

Portland State University

**PDXScholar**

---

Dissertations and Theses

Dissertations and Theses

---

5-1-1970

# Nutritional requirements for protease production by *Pseudomonas aeruginosa*, Ps-1C

Richard Myer Avedovech  
*Portland State University*

Follow this and additional works at: [https://pdxscholar.library.pdx.edu/open\\_access\\_etds](https://pdxscholar.library.pdx.edu/open_access_etds)

**Let us know how access to this document benefits you.**

---

## Recommended Citation

Avedovech, Richard Myer, "Nutritional requirements for protease production by *Pseudomonas aeruginosa*, Ps-1C" (1970). *Dissertations and Theses*. Paper 629.

<https://doi.org/10.15760/etd.629>

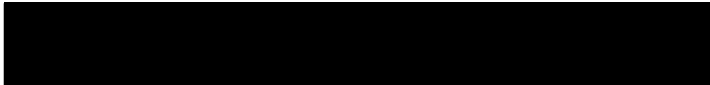
This Thesis is brought to you for free and open access. It has been accepted for inclusion in Dissertations and Theses by an authorized administrator of PDXScholar. Please contact us if we can make this document more accessible: [pdxscholar@pdx.edu](mailto:pdxscholar@pdx.edu).

AN ABSTRACT OF THE THESIS OF Richard Myer Avedovech Jr. for the Master of Science in Biology presented May 22, 1970.

Title: Nutritional Requirements for Protease Production by Pseudomonas aeruginosa, Ps-1C

APPROVED BY MEMBERS OF THE THESIS COMMITTEE:

  
Earl Fisher, Jr., Chairman

  
W. Herman Taylor

  
John W. Myers

Pseudomonas aeruginosa Ps-1C produces an extracellular proteolytic enzyme which from preliminary studies appears to be inducible, and responsible for corneal destruction in injured eyes.

In the present study the nutritional requirements for this bacterium to produce the proteolytic enzyme(s) were investigated.

Preliminary studies indicated that proteose peptone offered the required nutrients for good enzyme production. The separation of the components of proteose peptone by Sephadex G-10 and Sephadex G-75 descending column chromatography was undertaken to illucidate the nutritional requirements.

It was also noted that caseamino acids hydrolysate served as a good

substrate for Pseudomonas aeruginosa to produce this enzyme. The separation of amino acid groups was undertaken using paper and Geon electrophoresis and various types of thin layer chromatography. The three amino acids found to be required for good protease production were, phenylalanine, isoleucine, and valine in their respective concentrations of 0.5 mg/ml, 1.0 mg/ml, and 2.0 mg/ml. Isoleucine was found to be inhibiting at higher concentrations. Dextrose also inhibited protease production, but not growth, at concentrations greater than 0.05%.

Divalent metal ions in varying concentrations were tested as nutritional requirements for enzyme production. Magnesium ion provided very good enzymatic activity at a concentration of 0.01 M, whereas cobalt, copper, calcium and zinc ions did not allow appreciable enzyme activity and even in some cases were inhibitive.

NUTRITIONAL REQUIREMENTS FOR PROTEASE PRODUCTION BY  
PSEUDOMONAS AERUGINOSA, PS-1C

by

RICHARD MYER AVEDOVECH, JR.

A thesis submitted in partial fulfillment of  
the requirements for the degree of

MASTER OF SCIENCE  
in  
BIOLOGY

Portland State University  
1970

TO THE OFFICE OF GRADUATE STUDIES:

The members of the Committee approve the thesis of  
Richard Myer Avedovech, Jr. presented May 21, 1970.

[REDACTED]  
Earl Fisher, Jr., Chairman

[REDACTED]  
Herman Taylor

[REDACTED]  
John W. Myers

APPROVED: [REDACTED]

[REDACTED]  
Earl Fisher, Jr., Head, Department of Biology

[REDACTED]  
Frank L. Roberts, Acting Dean of Graduate Studies

May 21, 1970

## TABLE OF CONTENTS

	PAGE
LIST OF TABLES . . . . .	vi
LIST OF FIGURES . . . . .	viii
INTRODUCTION . . . . .	1
Definition of Terms . . . . .	1
Background . . . . .	1
Clostridial Collagenase	
Other Nutritional Studies	
Role of Amino Acids . . . . .	7
Inhibition . . . . .	8
METHODS AND MATERIALS . . . . .	10
Organism . . . . .	10
Starter Cultures . . . . .	10
Culture Media . . . . .	10
Proteolytic Assay for Ps-1C Protease . . . . .	11
Glucose Inhibition Determination . . . . .	11
Proteose Peptone Analysis . . . . .	13
Preparation of Proteose Peptone for Substrate Testing	
Casamino Acid Analysis . . . . .	14
Preparation of Casamino Acids for Substrate Testing	
Paper electrophoresis of Casamino Acids	

Determination of the Effect of $10^{-4}$ % Casamino Acid Dialysate for Substrate Testing	
Geon Electrophoresis of Casamino Acid Dialysate	
Chromatography of Casamino Acids	
Specific Amino Acid Analysis . . . . .	18
Amino Acid Combinations Tested as Nutritional Requirements	
Amino Acid Concentration Requirement	
Determination of Dextrose Concentration and Repression . . . . .	21
Growth Curves. . . . .	22
Growth Curve Vs. Optical Density at $\lambda 660$ nm	
Growth and Protease Production Versus Time	
Divalent Metal Ion Concentration Requirement . . . . .	26
RESULTS . . . . .	27
Glucose Inhibition Determination . . . . .	27
Protease Peptone Analysis . . . . .	27
Casamino Acid Analysis . . . . .	31
Amino Acid Combinations Tested as Substrate for Ps-1C Protease Production	
Divalent Metal Ion Requirement . . . . .	49
Growth Curves. . . . .	49
Growth Curve of <u>Pseudomonas aeruginosa</u> Ps-1C as Correlated to Optical Density at Wavelength 660 nm	
Growth and Protease Production Vs. Time	

DISCUSSION . . . . .	63
Glucose Inhibition of Protease Production. . . . .	63
Protease Peptone Analysis . . . . .	63
Amino Acid Analysis . . . . .	63
Divalent Metal Ion Requirement . . . . .	65
Enzyme Production Versus Growth . . . . .	65
SUMMARY . . . . .	67
REFERENCES . . . . .	68



# LIST OF TABLES

TABLE		PAGE
I	Hershey's M-9 Growth Medium . . . . .	12
II	Chromatography Systems Used on Casamino Acids . . . .	19
III	Growth Curve of <u>Pseudomonas aeruginosa</u> versus Optical Density at Wavelength 660 nm . . . . .	23
IV	Casamino Acid Dialysate Substrate Experiment . . . . .	34
V	Substrate Tests of Casamino Acids, Geon Electrophoresis Pools . . . . .	37
VI	Substrate Tests of Casamino Acids, Geon Electrophoresis II, Sephadex G10 Pools . . . . .	40
VII	Substrate Tests of General Amino Acid Groups for Enzyme Production . . . . .	50
VIII	Amino Acids Combinations-I Tested as Nutritional Requirements for Ps-1C Protease Production . . .	51
IX	Amino Acid Combinations-II Tested as Nutritional Requirements for Ps-1C Protease Production . . .	52
X	Amino Acid Combinations-III Tested as Nutritional Requirements for Ps-1C Protease Production . . .	53
XI	Amino Acid Combinations-IV Tested as Nutritional Requirements for Ps-1C Protease Production . . .	54
XII	Isoleucine Concentration Requirement for Ps-1C Protease Production . . . . .	55

## TABLE

## PAGE

XIII	Phenylalanine Concentration Requirement for Ps-1C Protease Production . . . . .	56
XIV	Valine Concentration Requirement for Ps-1C Protease Production . . . . .	57
XV	Tests for Inhibition by Excess Required Amino Acid	58

# LIST OF FIGURES

FIGURE		PAGE
1	Schematic of the breakdown of casamino acids for determination of amino acid requirements for Ps-1C protease production . . . . .	20
2	Dextrose concentration determination for the best Ps-1C protease production . . . . .	28
3a	Sephadex G10 column chromatography of 10% proteose peptone . . . . .	29
3b	Sephadex G75 column chromatography of proteose peptone, Sephadex G10, Pool I . . . . .	30
3c	Sephadex G75 column chromatography of 10% proteose peptone . . . . .	32
3d	Sephadex G10 column chromatography of 10% proteose peptone, sephadex G75, pool II . . . . .	33
4	Geon electrophoresis of casamino acids using a 0.05 M phosphate buffer, pH 6.02 . . . . .	36
5	Standard spot-o-gram for casamino acids, Geon electrophoresis II, sephadex G10 fractions . . .	38
6	One dimensional paper chromatography of the five pools from sephadex G10 treatment of casamino acids, Geon electrophoresis pool II . . . . .	41
7	One dimensional thin layer chromatography of casamino acids, Geon electrophoresis II, sephadex G10 II	42

## FIGURE

## PAGE

8	One dimensional thin layer chromatography of casamino acids, Geon electrophoresis II, sephadex G10 II . .	44
9	One dimensional thin layer chromatography of casamino acids, Geon electrophoresis II, sephadex G10 II . .	45
10	Two dimensional, thin layer chromatography of casamino acids, Geon electrophorsis II, sephadex G10 II . .	46
11	Two dimensional thin layer chromatography of casamino acids, Geon electrophoresis II, sephadex G10 II . .	47
12	Two dimensional thin layer chromatography of casamino acids, Geon electrophoresis II, sephadex G10, pool II . . . . .	48
13	Comparison of metal ion concentration requirements for Ps-1C protease production . . . . .	59
14	Growth versus optical density at wavelength 660 nm, for <u>Pseudomonas aeruginosa</u> , Ps-1C in nutrient broth-yeast extract. . . . .	60
15	Growth and protease production versus time in <u>Pseudomonas aeruginosa</u> , Ps-1C . . . . .	62

## INTRODUCTION

The purpose of this work is to determine some of the nutritional requirements for the bacterium Pseudomonas aeruginosa to produce a proteolytic enzyme(s) which is apparently inducible, and possibly has collagenase activity.

### Definition of Terms

Proteolytic enzymes or proteinases have been defined as enzymes that can hydrolyze a peptide bond when it is situated within a large molecule, (Thimann, 1963). Proteolytic enzymes include the serine proteinases such as chymotrypsin, trypsin, elastase, and thrombin; the cysteine proteinases such as papain, ficin, and bromelain; the metal containing peptidases such as the aminopeptidases and carboxypeptidases; and peptidases active at acid pH's such as pepsin and rennin, (Bender and Kezdy, 1965). The enzyme of this study probably falls under the category of a metal containing protease.

