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## OFFICE OF RESEARCH ADMINISTRATION

## RESEARCH PROJECT INITIATION

Date: June 10, 1974

Project Title: X-Ray Study of Enzyme and Small Molecule Interactions

Project No: G-33-681 (Continuation of G-33-667)

Principal Investigator: Dr. James C. Powers

Sponsor: Public Health Service

Agreement Period: From June 1, 1974 Until May 31, 1976

Type Agreement: Grant No. 2 R01 GM18292-04

Amount: $57,319 PHS
8,522 GIT (G-33-360)
$65,841 Total

Reports Required:
Interim Progress, with renewal application.
Final, if project is not to be renewed.

Sponsor Contact Person (s):

Dr. Walter L. Newton
Deputy Associate Director
for Program Activities
Nat. Inst. of Gen. Med. Sciences
Bethesda, Maryland 20014

Assigned to: Chemistry

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RA-3 (6-71)

**GEORGIA INSTITUTE OF TECHNOLOGY**
OFFICE OF CONTRACT ADMINISTRATION

## SPONSORED PROJECT TERMINATION

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Date: 1/24/78

Project Title: X-Ray Study of Enzyme and Small Molecule Interactions

Project No: G-33-681

Project Director: Dr. J. C. Powers

Sponsor: DHEW/PHS/NIH - National Institute of General Medical Sciences

Effective Termination Date: 5/31/77 (end of 05 budget period)

Clearance of Accounting Charges: by 5/31/77

Grant/Contract Closeout Actions Remaining:

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_ Final Invoice and Closing Documents
_ Final Fiscal Report
X Final Report of Inventions due by 8/31/77.
_ Govt. Property Inventory & Related Certificate
_ Classified Material Certificate
_ Other _____

Assigned to: Chemistry (School/Laboratory)

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CA—4 (3/76)

G 33-681

| APPLICANT: REPEAT GRANT NUMBER SHOWN ON PAGE 1 ——→ | GRANT NUMBER | |
|---|---|---|
| SECTION IV—SUMMARY PROGRESS REPORT | GM- 18292-04 | |
| PRINCIPAL INVESTIGATOR OR PROGRAM DIRECTOR (Last, First, Initial) James C. Powers | PERIOD COVERED BY THIS REPORT | |
| | FROM | THROUGH |
| NAME OF ORGANIZATION Georgia Institute of Technology | June 1, 1974 | July 1, 1975 |

TITLE (Repeat title shown in Item 1 on first page)

X-RAY STUDIES OF ENZYME AND SMALL MOLECULE INTERACTIONS

1. List publications: (a) published and not previously reported; (b) in press. Provide five reprints if not previously submitted.
2. List all additions and deletions in professional personnel and any changes in effort.
3. Progress Report. (See Instructions)

1a. Publications not previously reported.

"Active-Site Specific Inhibitors of Elastase," James C. Powers and Peter M. Tuhy, Biochemistry, 12, 4767 (1973).

"Inhibition of Chymotrypsin $A_\alpha$ with N-acyl and N-Peptidyl-2-phenylethylamines. Subsite Binding Free Energies," James C. Powers, B. Lee Baker, J. Brown and B. K. Chelm, J. Am. Chem. Soc., 96, 238 (1974).

"Synthetic Active Site-Directed Inhibitors of Elastolytic Proteases," James C. Powers, D. L. Carroll and P. M. Tuhy, Ann. N.Y. Acad. Sci., 256, 420 (1975).

1b. Publications in Press

"Modification of Chymotrypsin with the Cyanate Reagent Ac-Phe(OCN)-OEt", James C. Powers, P. M. Tuhy and F. Witter, Submitted to Biochemistry.

"Reactions of Acyl Carbazates with Proteolytic Enzymes," James C. Powers and D. L. Carroll, Submitted to J. Am. Chem. Soc.

2. Professional Personnel Changes

Norikazu Nishino - Postdoctorial Fellow-added

David L. Carroll - Graduate Research Assistant - deletion (took job at Colgate Palmolive)

Ronald Whitley - Graduate Research Assistant - deletion at end of summer (writing Ph.D. thesis).

3. Progress Report and Research Goals

The object of this research is to study the interaction of small molecules such as inhibitors or virtual substrates with enzymes in order to provide information about binding modes, recognition sites and the mechanism of enzymatic catalysis. One goal is to obtain difference Fourier electron density maps of enzyme-small molecular complexes to elucidate the structural details of the interactions. A second goal is to correlate the reactivity of enzymes with substrates and inhibitors in solution with the structure of the crystalline enzyme as observed by x-ray crystallography.

Peptide Carbazates. One goal set for the current year was to obtain a difference electron density map of chymotrypsin $A_\alpha$ inhibited by the carbazate Ac-Ala-Bzc-ONp (Bzc = $-NHN(CH_2C_6H_5)CO-$)


The carbazyl enzyme is simply an acyl enzyme in which the alpha carbon atom has been replaced by a nitrogen atom. A structural study of this analog of the acyl enzyme intermediate in hydrolysis of peptide by serine proteases such as chymotrypsin, was expected to yield information on the mechanism of enzymatic peptide bond hydrolysis. Dave Carroll collected x-ray data on the carbazyl enzyme and calculated a low resolution electron density map. The peaks of highest electron

density were in the active site region in line with our expectations. At this point, Dave left Georgia Tech to take a job and we have done no work on this derivative since that time. A summary of our work on the solution reactivity of carbazates is contained in the attached manuscript "Reaction of Acyl Carbazates with Proteolytic Enzymes" which has been submitted to J. Am. Chem. Soc. Reprints not yet received.

At present, it appears that obtaining a high resolution electron density map of the carbazoyl derivative of chymotrypsin will be difficult for two reasons. First, the derivative appears not to be strictly isomorphous and so considerable crystallographic refinement will be required. Secondly, I have been unable to convince another graduate student to undertake this problem.

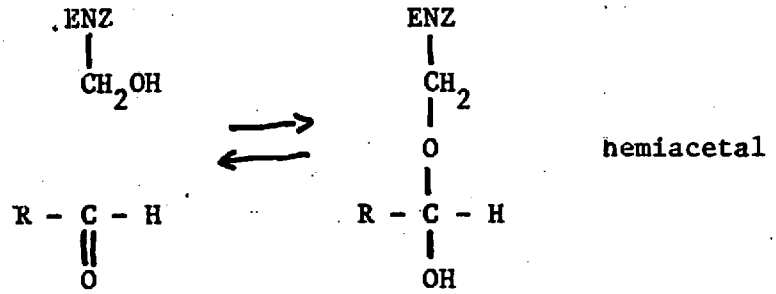
Recently, I have begun some collaborative studies with Greg Petsko (Wayne State Univ.) with the serine protease Subtilisin Carlsberg. One goal for the next year is to study the stability of carbazyl derivatives of subtilisin Carlsberg to ascertain if a crystallographic study is feasible with this enzyme.

