, pro-gly does not appear to act as a nucleophile, while glu-gly and phe-gly are very poor nucleophiles. Indeed, glu-gly is a strong competetive inhibitor of CLN hydrolysis (K_I = .009 M). In contrast, leu-gly, gly-phe, met-gly and gly-ser are good nucleophiles in the deacylation step, with k_4 for these dipeptides ranging from 2 to 5×10^4 sec^{-1}. The tripeptide, glyglygly, has no effect on the cathepsin B1 catalyzed

Table 17

The Effect of Dipeptides on the

Cathepsin B1 Catalyzed Hydrolysis of CLN[a]

	Dipeptide[b]	k_{cat} (sec^{-1})	K_m/k_{cat} (x 10^7 M sec)	k_4 (x 10^{-2} $M^{-1}sec^{-1}$)	K_I (M^{-1})
1.	None	6.5	2.62	--	--
2.	gly-gly	19.1	2.82	1.48	1.20
3.	ala-gly	69.2	3.66	8.79	.23
4.	leu-gly	201.1	7.19	55.24	.053
5.	ser-gly	61.6	3.66	7.51	.23
6.	met-gly	121.6	11.09	20.21	.028
7.	pro-gly	6.5	3.43	(0)	.30
8.	phe-gly	6.9	42.1	< .1	.015
9.	tyr-gly	88.7	7.56	12.46	.049
10.	glu-gly[c]	6.9	5.66	.36	.009
11.	lys-gly	15.3	7.06	1.02	.054

Table 17 (Cont'd)

The Effect of Dipeptides on the

Cathepsin B1 Catalyzed Hydrolysis of CLN

	Dipeptide	k_{cat} (sec^{-1})	K_m/k_{cat} (x 10^7 M sec)	k_4 (x 10^{-2} $M^{-1}sec^{-1}$)	K_I (M^{-1})
12.	leu-phe[d]	34.47	9.57	159.20	.0008
13.	gly-ala	108.2	3.03	16.78	.59
14.	gly-leu	92.1	4.68	13.16	.12
15.	gly-ser	120.8	3.40	20.00	.31
16.	gly-met	73.4	8.00	9.54	.045
17.	gly-pro	21.8	2.62	1.81	> 10
18.	gly-phe	164.0	7.22	34.71	.052
19.	gly-tyr	86.0	4.13	11.9	.16
20.	gly-asn	28.3	3.61	2.64	.24
21.	gly-glu	15.7	4.71	1.07	.12
22.	gly-lys	18.0	4.63	1.35	.12

Table 17 (Cont'd)

The Effect of Dipeptides on the

Cathepsin B1 Catalyzed Hydrolysis of CLN

	Dipeptide	k_{cat} (sec^{-1})	K_m/k_{cat} (x 10^7 Msec)	k_4 (x 10^{-2} $M^{-1}sec^{-1}$)	K_I (M^{-1})
23.	gly-gly-gly[e]	8.4	7.58	.05x3	.20
24.	D,L-leu-D,L-phe[f]	15.7	4.23	37.94	.005

[a] The buffer was 0.01 M potassium phosphate, pH 6.5, containing 1.1 mM EDTA, 0.9 mM DTT, 0.2 M NaCl and 1.6% (v/v) DMSO at 25°C.

[b] All dipeptides were tested at a conc. of 0.092 M except where indicated.

[c] Present at a conc. of .011 M.

[d] Present at a conc. of 2.00 X 10^{-3} M.

[e] Present at a conc. of 0.37 M.

[f] Present at a conc. of 2.60 X 10^{-3} M.

hydrolysis of CLN while the racemic mixture D,L-leu-D, L-phe had a value of k_4 which was one-fourth that of the L-isomer.

2. Substrate Specificities of Cathepsin B1

The specificity of cathepsin B1 toward a variety of p-nitrophenyl esters was also studied. In Table 18 is presented values of S_o, k_{cat}, K_m and k_{cat}/K_m for these substrates. Of all the p-nitrophenyl esters studied, CLN proved to have the best k_{cat} and K_m. It is also of interest to note that cathepsin B1 hydrolyzed all of the p-nitrophenyl esters studied. Thus, the second-order rate constant, k_{cat}/K_m varied by only a factor of 350 between the best and poorest substrates studied. In general, k_{cat} values for the t-Boc substituted nitrophenyl esters were similar to those of the corresponding CBZ-derivatives while K_m values were a factor of 6 to 8 poorer.

3. The Effect of Bromelain Inhibitor V on Cathepsin B1

The plant sulfhydryl proteinases, papain, ficin and bromelain, as well as the pancreatic proteinase, trypsin, are strongly inhibited by a polypeptide inhibitor, Bromelain Inhibitor V, isolated from pineapple stem. Through the generous gift of Dr. Ferenc Kézdy of the University of Chicago, it became possible to study the effect of Bromelain Inhibitor V on the cathepsin B1 catalyzed hydrolysis of

Table 18

Summary of the Substrate Specificity of Cathepsin B1[a]

Substrate[b]	S_0 ($\times 10^5$ M)	k_{cat}[c] (sec^{-1})	K_m ($M \times 10^{-6}$)	k_{cat}/K_m ($M^{-1}sec^{-1} \times 10^{-6}$)
p-Nitrophenyl Esters				
CBZ-glycine	5.71	1.23	27.95	0.044
CBZ-alanine	4.91	3.13	5.28	0.593
CBZ-valine	5.25	0.14	8.43	0.017
CBZ-leucine	4.35	1.44	8.93	0.161
CBZ-isoleucine	4.26	0.71	2.44	0.291
CBZ-phenylalanine	4.94	0.52	3.26	0.160
CBZ-tyrosine	4.37	0.62	5.96	0.104
CBZ-tryptophan	7.82	0.58	1.76	0.330
CBZ-asparagine	19.18	3.58	80.25	0.045
CBZ-lysine	5.91	6.53	2.39	2.732

Table 18 (Cont'd)

Summary of the Substrate Specificity

of Cathepsin B1

Substrate	S_0 ($\times 10^5$ M)	k_{cat} (sec^{-1})	K_m ($M \times 10^{-6}$)	k_{cat}/K_m ($M^{-1}sec^{-1} \times 10^{-6}$)
t-Boc-glycine	5.98	1.08	198.85	0.005
t-Boc-tryptophan	5.70	5.04	118.5	0.043
t-Boc-glutamine	4.66	5.10	79.91	0.064
t-Boc-asparagine	5.20	1.54	206.7	0.007

[a] The buffer was 0.01 M potassium phosphate, pH 6.5, containing 1.1 mM EDTA, 0.9 mM DTT, 0.2 M NaCl and 1.6% (v/v) DMSO at 25°C.

[b] Stock solutions contained acetonitrile except CBZ-lysine which contained DMSO.