An operational definition or classification of an extracellular enzyme is that it must demonstrate that the enzyme occurs in the medium, separated from the cells, and that the appearance of the enzyme in the medium does not depend upon irreversible damage to the cell structure. (Pollock, 1962).

Induced enzyme formation is when increases in enzyme activity correspond to increases in the amount of an enzymically active protein, after exposure of the cells with the specific substrate or substances chemically closely related to the substrate. Specific inducer molecule(s)

is shown to be necessary at some stage in the chain of events leading to formation of a particular enzyme.

Maschmann (1938) proposed the name collagenase for the extracellular gelatin-splitting enzyme. However, Jennison (1945) found several species of bacteria which cleave gelatin but are unable to attack collagen in the form of untreated beef tendon (tendo Achillis), and finely fibrous, highly purified collagen from steer hide; therefore he restricts the name collagenase to the "collagen-splitting" enzyme.

#### Background

Narayanan, Devi, and Menon (1953) tested various isolates of Vibrio cholera and other organisms including a pseudomonad (Pseudomonas pyocyanea) for the enzymatic activity of collagenase, elastinase, and mucinase. The collagen substrate was buffalo tendo Achillis. It was noted that the pseudomonad had appreciable collagenase and elastinase activity.

In 1958, Fisher and Allen (1958) determined that cell-free extracts of Pseudomonas aeruginosa produced corneal ulcers. A proteolytic enzyme fraction was obtained and partially purified which enhanced both proteolytic activity and severe corneal destruction of rabbit eyes. It was inferred that growth of the organisms in the presence of organic materials, probably proteins, is essential for proteolytic enzyme production in corneal destruction.

Neither corneal destructive factor nor protease enzyme activity could be detected when the organism was grown in a synthetic medium. This suggests that the protease is an adaptive (inducible) enzyme

requiring the presence of an appropriate protein substrate before the enzyme is operative.

Later, Shoellmann and Fisher (1966) provided evidence that this enzyme from Pseudomonas aeruginosa which may be responsible for the corneal damage is probably a collagenase. It is known that in clostridial collagenase, only the amino acid sequence of the general structure (amino group)-proline-r-glycine-proline-(carboxyl group) are susceptible to enzyme hydrolysis by cleavage of the r-gly bond. A synthetic substrate of carbobenzocy-gly-pro-gly-gly-pro-alanine was used since if a collagenase is present this hexapeptide should be split at the gly-gly- bond forming cbz-gly-pro-gly-/ and gly-pro-alanine. Silica gel thin layer chromatography demonstrated that these expected peptides did occur. Parallel experiments with the collagenase from Clostridium histolyticum also cleaved the hexapeptide at the identical bond.

Clostridial Collagenase. Using hide powder disintegration as a general indicator for collagenase action it was determined that enzymes produced by various clostridial species which affect hide powder is not related to pathogenicity but possibly is associated with muscle destruction, (Evans, 1948).

Thimann (1963) states that the collagenase of Clostridium histolyticum is the most active of all collagenases, and is activated both by cysteine and by  $\text{FeSO}_4$ . Also that there are several aerobes that have proteinases that are inhibited by cysteine but activated by  $\text{Fe}^{++}$  or by cysteine plus  $\text{Fe}^{++}$ . However it is now known that Clostridium histolyticum forms at least two physicochemically and enzymically distinct extracellular proteinases, one of which can be regarded as a

specific collagenase which is not activated by  $\text{Fe}^{++}$  and cysteine, (Pollock, 1962). This differs from the proteinases studied by Kochalaty and Krejci (1948).

The  $\kappa$ -toxin of Clostridium welchii is specific for collagen and gelatin which has a high content of proline and hydroxyproline. However the narrow specificity of collagenase does not appear to be connected with these amino acids since it does not seem to have prolidases nor prolinase activity. (Bidwell, van Heyningen, and Charlwood, 1948).

Bidwell (1948) also noted that "true" collagenase began to be unstable in the cold at pH 8.5 - 9.0 and was completely destroyed at pH 10.3. Warmer temperatures accelerated the destruction.

It has been shown that the collagenase from Clostridium histolyticum requires  $\text{Ca}^{++}$  for activation. This requirement has been tested by the removal of metal ions through dialysis and by treatment with the chelating agent Na-EDTA which inactivates the enzyme. Complete enzyme activity is restored by adding back  $\text{Ca}^{++}$ , whereas  $\text{Mg}^{++}$  did not activate nor inhibit the enzyme. It appears that the  $\text{Ca}^{++}$  is required for collagenase to be able to adsorb to a suspension of ichthyocol. (Gallop, Seifter, and Meilman, 1957)

There may be more than one metal involved in collagenase activity. Seifter, Gallop, Klein, and Meilman (1959) determined that the purified enzyme is inhibited by sulfhydryl-containing agents, where the effect of these compounds seem to be on a non-calcium metal component of collagenase.

Mandl, Keller, and Manahan (1964) obtained from Clostridium histolyticum collagenase preparation two distinct collagenolytic enzyme fractions. Collagenase I will not attack unspecific substrate azocoll.



and has minimal activity against gelatin; is not associated with proteolytic activities of crude collagenase, and has a high specific rate of synthetic substrate hydrolysis. Collagenase II is equally free of non-specific activities, but is very active against both azocoll and gelatin.

Both enzymes require  $\text{Ca}^{++}$  for stability as well as for activity. EDTA (ethylenediaminetetracetate) and exhaustive dialysis irreversibly inactivates the purified collagenase, but in the more stable crude form, they can be reactivated by addition of  $\text{Ca}^{++}$ .

Other Nutritional Studies. Keen and Williams (1967) did a nutritional study on Pseudomonas lachrymans and its production of a constitutive extracellular protease which is involved with the pathogenicity of angular leaf spot on cucumbers. The bacterium requires an organic form of nitrogen for growth in culture. Bacteria grown on synthetic media supplemented with 0.5% concentration of gelatin, casein, peptone, or lactalbumin grew better than non-supplemented control cultures. Gelatin and peptone slightly stimulated protease production, whereas casein and lactalbumin resulted in little or no production. Growth rates were greater in aerated cultures than in non-aerated cultures and protease activities were considerably higher in the aerated cultures.

There was no significant protease activity detected in cultures at 0.01% glutamic acid, but activity did correlate to growth at glutamic acid concentrations between 0.05% and 0.2%. At glutamic acid concentrations above 0.2%, however, enzyme synthesis was apparently repressed.

When bacteria were grown on a synthetic media with 0.2% glutamic acid and various concentrations of sucrose, growth increased proportionally with sucrose concentration. At 10% sucrose with 0.2% glutamic acid, growth was highest, but protease production was lowest. When the media

contained 1% glutamic acid, the maximum protease synthesis was attained at 0.2% sucrose concentration, whereas the growth increased up to the 5% sucrose level.

No growth or enzyme synthesis was observed when solely inorganic nitrogen was supplied to the bacteria. In cultures supplied with 0.16% glutamic acid in combination with inorganic nitrogen, better growth occurred than when glutamic acid was supplied alone.

Bacterial growth was limited by various concentrations of glutamic acid, inorganic nitrogen, or sucrose; but only glutamic acid indicated a consistent correlation with protease production. However, high concentrations of glutamic acid apparently repressed enzyme production and the repression was more pronounced when glutamic acid was supplemented with sucrose or inorganic nitrogen. These facts suggest that rapid assimilation of carbon by the bacteria represses protease production and counteracts the stimulation of synthesis by organic nitrogen.

Apparently it is rather common for extracellular enzymes to require a divalent ion as was demonstrated earlier with the clostridial collagenase. Casa and Zimmerman (1969) determined that an extracellular protease from Streptococcus faecalis var. liquefaciens requires  $Zn^{++}$ , for the secretion of this enzyme was inhibited by EDTA. If  $Zn^{++}$  is added back within a 45 minutes period, then a fraction of the enzyme activity is restored. Various results indicate that the energy for this proteolytic enzyme synthesis is apparently from arginine which is also required to stimulate proteolytic activity after 60 minutes of EDTA treatment.

The early works of Berger, Johnson, and Peterson (1938 a,b) have

shown that the four gram negative rods, E. coli, Proteus, sp., Pseudomonas fluorescens, and Pseudomonas tumefaciens, produced peptidases which all were activated by  $Mg^{++}$ .

Litchfield and Prescott (1970) working with proteolytic enzyme production by Aeromonas proteolytica determined that the amino acids asparagine, histidine, glutamic acid, alanine and proline could individually support appreciable growth and enzyme synthesis. Asparagine and histidine, individually and as a mixture of the two, allowed good enzyme elaboration.

When glycerol was present, there was a decrease in endopeptidase production. Glucose, sucrose, and acetate also caused a reduction in enzyme synthesis, although growth was not enhanced.

When acid-hydrolyzed casein was used as the carbon, nitrogen and energy source, proteinase production was twice that obtained in 0.2% asparagine.

Marvin, et. al. (1969) isolated and purified an exoenzyme, which was bacteriolytic on Staphylococcus aureus, from Pseudomonas aeruginosa. This enzyme is stabilized in the presence of  $Mg^{++}$ , and acts as an endopeptidase on peptidoglycans with poly-glycine bridges.

#### Role of Amino Acids

Sometimes amino acid analogues can stimulate enzyme production, although the analogue will not be detected in purified enzyme samples, (Hammel and Zimmerman, 1966).

It has been shown with Pseudomonas aeruginosa that a number of amino acids can serve as a good nitrogen source and/or as a good carbon source. Kay and Gronlund (1969) observed that alanine, arginine, aspar-

agine, aspartate, glutamate, glutamine, glycine, isoleucine, leucine, proline, serine, tryptophane, tyrosine, and valine were good nitrogen sources. Among those amino acids which served as good carbon sources were included isoleucine and valine.

Norton and Sokatch (1966) working with the oxidation of D-and L-valine by enzymes of Pseudomonas aeruginosa determined that D-valine was oxidized directly to 2-oxo-isovalerate. L-valine was deaminated by transamination with 2-oxoglutarate. It was not possible to demonstrate either the direct oxidation of L-valine or the conversion of L- to D-valine.

Sokatch (1966) did labeling experiments which indicated that Pseudomonas aeruginosa catabolized DL-valine by oxidation to isobutyrate and propionate. Alanine formed during growth on DL-valine-4-4'-C<sup>14</sup> was labeled in carbons 1 and 3, which suggests the direct oxidation of propionate derived from the isopropyl carbons of valine to pyruvate for alanine biosynthesis.