Protease Subsite Specificity. We are just completing a computer analysis of the amino acid sequence around cleavage sites of polypeptide substrates by proteolytic enzymes. The source of our information was all papers which sequenced proteins or peptides which appeared in the literature over a 5 year period. We were able to obtain statistically significant information for three proteases (chymotrypsin, pepsin and thermolysin) and some data for other proteases such as elastase. The data is too extensive to discuss in a short space, so I will concentrate only on elastase. Elastase, on basis of a large number of papers in the literature, is thought to prefer primarily alanine as the $P_1$ residue in a substrate or inhibitor. In contrast, our data showed a high preference for valine or isoleucine. To follow up this result, we synthesized the inhibitors Ac-Ala-Ala-Pro-Val$CH_2Cl$ to compare with our best elastase inhibitor Ac-Ala-Ala-Pro-Ala$CH_2Cl$. With porcine pancreatic elastase, both the valine and isoleucine chloromethyl ketone inhibitors were as effective or slightly more effective than our standard inhibitor Ac-Ala-Ala-Pro-Ala$CH_2Cl$.

The inhibition of human leukocyte elastase, the enzyme probably responsible for emphysema and some inflammatory diseases, by these same chloromethyl ketones was then studied. To our great surprise, Ac-Ala-Ala-Pro-Ile$CH_2Cl$ was 47 times more effective! I believe this is an important result since it is leading us closer to a treatment for this important disease.

Our goals in this area for the future are primarily concerned with analyzing the data and preparing it for publication.

Peptide Aldehydes. Peptide aldehydes can be considered to be virtual substrates of serine proteases due to their close resemblance to nature substrates. It is likely that they are transition state analogs due to the similarity of the hemiacetal, which is probably




formed as the aldehyde complexes with the enzyme, to the tetrahedral transition state involved in normal peptide bond hydrolysis. Our goal is to obtain a difference electron density map of a chymotrypsin - peptide aldehyde complex to elucidate the structure of this important intermediate in the catalytic mechanism.

We have suceeded in synthesizing several peptide aldehydes including Z-PheH and

Ac-Ala-PheH (PheH = $NHCH(CH_2C_6H_5)CHO$) which are competitive inhibitors of chymotrypsin. Crystals of chymotrypsin $A_\gamma$ into which the aldehydes have diffused, are isomorphons with native enzyme. We have this far taken zero level x-ray photographs of the complexes.

Our immediate goal is to collect enough data on one or both of these complexes to allow calculation of a low resolution electron density map.

If one of the derivatives seems suitable, we will then try to calculate a high resolution electron density map. In addition, we will probably shortly begin a similar x-ray crystallographic study using subtilisin Carlsbery in collaboration with Greg Petsko (Wayne State Univ.).

Acid Proteases. David Davies (NIH) is working on the crystal structure of an acid protease from Rhizopus Chinensis. The enzyme, related to pepsin, will probably be the first acid protease to be solved to atomic resolution. The goal of our initial research is to synthesize heavy atom derivatives which might be useful in the x-ray structure determination of this enzyme.

Recently we have completed the synthesis of two useful inhibitors: $CH_3C_6H_4SO_2$-PheCHN$_2$ and I-$C_6H_4SO_2$-PheCHN$_2$. We are currently studying the kinetics of their reaction with the Rhizopus Chinensis acid protease and pepsin. We will then supply these compounds to David Davies for crystallographic studies.

Our future studies in this area will be determined by the initial crystallographic results. If no further heavy atom derivatives are required, then we will begin the synthesis of virtual substrates for this enzyme.

3. Significance

From a study of the difference Fourier maps of small molecule-protein complexes, we expect to learn significant structural information about binding modes and recognition sites of serine and acid proteases. It is already evident that we are able to synthesize some highly reactive inhibitors for chymotrypsin, subtilisin and elastase using crystallographic results. Our results with chymotrypsin and subtilisin also show that it is possible to build selectivity into an inhibitor, even when dealing with closely related enzymes. Indeed, it should be possible to design an inhibitor which is not only enzyme specific, but is species specific. Thus we are approaching the day when we can design a drug to inhibit an enzyme system in only one particular type of all or one type of organism to the exclusion of all others.

Practical application of our elastase inhibitors is much closer to fruition. Pulmonary emphysema is currently thought to result from the uninhibited proteolysis of lung tissue by elastase and related neutral proteases derived from leukocytes, macrophages or other cellular sources. Synthetic inhibitors capable of specifically inhibiting elastase would be expected to be useful reagents both for the treatment of emphysema and for the study of the biological function of elastolytic enzymes. The peptide chloromethyl ketone inhibitors of elastase, developed during early years of this grant, have been in much demand by other investigators for these purposes. The Heart and Lung Institute funded a 1½ year contract to synthesize large quantities of these inhibitors and at present over two dozen investigators are utilizing these compounds in their own research. Thus, there is good reason to believe that these elastase inhibitors or related compounds will eventually find use, in the treatment of pulmonary disease.

6-33-681

APPLICANT: REPEAT GRANT NUMBER SHOWN ON PAGE 1 ——→

GRANT NUMBER

GM-18292-05

## SECTION IV—SUMMARY PROGRESS REPORT

PRINCIPAL INVESTIGATOR OR PROGRAM DIRECTOR (Last, First, Initial)

Powers, James C.

PERIOD COVERED BY THIS REPORT

FROM: July 1, 1975 THROUGH: March 1, 1976

NAME OF ORGANIZATION

Georgia Institute of Technology

TITLE (Repeat title shown in Item 1 on first page)

X-RAY STUDY OF ENZYME AND SMALL MOLECULE INTERACTIONS

1. List publications: (a) published and not previously reported; (b) in press. Provide five reprints if not previously submitted.
2. List all additions and deletions in professional personnel and any changes in effort.
3. Progress Report. (See Instructions)

Note: A summary progress report for the period June 1, 1974 to July 1, 1975 was submitted last year. This was half-way through the 2 year budget period of GM-18292-04. Therefore, this progress report covers the period since July 1, 1975 while the manpower and budget figures cover the whole period beginning June 1, 1974.

1a. Publications not previously reported

"Inhibition of Human Leukocyte Elastase by Peptide Chloromethyl Ketones", P. M. Tuhy and J. C. Powers, FEBS LETTERS, 50, 359 (1975).

"Reaction of Acyl Carbazates with Proteolytic Enzymes", J. C. Powers and D. L. Carroll, Biochem. Biophys. Res. Comm., 67, 639 (1975)

1b. Publications in Press

"Modification of Chymotrypsin with the Cyanate Reagent Ac-Phe(OCN)-OEt", James C. Powers, P. M. Tuhy, and F. Witter, Submitted to Biochem. Biophy. Acta.

2. Professional Personnel changes

Chin Min Kam - Half Time Postdoctoral Fellow - added

Frank Gupton - Graduate Research Assistant - added

During the last grant period, I utilized some of the personnel money to support four graduate research assistants for short periods of time (usually the summer quarter). Their names and the length of support are listed on the manpower report.