[c] $E_0 = 1.43 \times 10^{-6}$ M.

CLN and CGN. Under conditions which were effective in inhibiting the bromelain catalysis of CLN (93), the cathepsin B1 catalyzed hydrolysis of CLN and CGN were unaffected by Bromelain Inhibitor V at all pH's studied. Thus, it appears that cathepsin B1 differs from the plant sulfhydryl proteinases in its susceptibility to inhibition by Bromelain Inhibitor V.

CHAPTER IV

DISCUSSION

There are several important reasons for undertaking an investigation of the catalytic properties of cathepsin B1. First, cathepsin B1 is a major endopeptidase present in the lysosomes of mammalian cells (47). As such, it is been postulated to play an important role in protein homeostasis (23-25). In addition evidence has accumulated which suggests that cathepsin B1 may be involved in a variety of pathological conditions from acute and chronic inflammatory disorders to muscular dystrophy (26-38). Information obtained from studies of the mechanism and specificity of cathepsin B1 could, therefore, lead to the development of reagents which may be useful in studies of protein degradation in animal tissues and which may also possess potent pharmacological properties. Second, there is currently a strong general interest in the mechanism of sulfhydryl proteinases. The plant sulfhydryl proteinase, papain, has been the major focus of this research interest. Despite, the considerable joint efforts of many investigators, important details of its mechanism remain unclear. One problem which is frequently encountered in studying the catalytic properties of an enzyme is that it is frequently impossible

to distinguish between those characteristics which are related directly to the mechanism of the enzyme and those which are due to some other unique structural feature of the protein. By studying the catalytic properties of cathepsin B1, a protein only distantly related to papain, it was hoped that we could distinguish those catalytic properties which are shared by all sulfhydryl proteinases and which must, therefore, be intrinsic to their mechanism of catalysis. Before undertaking such a detailed comparison, it was first necessary to establish that cathepsin B1 and papain were functionally homologous. Therefore, the major goal of this research was to demonstrate that the pathway for hydrolysis of substrates by cathepsin B1 is essentially similar to that of papain.

A. Development of New Assays for Cathepsin B1

Before purifying and studying cathepsin B1, two esters were investigated to determine whether they could be used to monitor cathepsin B1 activity. These esters, CLN and CGN, were previously shown to be good substrates for papain (58, 67). The need to develop a new assay for cathepsin B1 became evident after a preliminary literature review. Most assays for cathepsin B1 are not specific (2), many require prolonged incubation times at elevated temperatures, and several are unable to measure cathepsin B1 at low enzyme concentrations. Based on the results with papain, both CLN

and CGN were expected to provide an easy, sensitive and specific assay of cathepsin B1 activity.

Cathepsin B1 was partially purified according to known procedures (9). The various fractions were assayed for their ability to hydrolyze CLN and CGN as well as for their ability to hydrolyze other known substrates of cathepsin B1: BAA, BAEE, BAPA and BANA. BAA is hydrolyzed by at least two distinct enzymes, cathepsin B1 and B2. BAEE was shown in this work to be hydrolyzed by at least three species separable by chromatography on Sephadex G-150. Although the distributions of BAPA and BANA activity coincided with that of CLN and CGN activity, BANA and BAPA are not hydrolyzed rapidly by cathepsin B1.

Results after the DEAE-Sephadex A-25 column showed that CLN and CGN activity corresponded to activity measurements with BANA and BAPA. However, when cathepsin B1 was purified to homogeneity with SP-Sephadex C-25, BANA activity was substantially diminished. These results support the conclusions of other investigators that the hydrolysis of BANA is catalyzed by an enzyme distinct from cathepsin B1 (2,19,20). Although such conclusions were based on experiments with rat tissue, the results obtained with bovine spleen homogenates widen the scope of such observations. It was also observed by Barrett (9) in the purification of cathepsin B1 from human liver, that BANA hydrolase activity was lost when enzyme samples were concentrated with an

Amicon Concentrator fitted with a PM-10 membrane. Similar studies in this laboratory confirmed these observations. BANA activity passed through the PM-10 membrane while BAPA and CLN activities remained in the concentrate. Thus, it appears that BANA is hydrolyzed at pH 5.1 by an enzyme other than cathepsin B1 which is present in bovine spleen homogenates.

The CLN and CGN assays developed for cathepsin B1 have proved to be very sensitive, permitting rapid measurements at very low enzyme levels. Because of the apparent high reactivity of cathepsin B1 with CLN, it is now possible to detect as little as 1 picomole of this enzyme. In contrast, the BAPA assay is about 3 orders of magnitude less sensitive.

B. Purification of Cathepsin B1

Cathepsin B1 was purified by a modification of other known procedures. These modifications include:

1. reducing the time and temperature of the autolysis step in order to reduce the possibility of artifically generating a heterogenious mixture of cathepsin B1 through partial proteolytic cleavage of the enzyme;

2. substituting SP-Sephadex at pH 4.1 for DEAE Sephadex at pH 6.8 since the former resulted in partial resolution of the isoenzymes; and

3. using an Organomercurial Sepharose column after

partial reaction with DTNB to separate isoenzymes with differing reactivities toward DTNB.

As a result of these modifications it is now possible to purify 3 isoenzymes of cathepsin B1 to homogeneity, with cathepsin B1 (II) being the predominant form. There are several possible explanations which may account for the observed microheterogeneity of cathepsin B1. These include:

1. The autolysis step generates several forms of cathepsin B1 by limited cleavage of the molecule;

2. The spleen as a heterogeneous tissue, composed of several distinct cell types, is responsible for the heterogeneity of the enzyme;

3. The carbohydrate content of cathepsin B1 is variable and contributes to its heterogeneity ; and

4. The heterogeneity of the enzyme reflects the expressions of multiple genes for cathepsin B1 in a single cell type.

Only the third possibility can be eliminated by our studies. The enzyme does not appear to contain any carbohydrate as determined with Alcian Blue or the periodic-Schiff reagent. These results were confirmed by other workers, who analyzed the protein for hexoses and hexosamines (91).

The results in Table 5 showed that when CLN was used to measure cathepsin B1 (II) activity, the final specific

activity of the enzyme was 11.97 nmoles/min/mg with a 99-fold purification. When BAPA was the substrate, the final specific activity of the enzyme was 45.2 μmoles/min/mg with a 85-fold purification. The yields were both 2%. The similarity between the recoveries of CLN activity and BAPA activity provides further evidence that both substrates monitor the same enzyme in tissue homogenates.

In order to resolve the isoenzymes of cathepsin B1 on SP-Sephadex C-25, a very shallow NaCl gradient was used to elute the enzyme from the resin. Although such a procedure resulted in well-resolved isoenzyme fractions, the length of time required for the NaCl gradient caused appreciable losses of cathepsin B1 activity. Thus, final yield was sacrificed for isoenzyme purity. Where distinctions between isoenzymes is not important, chromatography of cathepsin B1 on SP-Sephadex C-25 under conditions described in Figure 7 is preferred. Such a procedure produced only a 20% loss of cathepsin B1 activity.