#### Inhibition

Working with an acid proteinase from Pseudomonas aeruginosa, Morihara (1963) determined that most heavy metal ions and chelating agents would inactivate the enzyme. This indicates that a divalent ion may be required. Also oxidizing agents inactivated the proteolytic activity, although sulfhydryl groups are not necessarily essential for enzymic activity.

For the proteolytic enzymes of Clostridium welchii, Bidwell (1950) determined that citrate strongly inhibited the enzyme, and that there was no evidence for activation by Ca<sup>++</sup> or Mg<sup>++</sup>. The enzyme was slightly

inhibited by iodoacetic acid, and cyanide, and strongly inhibited by cysteine.

Five amino acids, isoleucine, leucine, phenylalanine, tryptophane, and valine, specifically and individually repressed the formation of the induced extracellular protease of Streptococcus faecalis var. liquefaciens. The protease, nor synthesis of other enzymes, nor growth of the organism were adversely affected by the presence of these amino acids.

It has also been noted by Somkuti and Babel (1967) in their studies on Mucor pusillus that there is an inverse relationship between the amount of proteinase and the amount of glucose present in the culture media.

Concerning the enzyme of this study, it has previously been demonstrated that this proteinase is inhibited by thiol groups, cysteine, glutathione, mercaptoacetate and mercaptoethanol. Sodium iodoacetate inhibits enzyme production while chelating agents greatly reduced enzymatic activity. This inhibition could not be relieved by the addition of various metal ions, (Fisher, 1960).

## METHODS AND MATERIALS

### Organism

The organism used in this study is Pseudomonas aeruginosa, Ps-1C, obtained from the stock cultures of Dr. E. Fisher, Jr., Portland State University Biology Department. This strain was originally isolated by Dr. Fisher from a corneal infection of a patient at Tulane Medical School. The Ps-1C strain has been steadily maintained on blood agar plates, prepared from whole sheep blood and Difco Bacto Blood Agar Base.

### Starter Cultures

Starter cultures for various experiments inoculated from a blood agar stock culture plate. One loop of Pseudomonas aeruginosa Ps-1C was transferred into 50 mls of sterile medium containing nutrient broth-yeast extract in a 150 ml Erlenmeyer flask stoppered with a cotton plug. This was then put on a Gyrotory Shaker, Model G-25, New Brunswick Scientific Co., for 12 - 18 hours, at 36 - 39° C, and the shaker speed set at 6.5. At the end of the incubation time the number of cells per ml of culture is determined by reading the optical density of the culture at 660 nm in a Gilford 2000 Automatic Spectrophotometer, and comparing the reading to a previously determined plot of viable cells versus optical density at 660 nm.

### Culture Media

The complete medium was composed of dehydrated 0.8% (w/v) nutrient broth, and 0.5% (w/v) yeast extract. The components are dissolved and sterilized in an American Sterilizer Autoclave, Model 57 CR, for 15 minutes at 250° C. These conditions were used consistently for the

sterilization of all media and equipment used where sterilization was required.

The synthetic salts medium used throughout this study was Hershey's M-9 minimal salts medium containing the salts listed in Table I, in which part A was mixed and sterilized as a unit, and in part B the individual solutions were separately sterilized and then added aseptically to part A.

#### Proteolytic Assay for Ps-1C Protease

The proteolytic activity was followed by means of a colorimetric procedure which depends on the enzymatic release of colored non-trichloroacetic acid (TCA) precipitated products from a diazotized protein. The method of diazotization was essentially that of Tomarelli, Charney, and Harding (1949). The method of assay is essentially that of Fisher and Allen (1958) with a slight modification.

The incubation mixture was composed of the following: 0.1 ml sample to test for enzyme activity; 0.9 ml distilled water; 0.5 ml of 0.05 M tris buffer (Tris(hydroxymethyl)aminomethane), pH 7.2; and 0.5 ml of 1% diazotized casein. This mixture was incubated in a water bath at 37° C for 10 minutes. The reaction was stopped by the addition of 5 ml of 5% TCA. The acidified mixture was filtered to remove the precipitated proteins and the color was intensified by adding 2.5 ml of 0.5 N NaOH to 2.5 ml of filtrate. The optical density was read at wavelength 420 nm on a Bausch and Lomb Spectronic 20 Colorimeter.

#### Glucose Inhibition Determination

Approximately  $10^9$  cells were inoculated into 50 ml aliquots of Hershey's M-9 medium, containing 10% Difco Bacto Proteose Peptone, but

TABLE I  
HERSHEY'S M-9 GROWTH MEDIUM

<u>Material</u>	<u>Amount</u>
Part A.	
Distilled water	992 ml
$\text{Na}_2\text{HPO}_4 \cdot 12 \text{ H}_2\text{O}$	15.0 g
(or)	
$\text{Na}_2\text{HPO}_4$	10.87 g
$\text{KH}_2\text{PO}_4$	3.0 g
$\text{NH}_4\text{Cl}$	1.0 g
Part B.	
1M $\text{MgSO}_4$	1.0 ml
$10^{-3}\text{M}$ $\text{FeCl}_3$	1.0 ml
0.1 M $\text{CaCl}_2$	1.0 ml
10% dextrose	5.0 ml



without dextrose. Protease activity was tested for in the above medium plus in media containing dextrose in the concentrations of 5%, 0.5%, 0.05%, 0.005%. Two controls were used: the first contained 10% proteose peptone, but no dextrose; the second contained no proteose peptone, but had 5% dextrose. A third flask contained only Hershey's M-9 medium without either dextrose or proteose peptone. An earlier study in this laboratory indicated that proteose peptone was a good non-specific medium for protease production by Pseudomonas aeruginosa, Ps-1C.

Test cultures were incubated for 14 - 16 hours on an incubator shaker water bath at 37° C, and were then assayed for proteolytic activity.

#### Proteose Peptone Analysis

Preparation of Proteose Peptone For Substrate Testing. Since proteose peptone provided a good substrate for the pseudomonal synthesis of the enzyme, it was thought that perhaps various components of proteose peptone could be separated and tested as inducers for protease formation. This might possibly give some general information as to the categories these nutritional requirements may belong.

Difco's Bacto Proteose Peptone, lot number 492472 was used throughout this experiment. A 5% (w/v) proteose peptone solution was dialyzed against large volumes of cold distilled water for two days, with changes of water twice a day. The dialysate remaining in the dialysis bag was lyophilized.

A 10% solution of this lyophilized proteose peptone in distilled water was percolated through a descending sephadex G-10 gel column, with

0.05M tris buffer, pH 7.5 as the eluant. Three ml aliquots were collected with a Gilson GME Volumetric Linear Fractionator, Model VL. Each fraction was read for optical density at wavelengths 260 and 280 nm with the Gilford 2000 Spectrophotometer. Fractions were pooled which represented the respective peaks, and labeled as pool I - pool V.

Five ml samples from each of the respective pools were added to 45 ml aliquots of Hershey's M-9 medium containing 0.05% dextrose. Each of these test flasks including a control flask with no sample were inoculated with Pseudomonas aeruginosa Ps-1C from a starter culture, incubated for eighteen hours and assayed for protease production.

Since the proteose peptone, (seph. G-10, I) from pool I indicated the best activity when tested as a substrate, this pool was lyophilized, and then a 10% solution was prepared. Five mls of this 10% solution was then percolated through a Sephadex G-75 gel column, and again three ml fractions were collected and read at wavelengths 260 and 280 nm with the spectrophotometer. Three peaks were indicated and the respective fractions were pooled and labeled as: Proteose peptone, Seph G-10 I, Seph G-75 (I, II, or III).

The above three pools from the sephadex G-75 column were tested as substrate possibilities for Ps-1C protease elaboration as were the previous pools, except that a flask containing 1% Bacto, Vitamin-Free Casamino Acids was also used as well as a flask containing 1% proteose peptone in the crude, undialyzed form.

#### Casamino Acid Analysis

Preparation of Casamino Acids for Substrate Testing. A 10% solution (w/v) of Difco Bacto Vitamin-Free Casamino Acids in distilled

water was dialyzed against distilled water in the cold for 24 hours.

Both the solution remaining in the dialysis tube and the outside dialysis water were lyophilized. One gram of each of these lyophilized portions was added to its respective flask containing 100 ml of Hershey's M-9 medium with 0.01% dextrose. These, along with controls were inoculated with Pseudomonas aeruginosa Ps-1C from a starter culture, incubated for 16 hours and assayed for proteolytic activity.

Paper Electrophoresis of Casamino Acids. One gram of casamino acid dialyzed-out material was added to 100 ml of 0.05 M tris buffer, pH 7.11 to serve as a stock sample to be tested on paper electrophoresis. A sheet of Whatman # 3M paper was presoaked in 0.05 M phosphate buffer at the pH of the desired run to be tested. The paper was placed on a plastic sheet laying on a metal plate cooled by circulating cold tap water. Each end of the paper rests in a buffer box where electrodes are connected to a Savant High Voltage Power supply, Model HV 1000 CR. The left buffer box was connected to the cathode and the right buffer box to the anode.

Before the sample was put on, a potential was applied to the system for a few minutes to equilibrate the buffer in the paper. With the system turned off, a one ml sample was applied to the center of the paper, and the potential was applied at a constant voltage for specified times.

At the end of the run, the paper was removed, air dried and cut lengthwise, into two strips; one was about 1/3 wide and the other about

2/3 wide. The narrower strip was treated with 0.3% ninhydrin in 95% ethanol (ETOH) to bring out the amino acid separation and make them visible.

Determination of The Effect of  $10^{-4}\%$  Casamino Acid Dialysate For Substrate Testing. One ml of 1% casamino acid dialysate was added to 99 ml of M-9 medium with 0.01% dextrose and inoculated with Pseudomonas aeruginosa Ps-1C starter culture. This was incubated on a shaker at 37° C for 18 hours and then assayed for proteolytic activity. A control flask which only contained the M-9 medium with the dextrose was also incubated and assayed.

Geon Electrophoresis of Casamino Acid Dialysate. A 500 ml beaker was filled to the 300 ml marker with dry powder Geon 428 Resin, lot number 5089. Added to the Geon was 0.05 M phosphate buffer of the pH of the electrophoresis run, until the mixture was thick, wet, and consistent, but not runny. This was applied to a plastic trough 62 cm x 6.5 cm x 1.3 cm so that the Geon was consistently even. The trough was placed on the cooling plate mentioned in the paper electrophoresis section, and the Geon was connected to the buffer boxes by buffer soaked, Whatman # 3M paper wicks. A center strip of Geon, 2 - 3 cm wide, was removed and replaced with Geon soaked in 10% Casamino acid dialysate. An electrical potential was applied for a specified time period to give a constant current of approximately 20 ma.