Change of Effort. During the last year, I have made the decision not to pursue protein crystallographic studies at Georgia Tech., but to pursue our research goals in collaboration with other protein crystallography groups. I will emphasize the synthetic and enzymatic aspects of the problems in my laboratory. There are two factors responsible for the decision. First, it is highly likely that the protease inhibitors which we have been synthesizing for crystallographic studies will be useful in the treatment of diseases such as emphysema and I thus decided to concentrate on this aspect of the problem. And secondly, I came to the realization that protein crystallographic studies could be more efficiently carried out in collaboration with full-time practicing crystallographers. I am at presently actively collaborating with two crystallographers, David Davies at the NIH and Greg Petsko at Wayne State University.

3. Progress Report and Research Goals. The object of this research is to study the interaction of small molecules such as inhibitors or virtual substrates with enzymes in order to provide information about binding modes, recognition sites and the mechanism of enzymatic catalysis. One goal is to correlate the reactivity of enzymes with substrates and inhibitors in solution with the structure of the crystalline enzyme as observed by x-ray crystallography. This has allowed us to design and synthesize more effective enzyme inhibitors.

Aza-Amino Acid Derivatives (Peptide Carbazates). Aza-amino acids are simply amino acid derivatives in which the α-carbon atom has been replaced by a nitrogen atom.

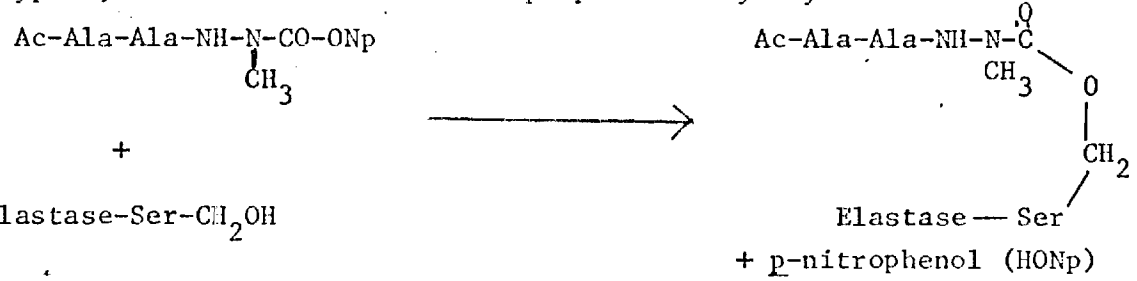
amino acid aza-amino acid



★ U. S. GOVERNMENT PRINTING OFFICE : 1974 [illegible]

I will use a nomenclature for aza amino acid residues which is based on the nomenclature for the corresponding amino acid. Thus, I will refer to aza phenylalanine (-NHN($CH_2C_6H_5$)CO-) as Aphe, aza alanine (-NHN($CH_3$)CO-) as Aala and so forth.

We have found peptides with aza-amino acid residues to be good inhibitors of serine proteases. For example, Ac-Ala-Aphe-ONp specifically acylates chymotrypsin, while Ac-Ala-Ala-Aala-ONp specifically acylates elastase.




With porcine pancreatic elastase, the acylated enzyme deacylates slowly and has a reasonable lifetime. Thus, the reagent Ac-Ala-Ala-Aala-ONp can be used as an effective inhibitor and as an active site titrant for elastase.

Two enzymes have been implicated in tissue damage in pulmonary emphysema: human neutrophil leukocyte elastase and cathepsin G (a chymotrypsin-like enzyme). In the past year we have synthesized a number of peptide aza-amino acid derivatives and have studied their reactivity toward the HNL elastase. The compounds are of the type Ac-Ala-Ala-Aaa-ONp where Aaa= Aala,Aleu,Aval,Aile,Anval,Agly, Anleu. We have measured rates of acylation and deacylation for the reaction of these compounds with both porcine pancreatic elastase and HNL elastase. The norvaline compound Ac-Ala-Ala-Anval-ONp appears to be the most effective inhibitor for the HNL elastase. It acetylates the enzyme instantaneously and the half-life for the deacylation reaction is ca. 12 hrs. This makes this compound a very effective inhibitor, although not irreversible like some of the chloromethyl ketones (Ac-Ala-Ala-Pro-Val$CH_2Cl$) which we prepared earlier.

We have recently obtained a sample of cathepsin G, but have not yet had an opportunity to carry out any studies with that enzyme. One of our goals for the next year is to investigate the reactivity of this enzyme with carbazates such as Ac-Ala-Aphe-ONp and Ac-Ala-Ala-Aleu-ONp.

Another of our goals for next year is to synthesize carbazates such as Ac-Ala-Aarg-ONp and investigate their reactivity toward trypsin-like enzymes. Many of the important enzymes involved in physiological processes like clotting and fertilization are trypsin like enzymes with specificity toward argimine residues. Thus, I feel it is worthwhile to investigate carbazates which react specifically with these enzymes in order to develop drugs which could be used to control or mediate the action of many of these proteases.

Acid Proteases. David Davies(NIH) is working on the crystal structure of an acid protease from Rhizopus Chinensis. The enzyme, related to pepsin, will probably be the first acid protease to be solved to atomic resolution. The goal of our initial research was to synthesize heavy atom derivatives which would be useful in the x-ray structure determination of this enzyme.

We synthesized two useful inhibitors: $CH_3C_6H_4SO_2$-PheC$HN_2$ and $IC_6H_4SO_2$-PheC$HN_2$ and measured the kinetics of their reaction with both pepsin and the Rhizopus protease. We sent samples of these compounds to David Davies who has done preliminary crystallographic studies with the iodo compound. In addition, we have given the iodo compound to Sine Laursen in Denmark for use in the structure determination of the acid protease chymosin.

In a continuation of this research, we are engaged in the synthesis of virtual substrates of acid protease for use in both kinetic and crystallographic studies. The synthesis of Ac-Phe-Aphe-ONp, an analog of the pepsin substrate Ac-Phe-Phe-OEt, has been synthesized. We expect this to be stable to hydrolysis

by the enzyme and to be a decent competitive inhibitor. We have not yet had the opportunity to carry out kinetic studies.

Our goals for the next year include the synthesis of other acid protease inhibitors such as Ac-Aphe-Phe-OEt and Ac-Aphe-Aphe-OEt and investigation of the kinetics of their reaction with pepsin and the Rhizopus Chinensis acid protease.

Significance. It is evident that we have been able to design and synthesize some highly reactive inhibitors for serine proteases using information about binding modes and recognition sites gained from x-ray crystallography and solution kinetics. Indeed, it now seems possible to design inhibitors which are not only enzyme specific, but are species specific. Thus, we are nearing the day when we can design a drug to inhibit an enzyme system in only one particular type of organism to the exclusion of all others.