Cathepsin B1 is extremely stable for a sulfhydryl enzyme. Although the enzyme is slowly inactivated in air, this inactivation can be reversed with the addition of DTT. Samples of cathepsin B1 that had been stored for 6 months at 4°C in 0.025 M sodium acetate buffer, pH 5.1, containing 1 mM EDTA, retained 90% of their activities. Occasionally, some sulfhydryl enzymes can undergo irreversible inactivation when allowed to undergo aerobic oxidation in the

presence of traces of small molecular weight thiols(90). Studies with cathepsin B1 and mercaptoethanol suggested that the enzyme is not irreversibly inactivated.

Two important physical characteristics of cathepsin B1 were determined in this work. First, cathepsin B1 from bovine spleen is shown by gel filtration to have an apparent molecular weight of 26,000. Based on active site titrations and absorbance measurements, its molar absorbtivity at 280 nm was found to be $4.03 \pm .13 \times 10^4$ $\underline{M}^{-1}cm^{-1}$. Using this value for the molar absorption coefficient, the concentration of solutions of pure cathepsin B1 can be determined with reasonable accuracy.

The concentration of cathepsin B1 can also be determined from active site titration data obtained with sulfhydryl modifying reagents such as DTNB and PCMB. These procedures have several drawbacks. DTNB titration requires relatively large amounts of enzyme and cannot be used to titrate cathepsin B1 (II) because of its poor reactivity toward this reagent (see below). Titrations cannot be performed on impure enzyme samples because the presence of sulfhydryl containing protein contaminants will result in erroneous values for the enzyme concentration. Titration with DTNB and PCMB are tedious to perform. However, once the molarity of a pure sample of cathepsin B1 has been established by titration with DTNB or PCMB, it is possible to calculate a turnover number or k_{cat} value for the enzyme,

according to the relationship, $k_{cat} = V_{max}/(E)$. In this equation V_{max} is the maximal velocity obtained with a particular substrate such as CLN and (E) is the enzyme concentration determined by titration. Once k_{cat} has been established under well-defined conditions it can be used to calculate the concentration of cathepsin B1 from V_{max} obtained with impure enzyme samples such as tissue homogenates as follows:

$$(E)_{unknown} = \frac{V_{max}}{k_{cat}}$$

k_{cat} for CLN hydrolysis in 0.01 M potassium phosphate buffer, pH 6.5, containing 1.1 mM EDTA, 2.2 mM DTT, 0.2 M NaCl and 1.6% (v/v) DMSO at 25°C is 6.54 sec^{-1}. The corresponding value for CGN is 1.23 sec^{-1}.

C. Titration of Cathepsin B1 with Sulfhydryl Modifying Reagents

The titration of cathepsin B1 (II) preparations with DTNB produced unexpected results. When this reaction was monitored by absorbance measurements at 412 nm it was found to be biphasic. One component, representing about 10% of the total enzyme, reacted rapidly with DTNB and was complete in 150 minutes. The second, slow reacting component required more than 24 hr to completely react with DTNB. These results suggested that cathepsin B1 (II) fractions from the SP-Sephadex column is composed of two species,

present in a molar ratio of 9:1. It is possible that the minor component represents cathepsin B1 (III).

In contrast to the spectrophotometric results, activity measurements indicated that greater than 80% of cathepsin B1 (II) reacted with DTNB within the first few minutes of the reaction. As discussed in the Results Section, these disparate observations are explained by the fact that the major cathepsin B1 (II) species can react with DTNB to form an unusually stable, non-covalent enzyme·DTNB complex. This complex then reacts slowly to form the modified TNB·enzyme and free TNB. This scheme is illustrated below.

$$E + DTNB \underset{\text{slow}}{\overset{\text{fast}}{\rightleftharpoons}} E\cdot DTNB \xrightarrow{\text{slow}} E\text{-}TNB + TNB$$

When excess DTNB is removed from the reaction mixture by gel filtration, the E·DTNB complex can slowly dissociate, even in the absence of simple thiols, to regenerate the active enzyme.

It proved possible to take advantage of the differential reactivities of the two species of cathepsin B1 (II) to isolate the major form of this enzyme. Samples of cathepsin B1 (II) were reacted with DTNB for 150 minutes. Excess DTNB was then removed by gel filtration and the resulting reaction mixture chromatographed on an Organomercurial Sepharose 4B column. The non-binding protein represented a mixture of the non-covalent DTNB·enzyme com-

plex and the covalent TNB-enzyme adduct. The protein which was bound to the column represented the free form of the major component of cathepsin B1 (II). This was then eluted from the mercurial column with L-cysteine as described in the Experimental Section.

The anomalous behavior of cathepsin B1 (II) toward DTNB prevented the use of this reagent to titrate the enzyme. Cathepsin B1 (II) was, therefore, titrated with PCMB. It was determined from the data with PCMB that the molar ratio of SH groups to protein is 1, indicating that cathepsin B1 (II) is pure.

D. Mechanism of Cathepsin B1

1. Demonstration of an Acyl-Enzyme Intermediate

A major thrust of this present work on the mechanism of cathepsin B1 was aimed at demonstrating that the reaction of this enzyme with substrates proceeds through the formation of an acyl-enzyme intermediate as illustrated by Equation 1.

$$E + S \underset{}{\overset{K_s}{\rightleftharpoons}} ES \xrightarrow{k_2} \begin{matrix} E' \\ + \\ P_1 \end{matrix} \xrightarrow{k_3'(H_2O)} E + P_2 \qquad (1)$$

This may be contrasted with the alternative mechanism involving a ternary complex between enzyme, substrate, and nucleophile (H_2O) which breaks down in a single step to yield products. The occurance of an acyl-enzyme intermedi-

ate in catalysis by cathepsin B1 leads to a number of predictions which may be readily tested.

For a reaction occurring according to Equation 1, it can be shown that the rate constant, k_{cat}, is given by (58)

$$k_{cat} = \frac{k_{acyl} \cdot k_{deacyl}}{k_{acyl} + k_{deacyl}} = \frac{k_2 k_3'(H_2O)}{k_2 + k_3'(H_2O)}$$

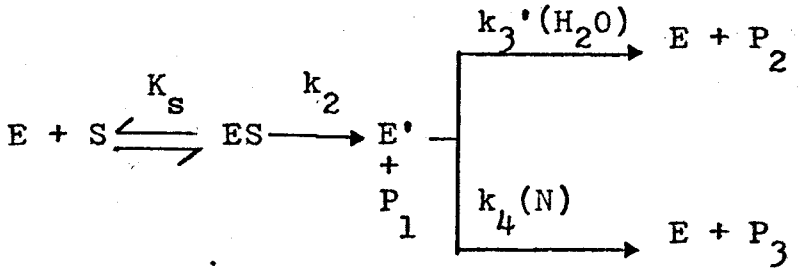
where k_2 and k_3 are the rate constants for acylation and deacylation, respectively.