At the end of each run, 2 - 3 cm wide Geon segments were removed and put into individual beakers, numbered in order from the cathode, labeled as I, through the center of origin, towards the anode. The amino acids were eluted with 10 ml of distilled water. A small sample

from each fraction was spotted onto a strip of Whatman #3M paper, air dried, and treated with 0.3% ninhydrin in ETOH to visually determine the migration of the amino acids and their separation in the buffer of the tested pH.

The fractions were pooled into three fractions: Pool I = those fractions that migrated towards the cathode under the specified pH conditions; Pool II = those fractions which remained at the origin, i.e. did not migrate at that pH; and Pool III = those amino acids which migrated towards the anode under the specified pH conditions.

These three pools were individually tested for containing nutritional requirements for protease production by Pseudomonas aeruginosa Ps-1C. A 150 mg sample of lyophilized casamino acid Geon pool was added to 100 mls of Hershey's M-9 medium with 0.05% dextrose in 250 ml Erlenmeyer flasks stoppered with cotton plugs. These were inoculated from a starter culture, incubated 15 hours at 37° C on a shaker, and assayed for proteolytic activity.

A concentration of 40 mg/ml of lyophilized casamino acids, Geon electrophoresis, pool II was applied to a sephadex G-10 gel chromatography column. Two ml fractions were collected, and a sample from each was spotted on Whatman # 1 chromatography paper, air dried, and treated with ninhydrin. Five pools were obtained according to spot intensity and color. These pools were lyophilized and individually tested as substrate containing the required nutritional factors for protease production. Samples of 250 mg each were applied to 100 ml aliquots of M-9 medium, inoculated, incubated, and assayed as previously explained.

Chromatography of Casamino Acids. A number of chromatography systems were tried on the casamino acid dialysate, Geon electrophoresis (II), sephadex G-10 (II), to help illucidate which amino acids were involved as nutritional requirements for the production of protease by Pseudomonas aeruginosa Ps-1C. Table II lists the types of systems used with their respective solvents. The paper chromatography techniques were essentially those of Smith (1958). The thin layer techniques were those listed by Pataki (1968). At the end of each run, the thin layer plate or paper was allowed to air dry before treatment with ninhydrin.

Thin layer plates of MN-Cellulosepulver 300, Macherey, Nagel and Co., 0.25 mm thick were prepared with a DESAGA Brinkmann layering device on glass plates.

In Figure 1, a schematic of the analysis of casamino acids as substrate for Ps-1C protease production is given.

#### Specific Amino Acid Analysis

Amino Acid Combinations Tested as Nutritional Requirements. All amino acids tested were of the L-form. Ammonia free glycine was obtained from Matheson Coleman and Bell, Co. The alanine, phenylalanine, leucine isoleucine, proline, hydroxyproline, and valine were obtained from Nutritional Biochemical Corporation. All other amino acids tested were from Sigma Chemical Company. The general procedure was to add 150 mg of the appropriate amino acid to 100 ml of Hershey's M-9 medium containing 0.05% dextrose, in 250 ml Erlenmeyer flasks with cotton plug stoppers. The test flasks were incubated for 14 to 16 hours at 37° C on a shaker after being inoculated with one ml of starter culture. At

TABLE II  
CHROMATOGRAPHY SYSTEMS USED  
ON CASAMINO ACIDS

Types of chromatography	Solvent systems	Sample
Thin layer, one dimensional, Cellulose MN 300	MeOH-H <sub>2</sub> O-Pyr (20:5:1) Bu-Ac-H <sub>2</sub> O (4:1:5) Pyr-H <sub>2</sub> O (4:1) Bu-Ac-H <sub>2</sub> O (12:3:5) CHCl <sub>3</sub> -MeOH-Am (2:2:1)	casamino acids, Geon electrophoresis II, sephadex G10, II.
Thin layer, two dimensional, Cellulose MN 300	1) Bu-Ac-H <sub>2</sub> O (4:1:5) 2) Ph-Am (4:1)	Casamino acids, Geon electrophoresis II, sephadex G10, II.
Thin layer, two dimensional, Cellulose MN 300	1) Bu-Ac-H <sub>2</sub> O (12:3:5) 2) Pyr-H <sub>2</sub> O (4:1)	Casamino acids, Geon electrophoresis II, sephadex G10, II.
Thin layer, two dimensional, Cellulose MN 300	1) MeOH-CHCl <sub>3</sub> -Am (2:2:1) 2) MeOH-H <sub>2</sub> O-Pyr (20:5:1)	Casamino acids, Geon electrophoresis, II, sephadex G10, II.
Paper, one dimensional Whatman #3M	Bu-Ac-H <sub>2</sub> O (4:1:5)	Casamino acids, Geon electrophoresis II, sephadex G10, (I, II, III, IV, and V).

The listing of more than one solvent system in the one dimensional chromatography refers to the fact that each one was used separately with a different sample. The ratios of the solvents are all in terms of (v/v/v).

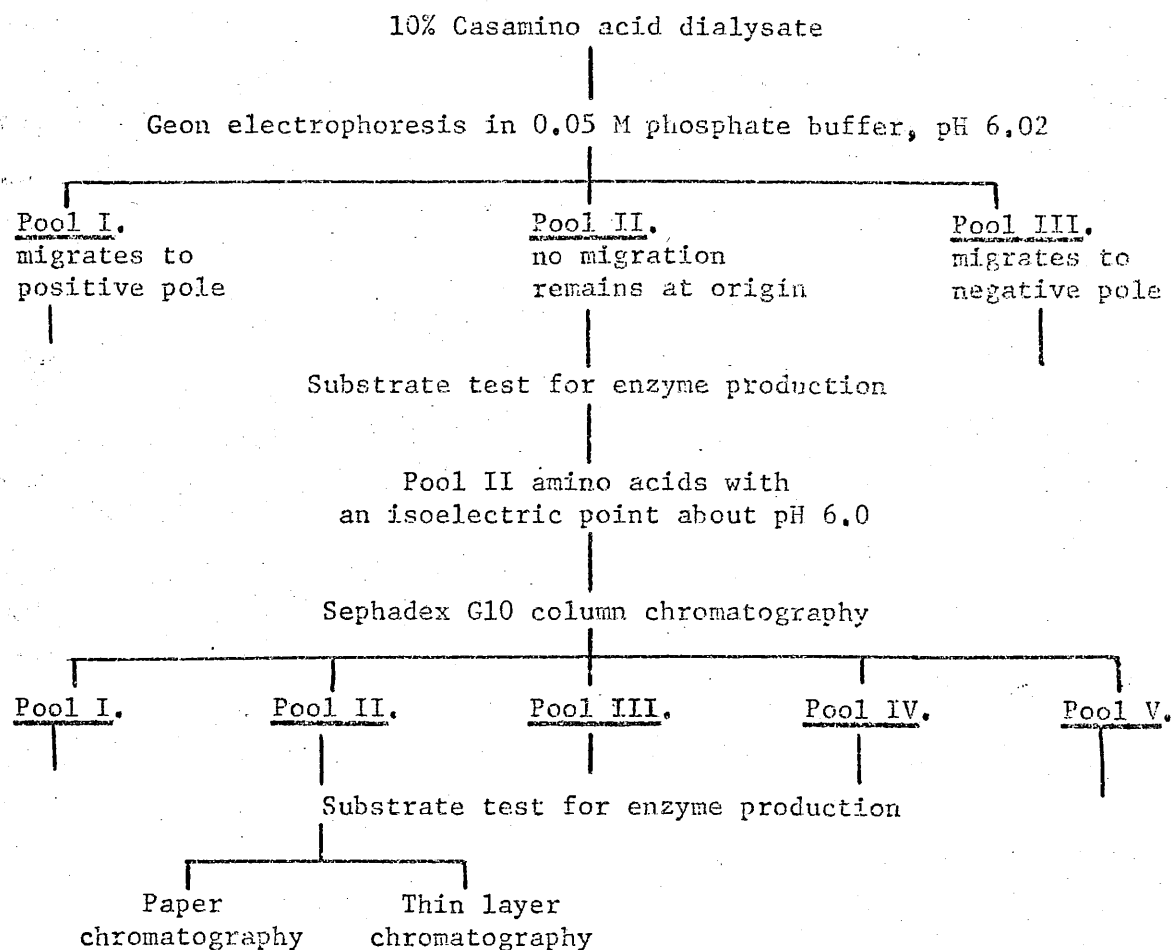


Figure 1. Schematic of the breakdown of casamino acids for determination of amino acid requirements for Ps-1C protease production.



the end of the incubation time the cultures were assayed for proteolytic activity.

In a few cases, 15 mg amounts of amino acids were added to 10 mls of M-9 medium in 30 ml test tubes. These were inoculated with 1/10 ml starter culture, incubated, and assayed as were the flasks.

Amino Acid Concentration Requirement. In all cases, L-amino acids were added in varying concentrations to 10 ml of Hershey's M-9 medium containing 0.05% dextrose. One tenth ml of starter culture was added to the experimental test tubes (30 ml size) which were incubated at 37° C on a water bath shaker for 14 to 16 hours. At that time they were then assayed for proteolytic activity.

All amino acid concentration experiments were performed in duplicate or triplicate, and the optical densities were averaged from the proteolytic assays.

#### Determination of Dextrose Concentration and Repression

The culture media contained 10 ml of Hershey's M-9 medium without dextrose, except that which was added at concentrations to be tested. The medium was supplemented with valine, 2.0 mg/ml; isoleucine, 1.0 mg/ml; and phenylalanine, 0.5 mg/ml. The dextrose used was Mallinckrodt dextrose Anhydrous; Mallinckrodt Chemical Works in concentrations of 1.0% to 0.001%. Two controls were employed; one had the amino acids, but no dextrose; and the other had neither dextrose nor amino acids.

The test tubes were incubated for 20 hours on an incubator shaker water bath at 37° C and then assayed for proteolytic activity.

### Growth Curves

Growth Curve Vs. Optical Density at 660 nm. A 50 ml aliquot of NBYE is inoculated with Pseudomonas aeruginosa Ps-1C and incubated 18 hours at 37° C on a Metabolyte Waterbath Shaker, New Brunswick Scientific Company, Inc., at 300 RPM. At zero time one ml of starter culture is inoculated into the test flask.

The culture medium consists of 99 ml of sterile NBYE in a 250 ml Erlenmeyer flask with a cotton plug stopper. This is incubated at 37° C on a Gyrotory Shaker, New Brunswick Scientific Company, Inc., RPM setting of 6.5.

All media including the 9 ml and the 99 ml dilution blanks were pre-sterilized by autoclaving for 15 minutes at 250° C.