Practical application of the elastase inhibitors which we synthesized earlier under this grant is in more advanced stage. Pulmonary emphysema is thought to result from the proteolysis of lung tissue by elastase and possibly cathepsin G derived from leukocytes. Several investigators have now begun to study the effect of peptide chloromethyl ketones such as Ac-Ala-Ala-Pro-Ala$CH_2Cl$ and Ac-Ala-Ala-Pro-Val$CH_2Cl$ on emphysema in animal models. In addition, I have now sent Ac-Ala-Ala-Aala-ONp to over 10 other investigators for studies involving HNL elastase. Thus, there is good reason to believe that these elastase inhibitors or related compounds will eventually find use in the treatment of pulmonary disease.

Invention Certification.

Inventor - James C. Powers
Title - Use of Aza-Amino Acid Derivatives for the Inhibition of Proteases and the Treatment of Emphysema, Arthritis and Related Diseases.

The undersigned agrees to accept responsibility for the scientific and technical conduct of the project and for provision of required progress reports if a grant is awarded as the result of this application.

March 17, 1976
Date

Principal Investigator or Program Director

# X-RAY STUDY OF ENZYME AND SMALL MOLECULE INTERACTIONS

Terminal Progress Report

NIH Grant 5 R01 GM18292-05

by

James C. Powers

School of Chemistry

Georgia Institute of Technology

Atlanta, Georgia 30332

October, 1977

Index

Research Goals. The object of this research was to study the interaction of small molecules such as inhibitors or virtual substrates with enzymes in order to provide information about binding modes, recognition sites and the mechanism of enzymatic catalysis. One goal was to correlate the reactivity of enzymes with substrates and inhibitors in solution with the structure of the crystalline enzymes as observed by x-ray crystallography. Another goal was to use this information to design and synthesize more effective enzyme inhibitors.

Summary of Progress. During the course of this research, our research emphasis shifted from crystallographic studies toward the design and synthesis of specific enzyme inhibitors. A number of reasons were responsible for this shift. First it appeared (and still appears) likely that the protease inhibitors which we were synthesizing for crystallographic studies will be useful in the treatment of diseases such as emphysema. I thus decided to concentrate on this aspect of the problem and am continuing with this research (supported by HL18679-Synthetic Protease Inhibitors). And secondly, I came to the realization that protein crystallographic studies could be more efficiently carried out in collaboration with full-time practicing crystallographers. At Georgia Tech I was never able to build up either enough interest in graduate students or enough equipment to seriously pursue protein crystallography. On the other hand we are actively collaborating with a number of protein crystallographers. We have sent inhibitors with heavy atoms to David Davies at NIH and Sine Laursen in Denmark for studies with acid proteases. We have sent chloromethyl ketones and aza-peptides to Greg Petsko (Wayne State) for studies with subtilisin Carlsberg. And

more recently we have sent some metalloprotease inhibitors to Brian Matthews (Oregon) for studies with thermolysin.

In the area of protease inhibitors, we have designed and synthesized some highly effective inhibitors for the granulocyte enzymes elastase and cathepsin G. Both of these enzymes digest lung elastin and have been implicated in the pathogenesis of pulmonary emphysema. Chloromethyl ketone inhibitors which we developed early in the course of this research are now being tested by several investigators in animal models of emphysema. A more promising type of inhibitor is aza-peptides. These compounds have the potential for being more specific than chloromethyl ketones. Aza-peptides which inhibit both elastase and cathepsin G have been prepared and studied. At present we are investigating changes in the leaving group portion of aza-peptide structures. Up to now we have used a p-nitrophenol group due to the ease of studying the reactions with enzymes. The leaving group is however not suitable for in vivo studies. Several of the aza-peptides which we developed have found use as active site titrants of various serine proteases.

The secondary specificity of the enzymes chymotrypsin, thermolysin, pepsin and papain have been investigated. In order to analyze the nature of the extended substrate binding site of these enzymes, we collected and analyzed the sites of proteolytic cleavage in a large number of proteins and polypeptides which were reported in the course of sequence determination. We were then able to determine the length of the extended binding region in each of these proteases, the preferences for individual amino acid residues at each subsite and the effect of terminal amino acid residues.

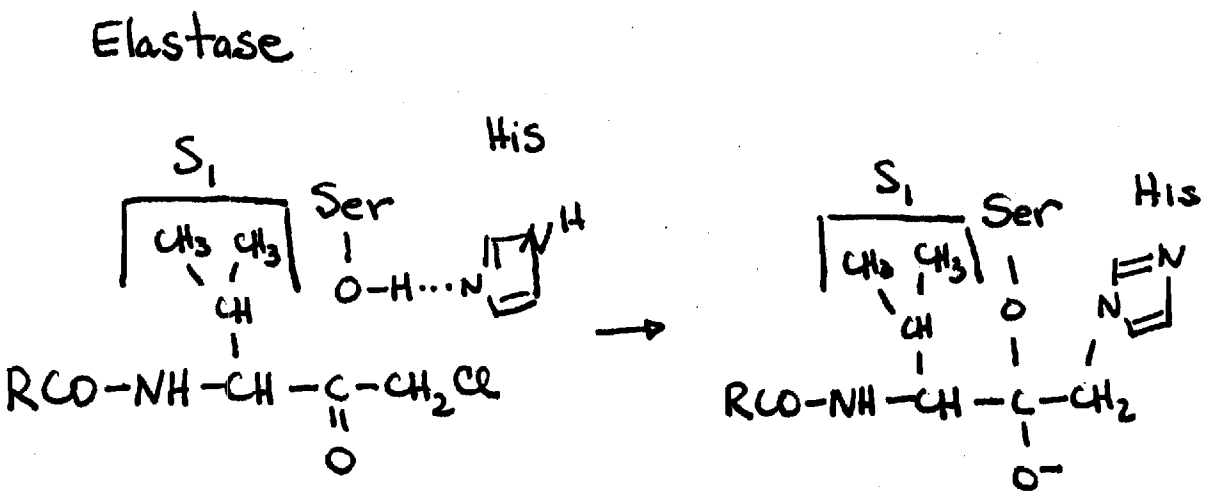
Detailed Progress Report. A number of neutral proteases have been isolated from human granulocytes. These include serine proteases such as elastase and cathepsin G. These proteases have attracted considerable interest recently since it is generally acknowledged that some or all of these enzymes are responsible for the tissue destruction that occurs in diseases such as emphysema. Since leukocytes are known to contain relative large amounts of elastase and due to its ability to attack elastic fibers, elastase is the prime candidate for the destructive agent. In addition purified human granulocyte elastase has been shown to induce experimental emphysema when instilled in dog lungs (Janoff et al, 1977).

Both elastase and cathepsin G are serine proteases as shown by their characteristic reaction with diisopropylfluorophosphate (DFP). The action of both enzymes on the oxidized B chain of insulin has been studied (Blow, 1977; Blow and Barrett, 1977). In addition kinetic studies with peptide p-nitroanilides have been reported (Zimmerman and Ashe, 1977).