This equation predicts that when the rate determining step in the overall reaction is deacylation (ie. $k_2 >> k_3$), k_{cat} approximately equals k_3. Thus, a series of different substrates which give rise to the same acyl-enzyme intermediate should have identical values for k_{cat} provided the deacylation step is rate determining. In contrast, mechanisms involving a ternary complex which breaks down into products in a single step predict different values for k_{cat} which depend upon the intrinsic reactivities of the different substrates.

In general, p-nitrophenyl esters such as CLN are between one and two orders of magnitude more reactive toward nucleophiles than their corresponding methyl esters, such as CLME (58). Furthermore, the reaction of these substrates with papain is known to be deacylation rate limiting (58). The observation that both CLN and CLME are hydrolyzed by cathepsin B1 with essentially identical values for k_{cat}

provides evidence for an acyl-enzyme intermediate in this reaction.

If nucleophiles other than water can participate in the deacylation step, then, in the presence of such a nucleophile, the acyl-enzyme intermediate will be partitioned between two products as shown in Equation 2.



(2)

The deacylation rate constant is now given by Equation 7

$$k_{deacyl} = k_3'(H_2O) + k_4(N) \qquad (7)$$

and therefore

$$k_{cat} = \frac{k_2(k_3'(H_2O) + k_4(N))}{k_2 + (k_3'(H_2O) + k_4(N))} \qquad (4)$$

Under conditions where deacylation is the rate determining step in substrate hydrolysis ($k_2 >> k_3$), k_{cat} will essentially be equal to $(k_3 + k_4(N))$. Thus, initial rates and maximal volocities for substrate hydrolysis will be dependent on the concentration of the second nucleophile (N). With substrates for which acylation is rate determining ($k_3 >> k_2$), k_{cat} will essentially equal k_2 and reaction velocities with these substrates will be independent of nucleophile concentration. In contrast, mechanisms involving a ternary complex between

enzyme, substrate, and nucleophile will give rise to rate equations which always contain terms for the nucleophile concentration. Rates of such reactions should always depend on the concentration of nucleophiles present and are independent of the nature of the substrate. The hydrolysis of BAPA by papain has been shown to be acylation rate limiting. Therefore, the observation that the reactions of cathepsin B1 with CLN, CLME, and CGN, but not with BAPA, are stimulated by nucleophiles, provides strong evidence for an acyl-enzyme intermediate on the reaction pathway.

A third consequence of an acyl-enzyme intermediate is that in the presence of a nucleophile, N, the ratio of products P_2/P_3, and the rate constants, k_3/k_4, should solely depend on the nature of the acyl-enzyme and the nucleophile (68). Thus, two different substrates giving rise to the same acyl-enzyme should show identical values for the ratio k_3/k_4. The observation that the ratios k_3/k_4 for the reaction of CLN and CLME with cathepsin B1 are identical is, thus, consistent with the formation of a common CBZ-lysyl-enzyme intermediate.

Finally, steady state rate equations (Equations 2-7) for an acyl-enzyme mechanism predict that Lineweaver and Burk plots of reaction velocity vs. substrate concentrations, obtained in the presence of varying nucleophile concentrations, should yield a series of parallel lines since the

slope of such plots, K_m/V_{max}, contain no terms for the nucleophile. In contrast, reaction mechanisms involving a ternary complex between enzyme, substrate and nucleophile almost always produce a series of intersecting lines in such a graphical analysis. The results obtained in Figure 23 for reaction rates with CLN in the presence of various concentrations of gly-gly provide very strong evidence for an acyl-enzyme intermediate.

2. Calculation of Individual Rate Constants

The ability of nucleophiles other than water to participate in the deacylation step of the cathepsin B1 catalyzed hydrolysis of substrates provides a means of measuring the rate constants for the individual steps in these reactions. Using Equations 9 and 12 in the Appendix, plots of $\left[\frac{V_o}{V_N - V_o}\right]$ vs. $\frac{1}{(N)}$ and $\frac{1}{(E)_o}\left[\frac{V_N V_o}{V_N - V_o}\right]$ vs. $\frac{1}{(N)}$, were constructed from data obtained with the nucleophiles gly-gly, leu-phe, gly-phe, and leu-gly.

In general, data with gly-gly, gly-phe and leu-phe conforms well to Equations 9 and 12. Data points for leu-gly, however, show a small systematic departure from a straight line at higher concentrations of this dipeptide. In Table 12 are collected the calculated values for k_4, k_3, and k_2 obtained with these four nucleophiles. Values for k_4 differ for the different dipeptides studied. These values

range from 140 ± 10 $\underline{M}^{-1}$ sec^{-1} for gly-gly, the poorest nucleophile in this series, to 5,610 ± 230 $\underline{M}^{-1}$ sec^{-1} for leu-gly. As expected, values for k_3 are independent of the nucleophile and are in excellent agreement. The range of values obtained for k_3 is 6.5 ± 1.8 sec^{-1} with leu-phe to 6.64 ± .07 sec^{-1} with gly-gly.

Accurate values for k_2 were more difficult to obtain. The departure from linearity of plots of $(V_o)/(V_N - V_o)$ vs. 1/N with leu-gly at high dipeptide concentration prevented an accurate estimate of the intercept, k_3/k_2. Leu-phe was not sufficiently soluble to permit studies at high nucleophile concentrations and the intercept, k_3/k_2, therefore, could not be obtained. For gly-gly and gly-phe values for k_3/k_2 and k_2 are in reasonable agreement given the larger experimental error inherent in their determinations. The values for k_2 with gly-gly and gly-phe are estimated to be 386 ± 151 sec^{-1} and 393 ± 85 sec^{-1} respectively.

3. pH Dependency of the Kinetic Constants

The pH dependency of k_{cat}, K_m, and k_{cat}/K_m were determined for the hydrolysis of CLN by cathepsin B1. These values are summarized in Table 10. Since k_{cat}/K_m is equal to k_2/K_s, the second order rate constant for the formation of the acyl-enzyme intermediate from free enzyme and substrate, the pH dependency of this constant provides information about ionizable groups on the free enzyme controlling

its reaction with substrate.