The agar plates were made with approximately 25 ml of NBYE agar, which consisted of the same concentrations of nutrient broth and yeast extract as previously mentioned plus 1.5% Difco Racto-Agar, Difco Laboratories. All agar plates were incubated for one day at room temperature and one day at 37° C before using in order to eliminate contaminated plates.

Five to eight ml samples were withdrawn from the test culture media at specified times and the optical density was read at wavelength 660 nm with a Bausch and Lomb Spectronic 20 colorimeter, using the red filter. The time intervals at which the culture density was read optically and the agar plates were inoculated are as follows: 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 14, 16, 18, 20, and 24 hours.

The agar plates were inoculated by making the specified dilutions (Table III) and withdrawing 0.1 ml from a 10 ml dilution tube with a

TABLE III

GROWTH CURVE OF PSEUDOMONAS AERUGINOSA  
 VERSES OPTICAL DENSITY AT  
 WAVELENGTH 660 NM

Incubation time in hours	Dilutions per Plate colony count				Optical density at wavelength 660 nm
0	$10^{-1}$ C	$10^{-2}$ C	$10^{-3}$ C	$10^{-4}$ C	0.010
1	$10^{-1}$ C	$10^{-2}$ C	$10^{-3}$ C	$10^{-4}$ 609	0.010
2	$10^{-2}$ C	$10^{-3}$ C	$10^{-4}$ C	$10^{-5}$ 194	0.022
3	$10^{-2}$ C	$10^{-3}$ C	$10^{-4}$ C	$10^{-5}$ C	0.060
4	$10^{-3}$ C	$10^{-4}$ C	$10^{-5}$ C	$10^{-6}$ 166	0.090
5	$10^{-3}$ C	$10^{-4}$ C	$10^{-5}$ C	$10^{-6}$ 350	0.118
6	$10^{-3}$ C	$10^{-4}$ C	$10^{-5}$ C	$10^{-6}$ 415	0.140
7	$10^{-4}$ C	$10^{-5}$ C	$10^{-6}$ 435	$10^{-7}$ 68	0.155
8	$10^{-4}$ C	$10^{-5}$ C	$10^{-6}$ 552	$10^{-7}$ 55	0.172
9	$10^{-5}$ C	$10^{-6}$ C	$10^{-7}$ 65	$10^{-8}$ 6	0.181
10	$10^{-5}$ C	$10^{-6}$ C	$10^{-7}$ 91	$10^{-8}$ 9	0.198
12	$10^{-5}$ C	$10^{-6}$ C	$10^{-7}$ 93	$10^{-8}$ 9	0.220

TABLE III

Continued

Incubation time in hours	Dilutions per plate colony count				Optical density at wavelength 660 nm
14	$10^{-5}$ C	$10^{-6}$ C	$10^{-7}$ 97	$10^{-8}$ 15	0.242
16	$10^{-5}$ C	$10^{-6}$ C	$10^{-7}$ 136	$10^{-8}$ 40	0.262
18	$10^{-5}$ C	$10^{-6}$ C	$10^{-7}$ 128	$10^{-8}$ 15	0.273
20	$10^{-5}$ C	$10^{-6}$ C	$10^{-7}$ 142	$10^{-8}$ 9	0.290
24	$10^{-5}$ C	$10^{-6}$ C	$10^{-7}$ 98	$10^{-8}$ 9	0.288

The symbol C indicates confluent growth.

pipette and applying this to the agar surface and spreading with an alcohol flamed glass spreading rod. The inoculated agar plates are incubated for 10 - 18 hours at 37° C in a Napco Incubator, Model 320. The colonies were counted and recorded on a Sansaur Bacti-Counter, Aloe Scientific Co. and a Klett Pressure Counter, Klett Mfg. Co.

Essentially the method of Eagon (1968) has been employed for the methods of the growth curve.

The number of cells per ml at a specified time are calculated by:

$$\frac{10^2 \times \text{sample dilution} \times \text{number of colonies/plate}}{10^2 \text{ mls of culture}} = n$$

Where n = number of cells per ml. (Eq. 1)

Log of the cell number is determined (Corliss and Berglund, 1958) and plotted against optical density at 660 nm.

Growth and Protease Production Versus Time. A starter culture was prepared as previously stated, and 1.0 ml was applied at zero time to 500 ml of test culture in a 2 liter Erlenmeyer flask with a cotton plug stopper.

The test culture medium contained Hershey's M-9 medium with 0.05% dextrose and supplemented with 0.5 mg/ml phenylalanine, 0.75 mg/ml isoleucine, and 2.0 mg/ml valine.

The inoculated test culture was incubated on a shaker at 37° C. At specified time intervals, three samples were removed. A sample of 3 ml was removed to determine the culture's optical density at 660 nm, and this was returned to the culture flask. When the readings were greater than 0.500, a 1/10 dilution in sterile Hershey's M-9 medium was made, and the O.D. was recorded after the value was multiplied by ten.

A second sample of 0.1 ml of culture was removed for proteolytic assay by previously described method.

A third sample of 1.0 ml of culture was removed for making appropriate dilutions in sterile NBYE for plating and determination of cell number/ml of culture as done in the growth curve above.

The log of the cell number, culture optical density at 660 nm, and proteolytic assay optical density at 420 nm were plotted against time.

Divalent Metal Ion Concentration Requirements. All divalent metal ions tested were analytical reagent grade chemicals from Mallinckrodt Chemical Works. The  $Mg^{++}$  solution was prepared from  $MgSO_4 \cdot 7H_2O$ ;  $Zn^{++}$  from  $ZnSO_4 \cdot 7H_2O$ ;  $Co^{++}$  from  $CoCl_2 \cdot 6H_2O$ ;  $Cu^{++}$  from  $CuSO_4 \cdot 5H_2O$ ; and the  $Ca^{++}$  from  $CaCl_2 \cdot 2H_2O$ .

For the  $Mg^{++}$  and  $Zn^{++}$  concentration experiments, the culture medium contained 100 ml of Hershey's M-9 part A only, with 0.05% dextrose and supplemented with 2.0 mg/ml valine, 1.0 mg/ml isoleucine, and 0.5 mg/ml phenylalanine. The tests for the other divalent ions employed 50 ml of the same medium. In all cases the medium was sterilized in 250 ml Erlenmeyer flasks. Varying concentrations of the ion to be tested were added to the experimental flasks before sterilization. All experiments were duplicated and averaged in the results.

Following inoculation from a starter culture the flasks were incubated on a shaker at 37° C for 14 - 16 hours, and then assayed for proteolytic activity.

## RESULTS

### Glucose Inhibition Determination

In both cases, the first using 10% proteose peptone in Hershey's M-9 medium and the second where Hershey's M-9 medium was supplemented with 0.05 mg/ml phenylalanine, 1.0 mg/ml isoleucine, and 2.0 mg/ml valine, there was an apparent inhibition of enzyme production at glucose concentrations greater than 0.05%. In Figure 2 both experimental conditions indicate that the best enzyme production occurred when the glucose concentration was maintained at 0.05%. It was noted visually, though not tested specifically that there was good growth at all concentrations.

### Proteose Peptone Analysis

A 10% proteose peptone solution, percolated through a descending sephadex G-10 column gave rise to five peaks as indicated by the optical density of the fractions read at wavelengths 260 and 280 nm. As indicated in Figure 3a, the largest amount of material came off in Pool I.

These five pools were tested for ability to stimulate protease production, and it was found that only pool I gave rise to any appreciable proteolytic activity under the the appropriate conditions.

Ten ml of pool I. was applied to a sephadex G-75 descending column and three sub-pools were obtained by this procedure, see Figure 3b. Five ml samples from each of these pools were tested as inducers for protease formation, as were also 5 mls of 10% casamino acids, and 5 mls of 10% crude proteose peptone. None of the pools from the sephadex G-75

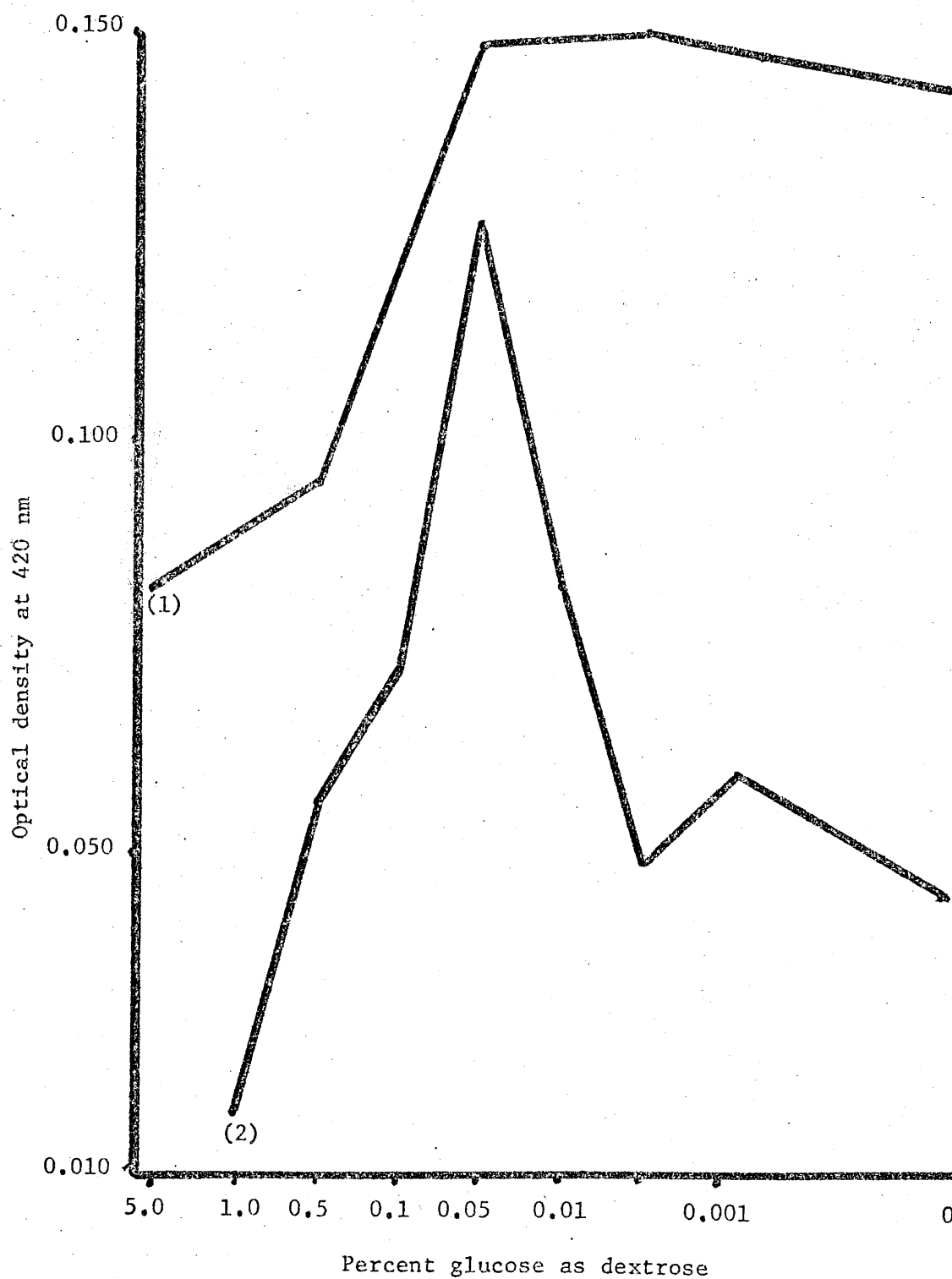


Figure 2. Dextrose concentration determination for the best Ps-1C protease production. The results are the average of two experiments. (1) M-9 medium plus proteose peptone, and (2) M-9 medium supplemented with valine, isoleucine, and phenylalanine.