Natural plasma inhibitors such as $\alpha_1$-protease inhibitor ($\alpha_1$-antitrypsin) ordinarily remove elastase or cathepsin G from the circulation whenever they are released from the granulocyte. Individuals lacking this inhibitor are predisposed toward emphysema. However, there are probably many other mechanisms for initiating of the disease since only a small percentage of emphysema patients are homozygotes in an $\alpha_1$-protease inhibitor deficiency.

Chloromethyl Ketone Inhibitors. Peptide chloromethyl ketone inhibitors are a type of affinity label that have been extensively studied as inhibitors for serine proteases. A fairly detailed picture of the chloromethyl ketone

inhibition reaction has emerged from numerous chemical and crystallographic studies (Powers, 1977).




The rate at which a peptide chloromethyl ketone inhibits human granulocyte elastase is profoundly influenced both by its interaction with the primary substrate binding subsite ($S_1$, nomenclature of Schechter and Berger, 1967) of the enzyme and by its interaction at other subsites (Tuhy and Powers, 1975; Powers et al, 1977). A comparison of the effectiveness of individual peptide chloromethyl ketones can be made on the basis of their $k_{obsd}/[I]$ values, the equivalent of a second-order rate constant, where $k_{obsd}$ is the pseudo first-order rate constant for the inhibition reaction and [I] is the inhibitor concentration. Some representative $k_{obsd}/[I]$ values at pH 6.5 are listed in Table 1. The rates are approximately 1.4 to 4.4 times faster at pH 7.5.

Table 1. Inhibition of Human Granulocyte Elastase with Peptide Chloromethyl Ketones[a]

| Inhibitor | $k_{obsd}/[I]$ ($M^{-1}$ $s^{-1}$) |
|---|---|
| **$P_1$ effect** | |
| Ac-Ala-Ala-Pro-Thr$CH_2Cl$ | 0.15 |
| Ac-Ala-Ala-Pro-Ala$CH_2Cl$ | 3.4 |
| Ac-Ala-Ala-Pro-Ile$CH_2Cl$ | 133 |
| Ac-Ala-Ala-Pro-Val$CH_2Cl$ | 160 |
| **Effect of the chain length** | |
| Ac-Ala-Ala-Ala$CH_2Cl$ | 0.28 |
| Ac-Ala-Ala-Ala-Ala$CH_2Cl$ | 0.97 |
| Ac-Ala-Pro-Ala$CH_2Cl$ | 1.1 |
| Ac-Ala-Ala-Pro-Ala$CH_2Cl$ | 4.4 |
| **Effect of a proline residue** | |
| Ac-Ala-Ala-Ala$CH_2Cl$ | 0.28 |
| Ac-Ala-Pro-Ala$CH_2Cl$ | 1.1 |
| Ac-Pro-Ala-Ala$CH_2Cl$ | 0 |
| Ac-Ala-Ala-Ala-Ala$CH_2Cl$ | 0.97 |
| Ac-Ala-Ala-Pro-Ala$CH_2Cl$ | 4.4 |
| Ac-Ala-Pro-Ala-Ala$CH_2Cl$ | 2.2 |
| **Effect of the $P_5$ residue** | |
| Ac-Ala-Ala-Pro-Val$CH_2Cl$ | 160 |
| Suc-Ala-Ala-Pro-Val$CH_2Cl$[b] | 320 |
| MeO-Suc-Ala-Ala-Pro-Val$CH_2Cl$[c] | 922 |

[a] pH 6.5, 0.1 M phosphate, 0.06 M NaCl, elastase 10μM, 30°, 5% (v/v) methanol. The rate of inhibition of elastase by Ac-Ala-Ala-Pro-Ala$CH_2Cl$ was found to vary slightly when measured by different individuals on different batches of enzyme. Both numbers are used in the above table to allow reporting of data for each group of compounds which was measured under identical circumstances. [b] Suc = $HOCOCH_2CH_2CO$-. [c] MeO-Suc = $CH_3OCOCH_2CH_2CO$-.

A number of conclusions can be drawn concerning the extended substrate binding site of granulocyte elastase. The $S_1$ subsite prefers Val>Ile>Ala>Thr. This is in contrast to porcine pancreatic elastase where the Val, Ile, and Ala inhibitors are all equally effective. This would indicate that the $S_1$ subsite is somewhat larger in granulocyte elastase. However, it will not accommodate a phenylalanine residue since Z-Gly-Leu-Phe$CH_2$Cl will not inhibit the enzyme while Z-Gly-Leu-Ala$CH_2$Cl is a reasonable inhibitor.

Studies with substrates show the same pattern with respect to the $S_1$ specificity. Granulocyte elastase cleaves the B chain of insulin at 5 sites (2 major and 3 minor; Blow, 1977). Both of the major cleavages occur after a valine residue, while the minor cleavages occur after Cys($SO_3H$), Ser and Ala. A similar trend is observed in the granulocyte elastase hydrolysis of Ac-Ala-Ala-Pro-X-p-nitroanilides (X=Val, Ile or Ala). The $k_{cat}/K_M$ ($M^{-1}s^{-1}$) values for the Val, Ile and Ala peptides were respectively 27,000, 5400 and 3900. Thus a qualitative correlation is observed between chloromethyl ketone inhibition, p-nitroanilide hydrolysis and peptide bond cleavage.

Extension of the peptide chain of a chloromethyl ketone inhibitor from a tripeptide to a tetrapeptide results in a 3 to 4 fold acceleration in the inhibition rate indicating that granulocyte elastase has an extended substrate binding site to interact with the inhibitor. The $S_2$ subsite of the enzyme prefers an amino acid residue with a long alkyl side chain. Changing the $P_2$ residue from an Ala to Pro in both the case of the tripeptide and tetrapeptide inhibitors results a 4 fold increase in the inhibiton rate. In addition, the $P_2$ residue at both the major cleavage sites and one of

the minor cleavage sites of the oxidized insulin B chain is a leucine residue; the latter cleavage is not observed with porcine pancreatic elastase. In fact there is one other leucine residue in the B chain which is not at a cleavage site and that leucine is followed by a tyrosine residue ($P_1$) which probably prevents cleavage. Thus the evidence points to a strong secondary specificity for Pro or Leu at $P_2$.

The replacement of an alanyl residue at $P_3$ by a prolyl residue prevents inhibition in the case of tripeptides, but results in a small acceleration in the case of tetrapeptides. This is in contrast to porcine pancreatic elastase where this structural change prevents inhibition.