A plot of k_{cat}/K_m vs. pH yields a bell-shaped curve. The pK_a's of the groups controlling the activity of the free enzyme are 4.54 and 7.95. The value of $(k_{cat}/K_m)_{lim}$ is $3.88 \pm .70 \times 10^6$ $\underline{M}^{-1}$ sec^{-1}. The value of 7.95 for the basic group controlling the alkaline limb of the curve must be considered uncertain because it is largely determined by measurements of k_{cat}/K_m at only one pH, 7.5. Measurements of k_{cat}/K_m at higher pH's were not possible owing to the rapid spontaneous hydrolysis of substrate above pH 7.5. Nevertheless, the pH dependency of k_{cat}/K_m obtained for cathepsin B1 is remarkably similar to results with papain (56,57). In papain these pK values have been attributed to the ionization of the active site histidine and cysteine residues (56,57). It, therefore, appears that the composition of the active site of cathepsin B1 may be similar to that of papain. One important difference between these two enzymes, however, is the observation that k_{cat} for cathepsin B1 is relatively pH independent between pH 3 and 7.5. In this regard, cathepsin B1 appears to be similar to the plant sulfhydryl proteinase, bromelain (94).

A procedure has been developed which permits the calculations of the individual rate constants in the reaction of sulfhydryl proteinases with substrates from the pH dependency of K_m and k_{cat}/K_m (57). This procedure depends upon the fact that k_3 and K_s for these enzymes are usually pH

independent above pH 5.0 while k_2 is strongly pH dependent. Under these conditions, it is possible to show that the following relationship exists for K_m and k_{cat}/K_m (57);

$\frac{1}{K_m} = \frac{1}{K_s} + \frac{1}{k_3}\frac{k_{cat}}{K_m}$. Thus, plots of $1/K_m$ vs. k_{cat}/K_m for data obtained with papain above pH 5.0 have been shown to fall on a straight line with a slope equal to $1/k_3$ and an intercept on the ordinate equal to $1/K_s$. This work has shown that for the cathepsin B1 catalyzed hydrolysis of CLN, k_2 is very much greater than k_3. Thus, k_{cat} for this reaction is essentially equal to k_3 (Appendix A). Therefore, the observation that k_{cat} is pH independent between pH 3 and 7.5 suggests that k_3 may be similarly pH independent. On the assumption that K_s may be also pH independent in this pH range, we plotted the data in Table 10 according to Equation 15.

From Figure 20, it can be seen that these data points did approximate a straight line. The value of k_3 calculated from the slope was $7.0 \pm .5$ sec^{-1} in reasonable agreement with k_3 values obtained independently from studies with dipeptide nucleophiles. K_s was found to be $2.0 \pm 1.08 \times 10^{-5}$ M. The value of k_2 was then calculated from the relationship $k_2 = k_{cat}/K_m \times K_s$. The value of k_2 obtained was 79 ± 42 sec^{-1}. Given the very large experimental uncertainties in these techniques, the value is probably not very

different from the values of 386 ± 151 sec^{-1} and 393 ± 85 obtained independently with the dipeptides gly-gly and gly-phe.

4. The pH Dependency of the Individual Steps in the Reaction of CLN with Cathepsin B1

Values for the rate constants of the individual steps in the cathepsin B1 catalyzed hydrolysis of CLN between pH 4 and 7.5 were calculated according to Equations 19 through 22 as described in the Results Section. The values obtained for k_2, k_3 and k_4 at pH 4.0, pH 7.0 and pH 7.5 are considered the least reliable. From Table 15 and Figure 32 it can be seen that 0.092 and 0.37 M glycylglycine caused only a relatively small increase in V_{max} at pH 4.0. Accurate estimation of k_2, k_3 and k_4 require large differences in V_{max} in the presence and absence of glycylglycine. At pH's above 6.5, the rates of spontaneous hydrolysis of substrate in the presence of nucleophiles (rates obtained with enzyme omitted) were very rapid. For example, at pH 7.5 and in the presence of 0.37 M glycylglycine, the rate of spontaneous hydrolysis of CLN was on the order of 30% of the rate of CLN hydrolysis obtained with cathepsin B1 present. It was not possible to increase the enzyme's contribution to the rate of substrate hydrolysis by increasing the enzyme concentration since reaction rates were already so large as to be difficult to measure. These difficulties introduced significant error in the calculated values of V_N

at pH 7.0 and 7.5. The values of k_2 at pH 4.0, pH 7.0 and pH 7.5 are most affected by these experimental difficulties.

Values for k_2 were also calculated from the relationship, $k_2 = k_3 k_{cat} / (k_3 - k_{cat})$. These are also listed in Table 16 for a comparison. Accurate calculation of k_2 with this equation requires a reliable estimate of k_3. The average value of k_3 obtained from the nucleophile pH data in Table 16 between pH 4.5 and 7.5 is $7.00 \pm .11$ sec^{-1}, in good agreement with the value of $7.00 \pm .50$ sec^{-1} obtained from a plot of $1/K_m$ vs. k_{cat}/K_m (Figure 20). Nevertheless, even small errors in k_3 can produce larger systematic errors in the calculated values of k_2. This error increases with increasing pH, as the term $(k_3 - k_{cat})$ becomes progressively smaller. Thus, an error in k_3 of less than 2% (0.11 sec^{-1}) can produce a 10% error in k_2 at pH 5.5 and a 50% error in k_2 at pH 7.5. Nevertheless, the good agreement between k_2 values calculated by two independent methods, from data obtained between pH 4.5 and 6.5, provide some degree of confidence in their validity.

An estimate of the error in k_4 is more difficult to determine. The value of k_4 at pH 6.5 for gly-gly in this study is 173 and is in reasonable agreement with the value of 140 ± 10 obtained previously (Figure 23 and Table 12).

In studying the pH dependency of these kinetic constants we are far more interested in their trend than in their absolute values. Sample calculations using Equation

22 indicate that k_3 is remarkably insensitive to random errors in V_o and V_N. Furthermore, the trend in k_3 is not affected by a systematic error in either the enzyme or nucleophile concentration. Thus k_3 appears to be truely pH independent between pH 4 and 7.0. This is in sharp contrast to papain where k_3 progressively decreases below pH 5.5 (58). Similarly, sample calculations using Equation 21 suggest that k_4 is not substantially affected by moderate variations in V_o and V_N and the trend in the data is unaffected by a systematic error in either the enzyme concentration, the nucleophile concentration or the value chosen for k_3. Thus, it is possible to conclude that k_4 is essentially pH independent between 5.0 and 7.5. This requires that the active form of glycylglycine in the deacylation reaction is the zwitterion. This conclusion posses a serious problem. The reaction of a protonated amine with a thiol ester to yield a peptide requires removal of two protons from the amino group during the reaction. This cannot be easily explained by any of the proposed mechanisms for papain.