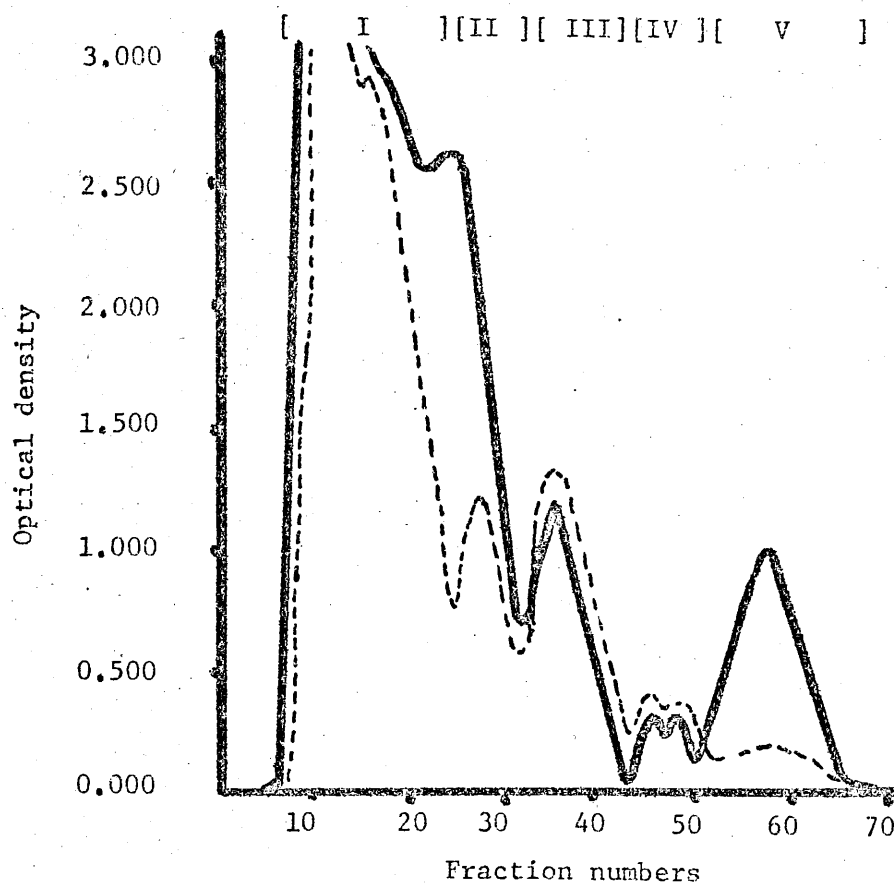


Figure 3a. Sephadex G10 column chromatography of 10% proteose peptone. Solid line indicates readings at wavelength 260 nm, and dotted line at 280 nm.

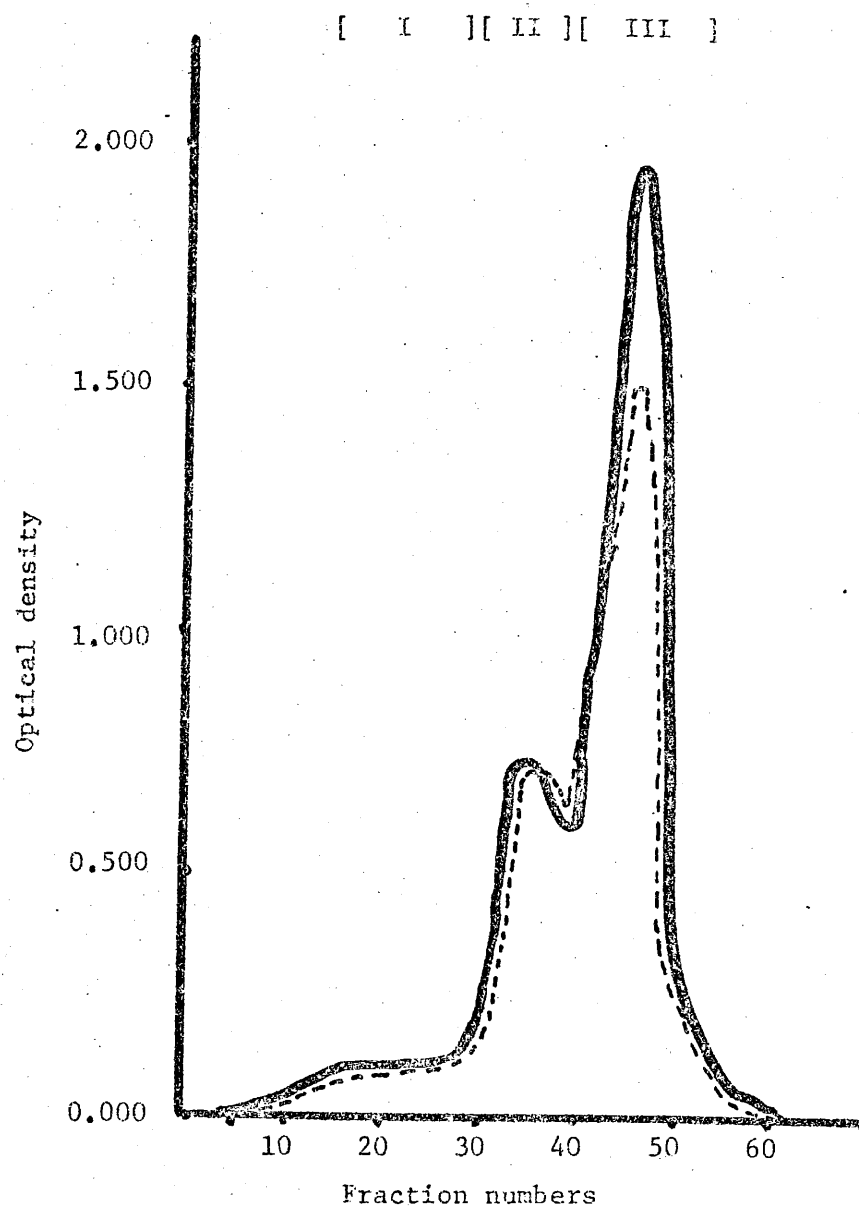


Figure 3b. Sephadex G75 column chromatography of proteose peptone sephadex G10, pool I. Solid line indicates readings at wavelength 260 nm, and dotted line at 280 nm.

treatment gave rise to appreciable protease production, as indicated by enzyme activity, however the casamino acid sample and proteose peptone control were active.

The proteose peptone sephadex G-10 pools and the sephadex G-75 pools were lyophilized and retested as 10% solutions in Hershey's M-9 media. Although there appeared to be good growth, there was not appreciable protease production in any of the pools.

The sephadex treatments were tried in reverse. Ten percent proteose peptone was first put through a descending sephadex G-75 column. This resulted into two peaks of material, see Figure 3c, and 100 mg of each lyophilized pool was put in 50 ml of M-9 medium and tested as inducer for Ps-1C protease production. Pool II. did give appreciable enzyme production.

A 10% solution of lyophilized proteose peptone, sephadex G-75, pool II. was chromatographed on a sephadex G-10 column. Three peaks of material (see Figure 3d) were separated, lyophilized and tested as inducer for protease production. None of these three pools gave rise to any proteolytic activity.

Casamino Acid Analysis. Since it had earlier been determined that casamino acids might serve as inducer for protease elaboration by Pseudomonas aeruginosa Ps-1C, a 10% solution was prepared and dialyzed against distilled water. The material remaining in the dialysis tubing and that which passed into the distilled water were lyophilized and used as inducers for enzyme production. Table IV indicates that the material which dialyzed into the distilled water provided the required nutrients for the bacterium to elaborate the enzyme.