Alterations in the $P_5$ group have profound effects on the inhibition rate. The inhibitors Suc-Ala-Ala-Pro-Val$CH_2$Cl and MeO-Suc-Ala-Ala-Pro-Val$CH_2$Cl, which were synthesized to increase water solubility, are 7-8 times more reactive than the acetyl compound at pH 7.5. MeO-Suc-Ala-Ala-Pro-Val$CH_2$Cl is the best granulocyte elastase chloromethyl ketone inhibitor thus far reported. It would appear that the $S_5$ subsite of granulocyte elastase contains some structural feature which interacts favorably with a succinyl or methyl succinyl grouping. This points out another significant difference between the granulocyte and porcine pancreatic enzymes, since the change from acetyl to succinyl or methyl succinyl has a relatively minor effect on the rate of inhibition of the porcine enzyme.

The subsite specificity of human granulocyte elastase can be summarized as follows: $P_1$, Val>Ile>>Ala; $P_2$, Pro(=Leu?)>Phe>Ala; $P_3$, Pro>Ala; $P_5$, MeO-Suc->Suc->Ac-.

Granulocyte Cathepsin G. Cathepsin G has been reported to be unreactive toward Tos-Phe$CH_2$Cl but could be inhibited slowly by Z-Phe$CH_2$Cl and Z-

Gly-Gly-Phe$CH_2Cl$ (Feinstein and Janoff, 1975). The effectiveness of more than a dozen chloromethyl ketone inhibitors of human cathepsin G were measured and are listed in Table 2 (Powers et al, 1977). The most effective inhibitor is Z-Gly-Leu-Phe$CH_2Cl$. Both the $P_1$ Phe (ratio of $k_{obsd}/[I]$ for Z-Gly-Leu-Phe$CH_2Cl$/Z-Gly-Leu-Ala$CH_2Cl$ = 20) and the $P_2$ Leu (ratio for Z-Gly-Leu-Phe$CH_2Cl$/Z-Gly-Gly-Phe$CH_2Cl$ = 12.5) are important features of this inhibitor. The $P_4$ benzyloxycarbonyl (Z) group has an insignificant effect (Z-Gly-Gly-Phe$CH_2Cl$/Ac-Ala-Gly-Phe$CH_2Cl$ = 1). Except for Z-Gly-Leu-Phe$CH_2Cl$, there is very little spread in the reactivities of the other inhibitors tested.

Table 2. Inhibition of Human Granulocyte Cathepsin G with Peptide Chloromethyl Ketones[a]

| Inhibitor | $k_{obsd}/[I]$ ($M^{-1}$ $s^{-1}$) |
|---|---|
| Z-Trp$CH_2Cl$ | 5.7 |
| Ac-Ala-Phe$CH_2Cl$ | 3.5 |
| Z-Leu-Phe$CH_2Cl$ | 4.7 |
| Z-Gly-Gly-Phe$CH_2Cl$ | 4.1 |
| Z-Gly-Leu-Ala$CH_2Cl$ | 2.6 |
| $(CH_3)_2CHCH_2CO$-Ala-Phe$CH_2Cl$ | 6.2 |
| Z-Gly-Leu-Phe$CH_2Cl$ | 51 |
| Ac-Ala-Gly-Phe$CH_2Cl$ | 4.0 |
| Ac-Ala-Ala-Pro-Ala$CH_2Cl$ | 2.0 |
| Ac-Ala-Ala-Pro-Val$CH_2Cl$ | 3.7 |
| Ac-Ala-Ala-Pro-Ile$CH_2Cl$ | 4.2 |
| Ac-Gly-Gly-Ala-Phe$CH_2Cl$ | 6.8 |
| Ac-Phe-Gly-Ala-Leu$CH_2Cl$ | 3.2 |
| MeO-Suc-Ala-Ala-Pro-Val$CH_2Cl$ | 0 |

[a] pH 7.5, 0.02 M phosphate, 1.0 M NaCl, cathepsin G 0.21μM, 30°C, 4.3%(v/v) methanol.

Cathepsin G cleavages of the oxidized insulin B chain occur at sites with a $P_1$ Phe, Leu or Tyr (Blow and Barrett, 1977). With the p-nitroanilides Ac-Ala-Ala-Pro-X-p-nitroanilides, only the X = Phe and Leu were cleaved, while those with Ala, Val and Ile were untouched (Zimmerman and Ashe, 1977). Thus a $P_1$ Phe is a preferred resiude in the case of chloromethyl ketone inhibitors, peptide p-nitroanilides and peptide substrates. However, in the case of chloromethyl ketone inhibitors subsite interactions can play a significant role. Z-Gly-Leu-Ala$CH_2$Cl is a reasonable inhibitor even though the $P_1$ residue is Ala. In addition, the change of a $P_5$ acetyl to a methyl succinyl group in the case of MeO-Suc-Ala-Ala-Pro-Val$CH_2$Cl prevents inhibition. This suggests that subsite interactions are effecting the catalytic site.

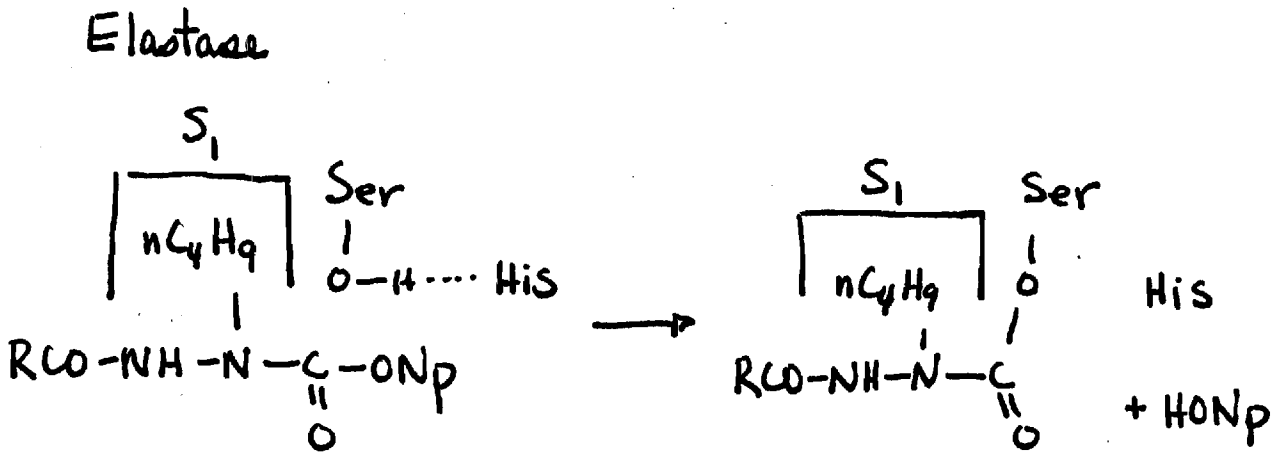
Specificity of the Chloromethyl Ketone Inhibitors. The specificity of several chloromethyl ketone inhibitors of elastase and cathepsin G are outlined in Table 3. It is possible to selectively inhibit cathepsin G with Z-Gly-Leu-Phe$CH_2$Cl and elastase with MeO-Suc-Ala-Ala-Pro-Val$CH_2$Cl. These inhibitors show no cross reactivity even after incubation for 2-4 days at pH 7.5. Other inhibitors such as Ac-Ala-Ala-Pro-Val$CH_2$Cl are relatively effective at inhibiting both enzymes, but will not touch other serine proteases such as bovine pancreatic trypsin.