Interpretation of the k_2 data is difficult. Sample calculations using Equation 19 indicate that k_2 is very sensitive to variations in V_N particularly at the higher values of V_N above pH 6.0. Furthermore, as described above, a systematic error in k_3 can affect the trend in k_2 calculated

from k_{cat} data in Table 16. Despite these difficulties, some conclusions can be made. The overall rate of a chemical reaction can never be greater than the rate of its individual steps. Thus, k_2 must always be greater than or equal to k_{cat}. From Table 10 it can be seen that k_{cat} does not decrease significantly below pH 4.0. Thus, the value for k_2 at pH 3.0 must be similar to its value at pH 4.0. Therefore, it appears that the low pH form of the cathepsin B1 substrate complex, ES, can react to form products. This is in sharp contrast to results obtained with papain which indicate that its low pH is inactive.

The data obtained for the pH dependency of k_{cat}, k_2, and k_3 for cathepsin B1 strongly support the conclusion that the low pH form of this enzyme is active. These observations cannot be easily explained by any of the current mechanisms proposed for papain and other sulfhydryl proteinases. These observations justify our proposal that sulfhydryl proteinases from a variety of sources must be studied in order to obtain a complete picture of the catalytic properties of these enzymes and before any conclusions can be made as to their catalytic mechanism.

E. Specificity

The ability of cathepsin B1 to hydrolyze a variety of p-nitrophenyl esters of N-benzyloxycarbonyl and N-Butoxycarbonyl substituted amino acids was studied. The enzyme displayed only a small specificity toward the nature of the

amino acid residue in the substrate. In particular, in the series of substrates studied, the largest difference in k_{cat} was observed between CLN and CBZ-L-valine-p-nitrophenyl ester, CVN. The k_{cat} for CLN was 40 times better than for CVN. Substitution of the t-Boc substituent for the CBZ-group had little effect on k_{cat} but increased K_m by about 6 to 8 fold.

The effect of dipeptides on the cathepsin B1 catalyzed hydrolysis of CLN is shown in Table 17. Since Equations 3 and 6 predict that K_m/k_{cat} will be constant and independent of nucleophile concentration, then, one should observe with a series of dipeptides similar values of K_m/k_{cat}. Whereas k_{cat} varies over a 30-fold range with the various dipeptide nucleophiles listed, K_m/k_{cat} are much more nearly similar. The only major exception is phe-gly. This dipeptide, however, does not act as an acyl-acceptor but is instead a competitive inhibitor of CLN hydrolysis. The small increase seen in K_m/k_{cat} with some of the other dipeptides may, thus, also be attributed to their ability to act as weak competetive inhibitors as well as acyl-acceptors in the reaction of cathepsin B1 with CLN. Values of K_I which are calculated for each dipeptide reflects this inhibition. Both glu-gly and leu-phe are strong competitive inhibitors. with K_I's of 9×10^{-3} $\underline{M}^{-1}$ and 8×10^{-4} $\underline{M}^{-1}$, respectively.

Inspection of Table 17 reveals that not all dipeptides are equally effective as nucleophiles in the cathepsin B1

catalyzed cleavage of CLN. From the limited number of dipeptides studied it is already possible to discern certain specificity patterns. Thus, in the dipeptide series X-gly k_{cat} is observed to increase when X is an aliphatic amino acid. Introduction of a polar group into X reduces or abolishes the ability of the dipeptide to act as an acyl-acceptor. The dipeptide, tyr-gly, appears to be an exception. Thus, lys-gly and glu-gly stimulate k_{cat} slightly or not at all, and ser-gly is less effective than ala-gly. Finally, X cannot be an aromatic amino acid as phe-gly is a competitive inhibitor of CLN hydrolysis. Similarly, if we consider the dipeptide series gly-Y we find that substituting amino acids with large non-polar side chains for Y produces a large stimulation in k_{cat}. On the basis of these studies we may predict that dipeptides containing leucine, isoleucine or valine in position X and phenylalanine, tryosine or tryptophan in position Y should be very efficient acyl-acceptors. Indeed, the stimulation of the cathepsin B1 catalyzed hydrolysis by leu-phe was greater than that observed for either leu-gly or gly-phe.

From the equation below, it is apparent that the transfer of the CBZ-L-lysyl group from cathepsin B1 to the dipeptide H_2N-XY-COOH is the microscopic reverse of the cleavage of the tripeptide CBZ-L-lysyl-XY-COOH by cathepsin B1

$$\text{CBZ-lysyl-cathepsin B1} + H_2NXYCOOH \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}}$$

$$\text{CBZ-lysyl-XYCOOH} + \text{cathepsin B1}$$

Thus, in peptide bond hydrolysis cathepsin B1 displays specificity for the amino acid residue donating the carboxyl group to the peptide bond, and for at least the first two amino acid residues on the amide nitrogen side of that bond (residues X and Y). By extending these studies it may eventually be possible to predict the specificity of cathepsin B1 toward polypeptide substrates.

CHAPTER V

SUMMARY

Cathepsin B1, a lysosomal sulfhydryl protease, was purified from homogenates of bovine spleen. The enzyme was shown to exist as a mixture of at least 3 isoenzymes. One isoenzyme, separable by ion-exchange chromatography on SP-Sephadex C-25 and polyacrylamide gel electrophoresis, was named cathepsin B1 (II) and represented about 70% of the material recoverable from bovine spleen homogenates.

All three isoenzymes of cathepsin B1 were shown to catalyze the hydrolysis of p-nitrophenyl benzyloxycarbonylglycinate, CGN, and p-nitrophenyl alpha-N-benzyloxycarbonyl-L-lysinate, CLN. Under the assay conditions, cathepsin B1 is the major enzyme present in bovine spleen homogenates hydrolyzing these substrates. The kinetic parameters for the hydrolysis of CGN and CLN were measured and compared with those obtained for other cathepsin B1 substrates. These results form the basis of an improved spectrophotometric assay for cathepsin B1.

Cathepsin B1 (II) was found to have a molecular weight of 26,000 and a molar absorption coefficient of $4.03 \pm .13 \times 10^{4}$ $1\underline{M}^{-1}$ cm^{-1}. Purified cathepsin B1 preparations gave negative results when tested for carbohydrate

and lipid.

The number of exposed sulfhydryl groups in the two major isoenzymes of cathepsin B1 (II) and (III) were determined by titration with DTNB and p-chloromercuribenzoate. Cathepsin B1 (III) reacted as expected yielding 1 mole of SH group per mole of enzyme. In contrast, cathepsin B1 (II) reacted with DTNB to form a stable non-covalent intermediate which only slowly released 5-nitrothiobenzoic acid. When this complex was separated from unreacted DTNB, by gel filtration, it underwent a slow spontaneous reactivation with concomitant release of DTNB. Cathepsin B1 (II) reacted rapidly with p-chloromercuribenzoate giving 1 mole of sulfhydryl group per mole of enzyme.