Paper electrophoresis of casamino acid dialysates were performed

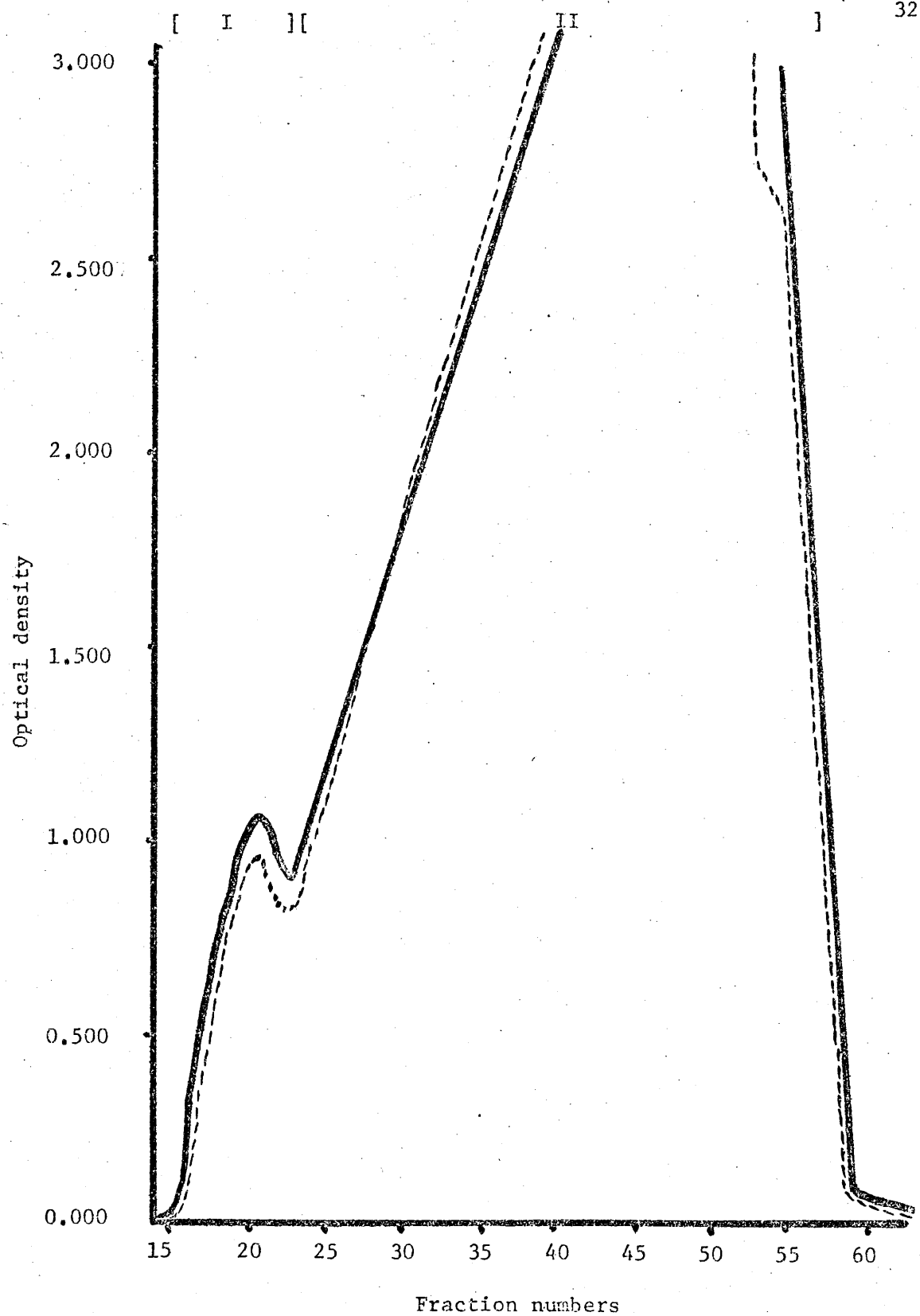


Figure 3c. Sephadex G75 column chromatography of 10% proteose peptone. Solid line indicates readings at wavelength 260 nm, and dotted line at 280 nm.

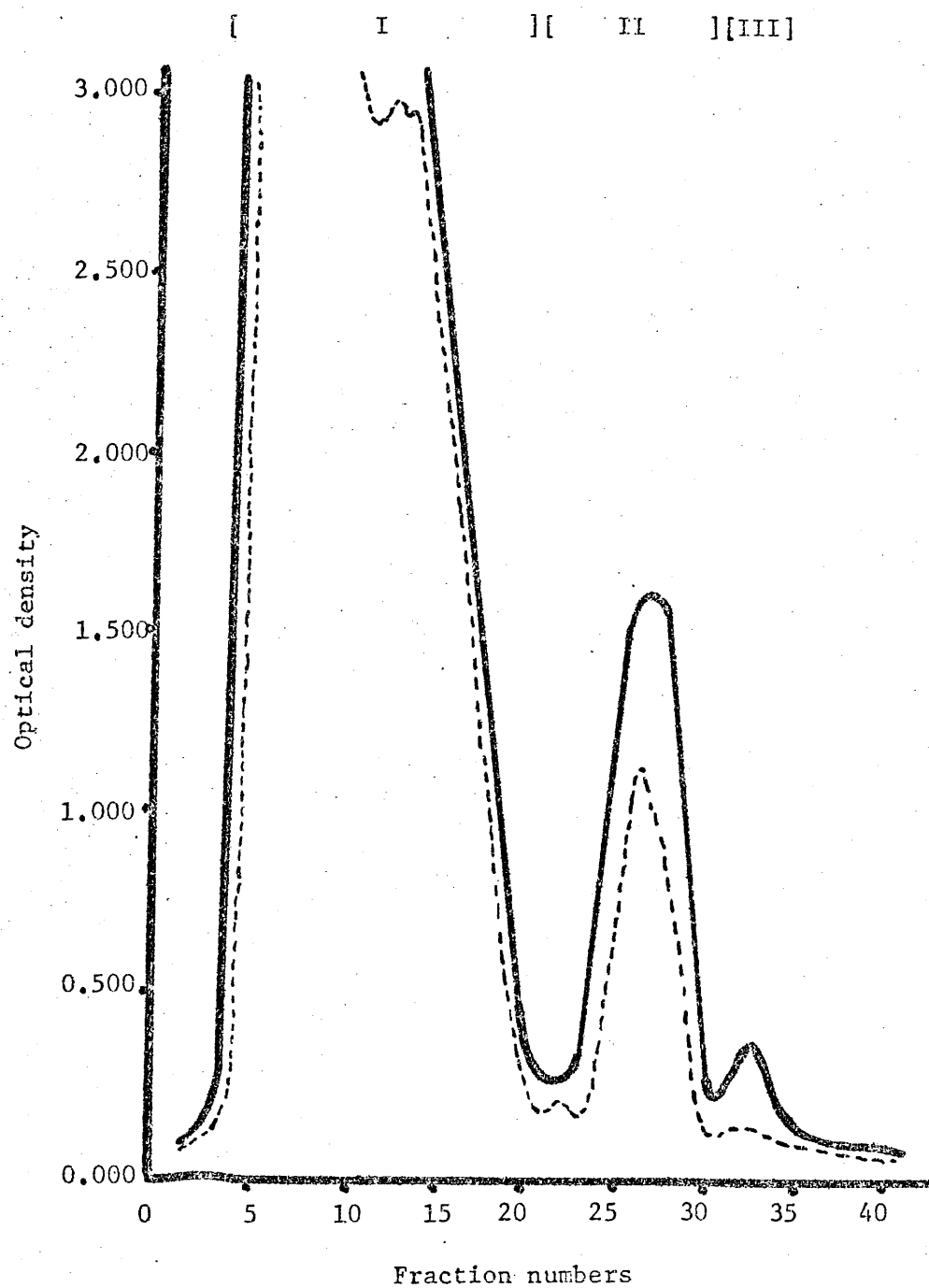


Figure 3d. Sephadex G10 column chromatography of 10% proteose peptone, sephadex G75, pool II. Solid line indicates readings of optical density at wavelength 260 nm, and dotted line at 280 nm.

TABLE IV  
CASAMINO ACID DIALYSATE  
SUBSTRATE EXPERIMENT

Flask	Amount of Hershey's M-9	Amount Dextrose	Casamino acids Amount	Preparation	Optical density
A	100 ml	0.01%	1.044	Material in the dialysis tube	0.060
B	100 ml	0.01%	1.000	Material in the dialysis water	0.115
C	100 ml	0.01%	1.000	Non-dialyzed, crude	0.025
D	100 ml	0.01%	none	-----	0.005

The optical density readings are at wavelength 420 nm, and the amount of casamino acids is in grams.

using 0.05 M tris buffer pH 7.11, and 0.05 M phosphate buffer with pH's of 6.0 to 8.0. The times of the runs were from 20 minutes to 180 minutes with voltages of 600 V and currents of about 30 ma. The acid pH's gave better separation than did the basic or neutral pH's. However substrate tests indicated that not enough material could be obtained by this method of separation in order to obtain meaningful data.

Geon electrophoresis of 10% casamino acid dialysate permitted larger quantities of separated material to be obtained. After the technique was developed, the average geon electrophoresis run was about 5 hours with 800 V and current from 15 to 20 ma. Figure 4. shows an average geon electrophoresis experiment and the respective sample spot paper to determine the separation of the amino acids.

When the lyophilized pools from the geon electrophoresis of casamino acids were tested as substrate for Ps-1C protease production, it was determined that pool II, or the material remaining at the origin best contributed to protease synthesis. This was true when pool II. was tested singularly or in combination with the other pools, as indicated in Table V. This experiment was repeated several times and the same results occurred.

To further separate the casamino acids, pool II from the Geon electrophoresis treatment was lyophilized and a sample was resuspended in the eluting buffer and applied to a sephadex G-10 column. A spot-o-gram was made to determine which 2 ml fractions contained the separated amino acids. An average spot-o-gram is shown in Figure 5. where the five pools were established. The pools, I through V were lyophilized and tested for substrate which contributes to protease production for

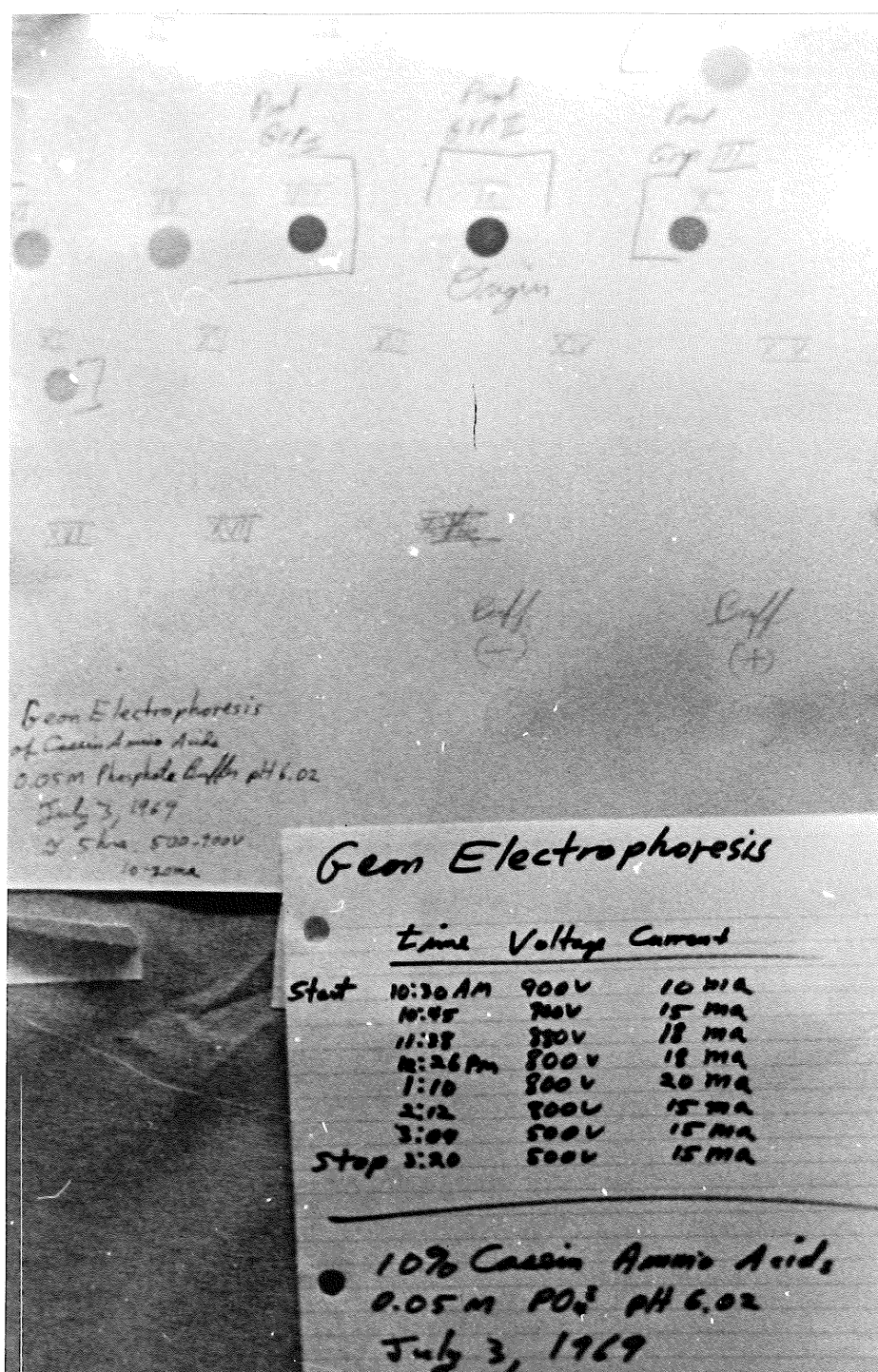


Figure 4. Geon electrophoresis of casamino acids using a 0.05 M phosphate buffer, pH 6.02.