Table 3. Specificity of the Chloromethyl Ketone Inhibitors at pH 7.5

| Inhibitor | $k_{obsd}/[I]$ ($M^{-1}$ $s^{-1}$) |
|---|---|
| Z-Gly-Leu-Phe$CH_2$Cl | |
| elastase | 0 |
| cathepsin G | 51 |
| MeO-Suc-Ala-Ala-Pro-Val$CH_2$Cl | |
| elastase | 1560 |
| cathepsin G | 0 |
| glutathione | 0.88 |
| Ac-Ala-Ala-Pro-Val$CH_2$Cl | |
| elastase | 219 |
| cathepsin G | 3.7 |

Under physiological conditions, chloromethyl ketones can react not only with serine proteases but with other nucleophilic groups. The alkylation of sulfhydryl groups could be a significant side reaction. In order to evaluate the significance of such side reactions, the rate of reaction of glutathione with MeO-Suc-Ala-Ala-Pro-Val$CH_2Cl$ was measured and is listed in Table 3. The rate is quite slow and the inhibitor would descriminate in favor of leukocyte elastase over glutathione by a factor of 1770 if the concentrations were equivalent. Thus MeO-Suc-Ala-Ala-Pro-Val$CH_2Cl$ appears to be a highly reactive and selective inhibitor for use in physiological situations. However, it should be noted that cellular thiol proteases such as cathepsin B1 can also react with chloromethyl ketones and it remains to be seen whether an inhibitor can be constructed with would be able to descriminate between elastase and cellular thiol proteases.

Aza-peptides. Aza-amino acid residues are analogs of amino acids in which the α-CH has been replaced by a nitrogen atom. This substitution has a profound effect on the reactivity of aza-peptides, i.e., those containing aza-amino acid residues. In particular, aza-peptide p-nitrophenyl esters are inhibitors and active site titrants of several serine proteases (Powers and Gupton, 1977). Inhibition of these enzymes is believed to arise from the acylation of the active site serine yielding an acylated enzyme.




The reaction of granulocyte elastase with a number of aza-peptide p-nitrophenyl esters has been studied and some of the results are listed in Table 4. Most of the aza-peptides acylate elastase rapidly, although the rates are variable. The reaction of many of aza-peptides such as Ac-ala-Ala-Anle-ONp with elastase are complete in less than 10 s. However, Ac-Ala-Aphe-ONp acylates elastase very slowly. The kinetics of the reaction are described in detail elsewhere (Powers and Gupton, 1977), but with most of the inhibitors, $k_{cat}$ is equal to the deacylation (or reactivation) rate of the acylated enzyme. Aza-peptides with low $k_{cat}$ values are more suitable inhibitors since the acylated enzyme formed will regain enzyme activity more slowly. The best inhibitor in the series is Ac-Ala-Ala-Anle-ONp which acylates elastase in less than 10 s and has no measurable $k_{cat}$ at either pH 6.0 or pH 7.0.

Table 4. Reaction of Granulocyte Elastase and Cathepsin G with Aza-peptide p-Nitrophenyl Esters.[a]

| | $k_{cat}(s^{-1}) \times 10^4$ | |
|---|---|---|
| | elastase | cathepsin G |
| Ac-Ala-Ala-Aval-ONp | 3500 | <0.8 |
| Ac-Ala-Ala-Aile-ONp | 59 | no reaction |
| Ac-Ala-Ala-Aala-ONp | 20 | no reaction |
| Ac-Ala-Ala-Anva-ONp | 14 | no reaction |
| Ac-Ala-Ala-Aleu-ONp | <1.9 | <0.8 |
| Ac-Ala-Ala-Anle-ONp | <1.9 | <0.8 |
| Ac-Ala-Aphe-ONp | -- | 33 |

[a]pH = 6.0, 0.1 M citrate. Aval = $-NHN(CH(CH_3)_2)CO-$; Aile = $-NHN(CH(CH_3)CH_2CH_3)CO-$; Aala = $-NHN(CH_3)CO-$; Anva = $-NHN(CH_2CH_2CH_3)CO-$; Aleu = $-NHN(CH_2CH(CH_3)_2)CO-$; Anle = $-NHN(CH_2CH_2CH_2CH_3)CO-$; Aphe = $-NHN(CH_2C_6H_5)CO-$.

The rates of Ac-Ala-Ala-Pro-X-p-nitroanilide hydrolysis (Zimmerman and Ashe, 1977) can be compared to the $k_{cat}$ values in Table 4. With the p-nitroanilides the order of reactivity was X=Val>Ile>Ala>Leu, while with aza-peptides the $k_{cat}$ values followed the same pattern of Aval>Aile>Aala>Aleu. The confirms our expectation that aza-peptides are interacting with elastase in a manner similar to substrates.

Cathepsin G was also inhibited by several aza-peptides (Table 4). Some specificity is observed since the Aile, Aala and Anva peptides will not react with cathepsin G, while Ac-Ala-Aphe-ONp reacts only very slowly with elastase. We believe it will be possible to increase the specificity of the aza-peptides by substituting other groups for the p-nitrophenyl leaving group.

Aza-peptide p-nitrophenyl esters can also be used as active site titrants for granulocyte proteases. A "burst" of p-nitrophenol is released in the reaction and under the proper conditions this is stoichiometric with the normality of active sites in the enzyme solution. In particular we have utilized Ac-Ala-Ala-Aala-ONp to titrate granuolcyte elastase and Ac-Ala-Ala-Anle-ONp to titrate cathepsin G.

Significance and Prospectives. A number of different types of inhibitors for granulocyte proteases are now available and new inhibitor structures such as cis-unsaturated fatty acids (Ashe and Zimmerman, 1977) are being uncovered. Some of the inhibitors are quite specific. There is considerable potential for the use of synthetic elastase inhibitors for the treatment of emphysema. They have the advantage of being readily synthesizable and may be able to diffuse to cellular sites which are inaccessible to larger molecules. In addition there is the prospect of relatively direct application at the site of tissue destruction via aerosol.

In the last 18 months we have shipped samples of our inhibitors to 50 other investigators. Several of these investigators have begun studies with elastase inhibitors in animal models of emphysema. There is a good possibility that the course of disease could be arrested by the use of the appropriate inhibitor. Whether lung healing would also occur is an unanswered question. Protease inhibitors could also be used to prvent the occurrence of the disease in high risk groups such as smokers. At present an obvious and urgent need is for more animal testing to evaluate the therapeutic possibilities

of inhibitors of granulocyte proteases. Only in this way can the limitations of the currently available protease inhibitors be uncovered so that more ideal reagents will be available in the future.