A number of observations were made which indicate that the reaction of cathepsin B1 with ester and amide substrates occurs through the formation of a covalent acyl-enzyme intermediate. First, under conditions where deacylation was rate-limiting, substrates derived from the same amino acids but differing in their leaving groups were hydrolyzed with identical rates. Second, nucleophiles were able to compete with water and enhanced the rate of cleavage of CLN. In the presence of dipeptide nucleophiles, the corresponding N-substituted tripeptides were formed. Third, in the presence of the dipeptide, L-leucylglycine, the ratio of the rates of formation of the product and the acyl-nucleophile adduct were identical for both the methyl ester and p-nitrophenyl

ester of N-benzyloxycarbonyl-L-lysine. Fourth, in the presence of increasing concentrations of glycylglycine, a Lineweaver and Burk analysis of steady state rate data for CLN hydrolysis yielded a series of parallel lines as expected for a mechanism involving an acyl-enzyme (ping pong bi bi). Fifth, under conditions where the acylation step was rate limiting, nucleophiles did not enhance the rate of cleavage of substrates by cathepsin B1. Finally, an analysis of rate data for CLN hydrolysis in the presence of nucleophiles permitted calculations of the rate constants for the individual steps in this reaction. The values for these constants were in good agreement with corresponding values obtained independently from studies of the pH dependency of CLN hydrolysis, supporting the formation of an acyl-enzyme intermediate.

The pH dependencies of the individual steps in the hydrolysis of CLN by cathepsin B1 were determined from kinetic data obtained between pH 3.0 and pH 7.5 in both the presence and absence of glycylglycine. The rate of hydrolysis of the acyl-enzyme, k_3, was found to be pH independent in this region. The rate of reaction of the acyl-enzyme with glycylglycine, k_4, was found to be independent of pH between 5.0 and 7.5, suggesting that the zwitterion form of this dipeptide was the active nucleophile. In contrast, the rate of acylation, k_2, appeared to increase between pH 4.5

and 7.0, approaching finite limits below pH 4.0 and above pH 7.5. Lastly, the pH dependency of the second-order rate constant, k_{cat}/K_m, appeared bell-shaped. The pK_a's of the ionizable groups controlling k_{cat}/K_m were 4.54 and 7.95.

The ability of cathepsin B1 to hydrolyze a variety of p-nitrophenyl esters of N-benzyloxycarbonyl and N-Butoxycarbonyl substituted amino acids was studied. The enzyme displayed only a small specificity toward the nature of the amino acid residue in the substrate. In particular, in the series of substrates studied, the largest difference in k_{cat} was observed between CLN and CBZ-L-valine-p-nitrophenyl ester, CVN The k_{cat} for CLN was 40 times better than for CVN. Substitution of the t-Boc substituent for the CBZ-group had little effect on k_{cat} but increased K_m by about 6 to 8 fold.

The ability of cathepsin B1 to transfer the acyl-portion of CLN to a variety of nucleophiles was also investigated. Dipeptides were found to be more effective than amines, amino acids, amino acid esters and tripeptides. Strong differences were also found in the abilities of various dipeptides to act as acyl-acceptors. Since transfer of the CBZ-L-lysyl residue to a dipeptide nucleophile is the microscopic reverse of the cleavage of a CBZ-L-lylsyl -containing tripeptide, it is inferred that cathepsin B1 displays a strong specificity toward the nature of the leaving group in peptide bond hydrolysis.

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APPENDIX A

APPENDIX A

1. Steady State Rate Equations in the Absence of an Added Nucleophile

The reaction of papain and other plant sulfhydryl proteases with ester and peptide substrates have been shown to proceed through the formation of an acyl-enzyme intermediate, as illustrated by Scheme 1.

$$E + S \underset{}{\overset{K_s}{\rightleftharpoons}} ES \xrightarrow{k_2} \underset{+\ P_1}{E'} \xrightarrow{k_3} E + P_2 \qquad (1)$$

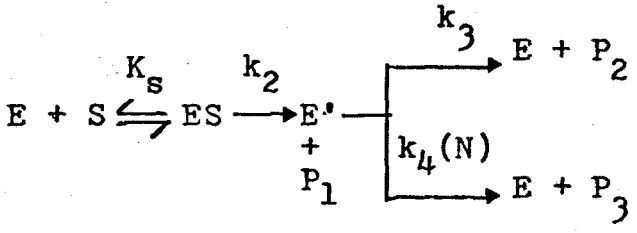
In this equation, K_s is the dissociation constant for the enzyme substrate complex, ES; k_2 is the first order rate constant for the conversion of the ES complex to the acyl-enzyme, E', k_3 is the pseudo-first order rate constant for the hydrolysis of the acyl-enzyme, E', and is equal to $k_3'(H_2O)$; P_1 is an alcohol in ester hydrolysis or an amine in amide hydrolysis; and P_2 is a carboxylic acid. The steady state rate equation is given by (57,58).

$$v = \frac{d(P_1)}{dt} = \frac{d(P_2)}{dt} = \frac{\dfrac{k_2 k_3 (E)_o}{k_2 + k_3}}{\left[\dfrac{k_3}{k_2 + k_3}\right] \dfrac{K_s}{(S)} + 1} \qquad (2)$$

Thus, $K_m = \left[\frac{k_3}{k_2 + k_3}\right] K_s$, and $k_{cat} = \frac{V_{max}}{(E)_o} = \frac{k_2 k_3}{k_2 + k_3}$. Under conditions where the acylation step is much faster than the deacylation step, $k_2 >> k_3$, $K_m \approx \frac{k_3}{k_2} K_s$ and $k_{cat} \approx k_3$. When the deacylation step is much faster than the acylation step, $K_m \approx K_s$ and $k_{cat} \approx k_2$. In all cases, $\frac{k_{cat}}{K_m} = \frac{k_2}{K_s}$.

2. Steady State Rate Equations in the Presence of an Added Nucleophile; Nucleophile does not Bind to the Acyl-enzyme

Papain has been shown to transfer the acyl portions of ester and amide substrates to nucleophiles other than water. This reaction appears to involve a direct second order reaction of the nucleophile with the acyl-enzyme intermediate, E' (95,96). In the presence of an excess of a second nucleophile, N, Scheme 1 can be rewritten as follows.