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Zimmerman, M.; Ashe, B. M.: Substrate Specificity of the Elastase and the Chymotrypsin-like Enzyme of the Human Granulocyte. Biochim. Biophys. Acta 480 (1977) 241-245

PUBLICATION LIST

a. Papers and Book Chapters Published since last report

"Inhibitors of Elastase and Pulmonary Emphysema," James C. Powers, Trends in Biochemical Sciences, 1, 211 (1976).

"Modification of Chymotrypsin with the Specific Cyanate Reagent N-Acetyl-p-Cyanato-L-Phenylalanine Ethyl Ester," James C. Powers, Peter M. Tuhy and Frank Witter, Biochem. Biophys. Acta, 445, 426 (1976).

"Inhibition of Subtilizin BPN' with Peptide Chloromethyl Ketones," James C. Powers, Mark O. Lively, III and James T. Tippett, Biochem. Biophys. Acta, 480, 246 (1977).

"Haloketone Inhibitors of Proteolytic Enzymes," James C. Powers, Chap. in Weinstein (ed.), "Chemistry and Biochemistry of Amino Acids, Peptides and Proteins," Vol. 4, Marcel Dekker, New York, pp 65-178, 1977.

"Reaction of Serine Proteases with Halomethyl Ketones," James C. Powers, Chap. in W. Jakoby and M. Weilchek (eds.), Meth. in Enzymology, Academic Press, Vol. 46, pp 197-208, 1977.

"Reaction of Serine Protease with Aza-Amino Acid and Aza-peptide Derivatives," James C. Powers and B. Frank Gupton, Chap. in W. Jakoby and M. Weilchek (eds.), Meth. in Enzymology, Academic Press, Vol. 46, pp 208-216, 1977.

b. Published Abstracts

"Inhibition of Elastase by Aza Amino Acid Derivatives," James C. Powers, Peter M. Tuhy, David L. Carroll, Norkazu Nishino and B. Frank Gupton, Fed. Proc., Fed. Am. Soc. Exp. Biol., 35, 1463 (1976).

"Studies on the Inhibition of Thermolysin with Phosphoramidates of Peptides and Amino Acids," Chih-Min Kam and James C. Powers, Fed. Proc., Fed. Am. Soc. Exp. Biol., 36, 766 (1977).

c. Papers in Press

"Specificity of Porcine Elastase, Human Leukocyte Elastase and Cathepsin G. Inhibition with Peptide Chloromethyl Ketones," Biochem. Biophys. Acta, in press.

"The Role of Chymotrypsin-like Protease (cathepsin G) of Human Polymorphonuclear Leukocytes in the Digestion of E. coli Proteins by Living Cells," Joanne Blondin, Aaron Janoff and James C. Powers, Proc. Exp. Soc. Biol. Med., in press.

"Subsite Specificity of Porcine Pepsin," James C. Powers, A. Dale Harley and Dirck V. Meyers, Chap. 9 in J. Tang (ed.), "Acid Proteases, Structure, Function and Biology," Plenum Press, New York, in press, 1977.

"Synthetic Inhibitors of Elastase and Cathepsin G," James C. Powers, Chap. in A. Janoff and K. Havemann (eds.), Neutral Proteases of Human Polymorphonuclear Leucocytes, Urban and Schwarzenberg, in press, 1978.

d. Future Publications

"Subsite Specificity of Chymotrypsin" - final draft written - to be submitted to Biochem.

"Subsite Specificity of Thermolysin - first draft written - to be submitted to Biochem.

"Subsite Specificity of Pepsin" - first draft written - to be submitted to Biochem.

"Subsite Specificity of Papain" - first draft written - to be submitted to Biochem.

"Reaction of Aza-peptides with Elastase" - data complete - but no drafts written.

"Reaction of Aza-peptides with Chymotrypsin and Subtilisin" - data complete - but no drafts written.

GEORGIA INSTITUTE OF TECHNOLOGY
ATLANTA, GEORGIA 30332

OFFICE OF
THE DIRECTOR OF
FINANCIAL AFFAIRS

November 30, 1977

National Institutes of Health
Div. of Financial Management
Grants Section, FAAB
Westwood Bldg., Room 405
5333 Westbard Avenue
Bethesda, Md. 20014

Gentlemen:

Enclosed is the Final Report of Research Grant Expenditures for Grant No. 5 ROI GM18292-05 covering the period June 1, 1976 through May 31, 1977.

If you have questions or desire additional information, please let us know.

Sincerely yours,

Evan Crosby
Associate Director of
Financial Affairs

EC/bs
Enclosure as stated
cc: Dr. J. A. Bertrand
Dr. J. C. Powers
Mr. E. E. Renfro
Mr. A. H. Becker
File G-33-681

U.S. GOVERNMENT PRINTING OFFICE 1977-234-057

Department of Health, Education, and Welfare

Grant No.
5 ROI GM18292-05

E AND ADDRESS OF GRANTEE INSTITUTION
orgia Institute of Technology
lanta, Ga. 30332

TRANSACTION NO.
(08)RIGMI8292A

INSTITUTIONAL ID NO.
G-33-681

DATE OF THIS REPORTING PERIOD
FROM 6/1/76 TO 5/31/77

PROJECT PERIOD
FROM 6/1/74 TO 5/31/77

☒ CHECK IF FINAL REPORT

xpenditures of DHEW Funds for this Reporting Period

| | | | |
|---|---|---|---|
| Personnel | $ 14,077.23 | h. Alterations and renovations | |
| Consultant services | 471.00 | i. Other | |
| Equipment | 1,377.04 | Publications | 86.42 |
| Supplies | 6,089.22 | j. Total direct costs | 22,835.25 |
| Travel, domestic | 734.34 | k. Indirect costs: Rate 68 % ☒ S&W ☐ TDC Base $ 13,522.96 | |
| Travel, foreign | | | 9,195.61 |
| Patient care costs | | l. TOTAL | $ 32,030.86 |

| | |
|---|---|
| xpenditures from Prior Periods (previously reported) | 61,427.00 |
| umulative Expenditures | 93,457.86 |
| otal Amount Awarded — Cumulatively | 94,207.00 |
| nexpended Balance (Item 4 less Item 3) | 749.14 |
| nliquidated Obligations | -0- |
| nobligated Balance (Item 5 less Item 6) | 749.14 |
| Cost Sharing Information — Grantee Contribution This Period | 8,855.00 |
| % of Total Project Costs (Item 8a divided by total of Items 1 and 8a) | % 21.66 |
| Interest/Income (enclose check) | |
| Other Refundable Income (enclose check) | |

Remarks

by certify that this report is true and correct to the best of my knowledge, and that all expenditures reported herein have been made in dance with appropriate grant policies and for the purposes set forth in the application and award documents.

Dr. J. C. Powers
Evan Crosby
SIGNATURE OF INSTITUTION OFFICER

11/28/77
11/30/77
DATE

oc. Dir. of Financial Affairs

489 (REV. 10/73)

REPORT OF RESEARCH GRANT EXPENDITURES

OMB 85R0219