(3)

In this equation, k_4 is the second order rate constant for the reaction of the nucleophile with the acyl-enzyme, E', and P_3 is a covalent acyl-nucleophile adduct. The steady

state rate equations are given by (95,96).

$$v_2 = \frac{d(P_2)}{dt} = \frac{\dfrac{k_2 k_3 (E)_o}{k_2 + k_3 + k_4(N)}}{\left[\dfrac{k_3 + k_4(N)}{k_2 + k_3 + k_4(N)}\right]\dfrac{K_s}{(S)} + 1} \qquad (4)$$

$$v_3 = \frac{d(P_3)}{dt} = \frac{\dfrac{k_2 k_4 (N)(E)_o}{k_2 + k_3 + k_4(N)}}{\left[\dfrac{k_3 + k_4(N)}{k_2 + k_3 + k_4(N)}\right]\dfrac{K_s}{(S)} + 1} \qquad (5)$$

$$v_1 = \frac{d(P_1)}{dt} = \frac{d(P_2)}{dt} + \frac{d(P_3)}{dt} = \frac{\dfrac{k_2 (k_3 + k_4(N))(E)_o}{k_2 + k_3 + k_4(N)}}{\left[\dfrac{k_3 + k_4(N)}{k_2 + k_3 + k_4(N)}\right]\dfrac{K_s}{(S)} + 1} \qquad (6)$$

In this present study, we have only measured reaction rates by monitoring the formation of the first product, P_1, Equation 6. Thus $K_m = \left[\dfrac{k_3 + k_4(N)}{k_2 + k_3 + k_4(N)}\right] K_s$, and $k_{cat} = \dfrac{V_{max}}{(E)_o} = \dfrac{k_2(k_3 + k_4(N)}{k_2 + k_3 + k_4(N)}$. When acylation is much faster than deacylation, $k_2 >> (k_3 + k_4(N))$, $K_m \approx \dfrac{k_3 + k_4(N)}{k_2}$ and $k_{cat} \approx$

$k_3 + k_4(N)$. When deacylation is much faster than acylation, $K_m \approx K_s$ and $k_{cat} \approx k_2$. In this later case, a nucleophile (N) would not be expected to increase k_{cat}. In all cases, however, $\frac{k_{cat}}{K_m} = \frac{k_2}{K_s}$.

When the rate of deacylation is of the same order of magnitude as the rate of acylation, there exists no linear relationship describing changes in the maximal velocity of formation of the first product, (P_1), with changes in nucleophile concentration. Such linear relationship can be derived, however, by comparing maximal velocities in the presence and absence of an added nucleophile.

$$\frac{V_N - V_o}{(E)_o} = \frac{k_2(k_3 + k_4(N))}{k_2 + k_3 + k_4(N)} - \frac{k_2 k_3}{k_2 + k_3} \qquad (7)$$

In Equation 7, V_N and V_o are maximal velocities of P_1 formation in the presence and absence of nucleophile, N. Expanding Equation 7, we have

$$\frac{V_N - V_o}{(E)_o} =$$

$$\frac{k_2k_3(k_2 + k_3) + k_2k_4(N)(k_2 + k_3) - k_2k_3(k_2 + k_3) - k_2k_3k_4(N)}{(k_2 + k_3)(k_2 + k_3 + k_4(N)}$$

Subtracting terms we have

$$\frac{V_N - V_o}{(E)_o} = \frac{k_2^2 k_4(N)}{(k_2 + k_3)(k_2 + k_3 + k_4(N))} \quad (8)$$

Taking the reciprocal of Equation 8 and multiplying by $V_o/(E)_o$, we have

$$\frac{V_o}{(E)_o} \times \frac{(E)_o}{V_N - V_o} = \frac{(k_2 + k_3)(k_2 + k_3 + k_4(N)}{k_2^2 k_4} \left[\frac{k_2 k_3}{k_2 + k_3}\right]$$

Simplifying we have

$$\frac{V_o}{V_N - V_o} = \frac{k_3(k_2 + k_3 + k_4(N)}{k_2 k_4(N)}$$

Rearranging terms yields Equation 9

$$\frac{V_o}{V_N - V_o} = \frac{k_3}{k_2}\left[\frac{k_2 + k_3}{k_4}\right]\frac{1}{(N)} + \frac{k_3}{k_2} \quad (9)$$

Equation 9 predicts a linear relationship between $\frac{V_o}{V_N - V_o}$ and $\frac{1}{(N)}$. From the intercept of the line on the ordinate, one can obtain the ratio k_3/k_2.

Alternatively, multiplying the reciprocal of Equation 8 by $V_N/(E)_o$ we obtain

$$\frac{V_N}{(E)_o} \times \frac{(E)_o}{V_N - V_o} = \frac{(k_2 + k_3)(k_2 + k_3 + k_4(N))}{k_2^2 k_4(N)} \left[\frac{k_2(k_3 + k_4(N))}{k_2 + k_3 + k_4(N)}\right]$$

Simplifying we have

$$\frac{V_N}{V_N - V_o} = \frac{(k_2 + k_3)(k_3 + k_4(N))}{k_2 k_4 (N)}$$

Rearranging terms yields Equation 10

$$\frac{V_N}{V_N - V_o} = \frac{k_3}{k_4}\left[\frac{k_2 + k_3}{k_2}\right]\frac{1}{(N)} + \frac{k_2 + k_3}{k_2} \qquad (10)$$

Equation 10 predicts a linear relationship between $\frac{V_N}{V_N - V_o}$ and $\frac{1}{(N)}$. From the slope of the line and its intercept on the ordinate, one can obtain the ratio k_3/k_4.

To obtain values for k_2 and k_4, one must independently obtain a value for k_3. This value can be obtained with the help of the following relationships. From Equations 2 and 6

$$\frac{(E)_o}{V_o} - \frac{(E)_o}{V_N} = \frac{k_2 + k_3}{k_2 k_3} - \frac{k_2 + k_3 + k_4(N)}{k_2(k_3 + k_4(N))}$$

Rearranging terms we have

$$\frac{(V_N - V_o)(E)_o}{V_N V_o} = \frac{1}{k_2} + \frac{1}{k_3} - \left[\frac{1}{k_2} + \frac{1}{k_3 + k_4(N)}\right]$$

Subtracting yields

$$\frac{(V_N - V_o)(E)_o}{V_N V_o} = \frac{1}{k_3} - \frac{1}{k_3 + k_4(N)}$$

Rearranging terms gives

$$\frac{(V_N - V_o)(E)_o}{V_N V_o} = \frac{k_3 + k_4(N) - k_3}{k_3(k_3 + k_4(N))}$$

$$\frac{(V_N - V_o)(E)_o}{V_N V_o} = \frac{k_4(N)}{k_3(k_3 + k_4(N)} \qquad (11)$$

Taking the reciprocal of Equation 11 yields

$$\frac{1}{(E)_o}\left[\frac{V_N V_o}{V_N - V_o}\right] = \frac{k_3^2}{k_4}\frac{1}{(N)} + k_3 \qquad (12)$$

Equation 12 predicts a linear relationship between $\frac{1}{(E)_o}\left[\frac{V_N V_o}{V_N - V_o}\right]$ and $\frac{1}{(N)}$. From the intercept of the line on the ordinate one can obtain k_3 and from its slope one can obtain k_4. Equation 12 together with either Equation 9 or 10 can be used to calculate the rate constants for the individual steps in Schemes 1 and 3.

APPROVAL SHEET

The dissertation submitted by Andrew S. Bajkowski has been read and approved by the following committee:

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The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the dissertation is now given final approval by the Committee with reference to content and form.

The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

April 24, 1979
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