TABLE V  
SUBSTRATE TESTS OF CASAMINO ACIDS  
GEON ELECTROPHORESIS POOLS

Flask number	Contents tested	Amount of lyophilized material	Amount of Hershey's M-9 medium	Optical density at wavelength 420 nm
1	Pool I	100.0 mg	100 ml	0.000
2	Pool II	152.0 mg	100 ml	0.105
3	Pool III	150.9 mg	100 ml	0.010
4	Pool I Pool II	150.8 mg 151.0 mg	100 ml	0.210
5	Pool II Pool III	150.8 mg 150.7 mg	100 ml	0.210
6	Pool I Pool II Pool III	150.0 mg 150.0 mg 150.0 mg	100 ml	0.146
7	Crude casamino acids	151.8 mg	100 ml	0.135
8	none	-----	100 ml	0.000

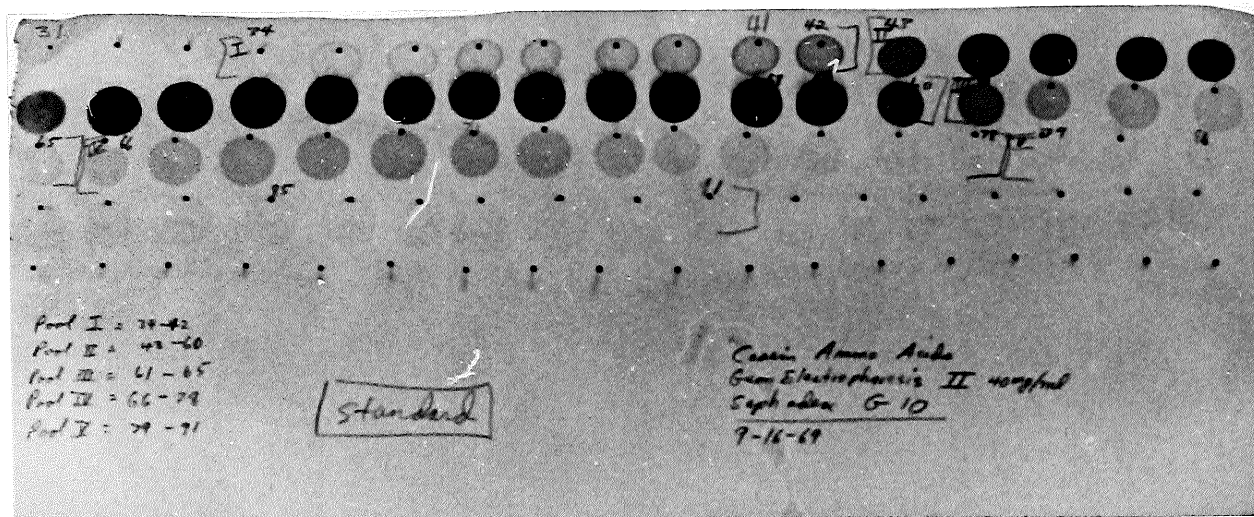


Figure 5. Standard spot-o-gram for casamino acids, Geon electrophoresis II, sephadex G10 fractions.

Pseudomonas aeruginosa Ps-1C. As seen in Table VI, pool II best contributed to enzyme production.

In order to determine which amino acids make up the pool II group from the sephadex treatment, a series of paper and thin layer chromatographs were made.

The first type of chromatography used was one dimensional paper chromatography of the five pools collected from the sephadex treatment of casamino acids, Geon electrophoresis, II. The results, as seen in Figure 6 show the comparison of the five pools, indicating a possible connection with the amino acids of glycine, hydroxy proline, alanine, proline, valine, phenylalanine, isoleucine and leucine, when compared to results of Smith (1958). Pool II obviously contains most of the material, however it appears that pool V contains amino acids not found in pool II.

One dimensional-thin layer chromatography of casamino acids, geon electrophoresis II, sephadex G-10 pool II apparently did contain the amino acids: proline, alanine, glycine, valine, leucine, hydroxyproline, aspartic acid, lysine, and glutamic acid as seen in Figures 7, 8, and 9.

Two dimensional-thin layer chromatography of the same sample, using different solvent systems according to Pataki (1968), also indicated the same general results. See Figures 10, 11, and 12.

Another attempt to determine which general group of amino acids contribute to the nutritional requirements for Ps-1C protease production, was made by testing the amino acid grouping as outlined by Conn and Stumpf (1964). The monoamino-monocarboxylic amino acids, dicarboxylic amino acids, basic amino acids, aromatic amino acids, and the heterocyclic amino acids were tested as substrate for enzyme production, and

TABLE VI  
SUBSTRATE TESTS OF CASAMINO ACIDS,  
GEON ELECTROPHORESIS II,  
SEPHADEX G10 POOLS

Flask set number	Sephadex G10 pool number	Amount of lyophilized material	Amount of Hershey's M-9 medium	Optical density at $\lambda$ 420 nm
1	I	250 mg	100 ml	0.005
2	II	250 mg	100 ml	0.065
3	III	250 mg	100 ml	0.010
4	IV	250 mg	100 ml	0.002
5	V	250 mg	100 ml	0.000
6	Control	none	100 ml	0.000

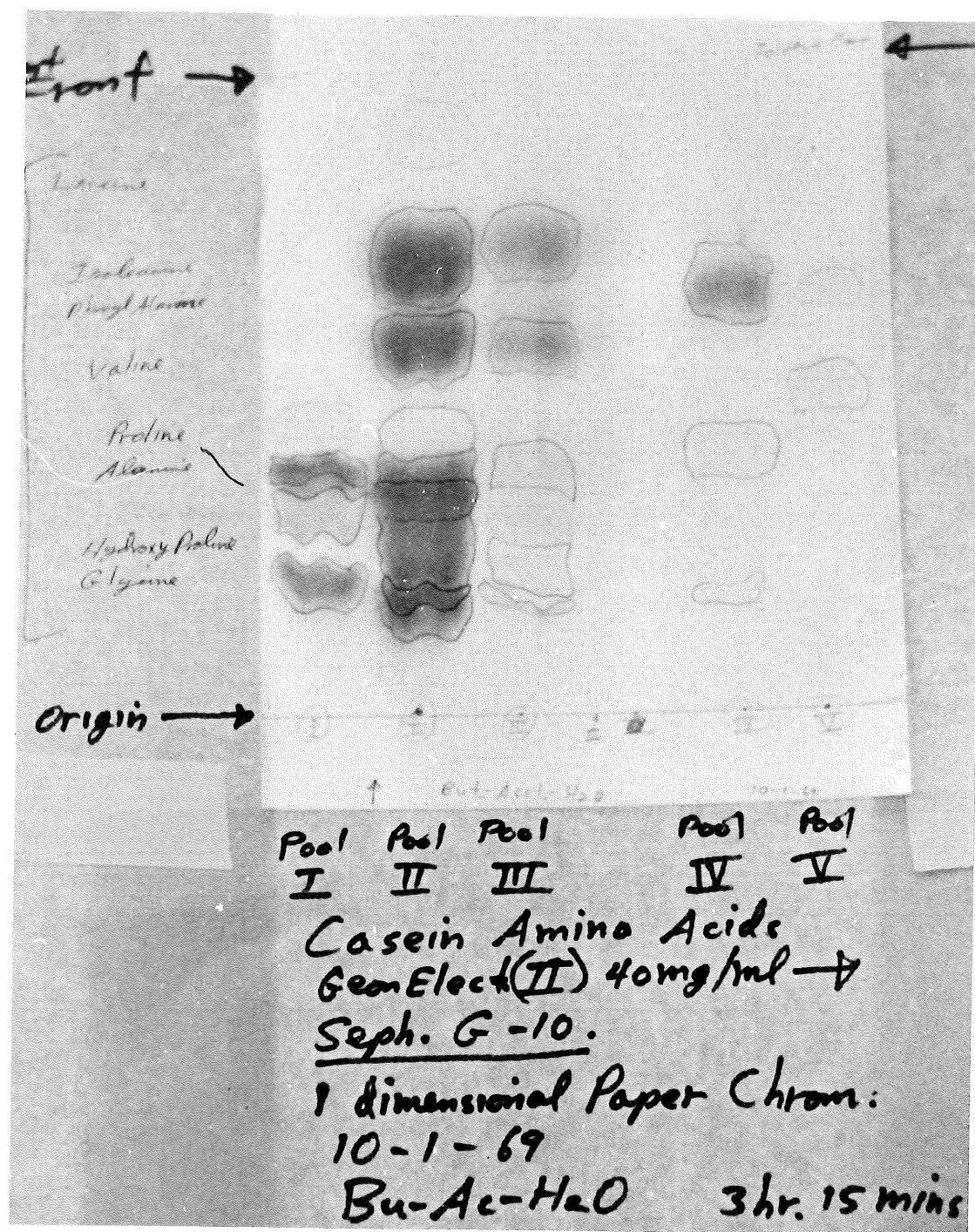


Figure 6. One dimensional paper chromatography of the five pools from sephadex G-10 treatment of casamino acids, Geon electrophoresis pool II.

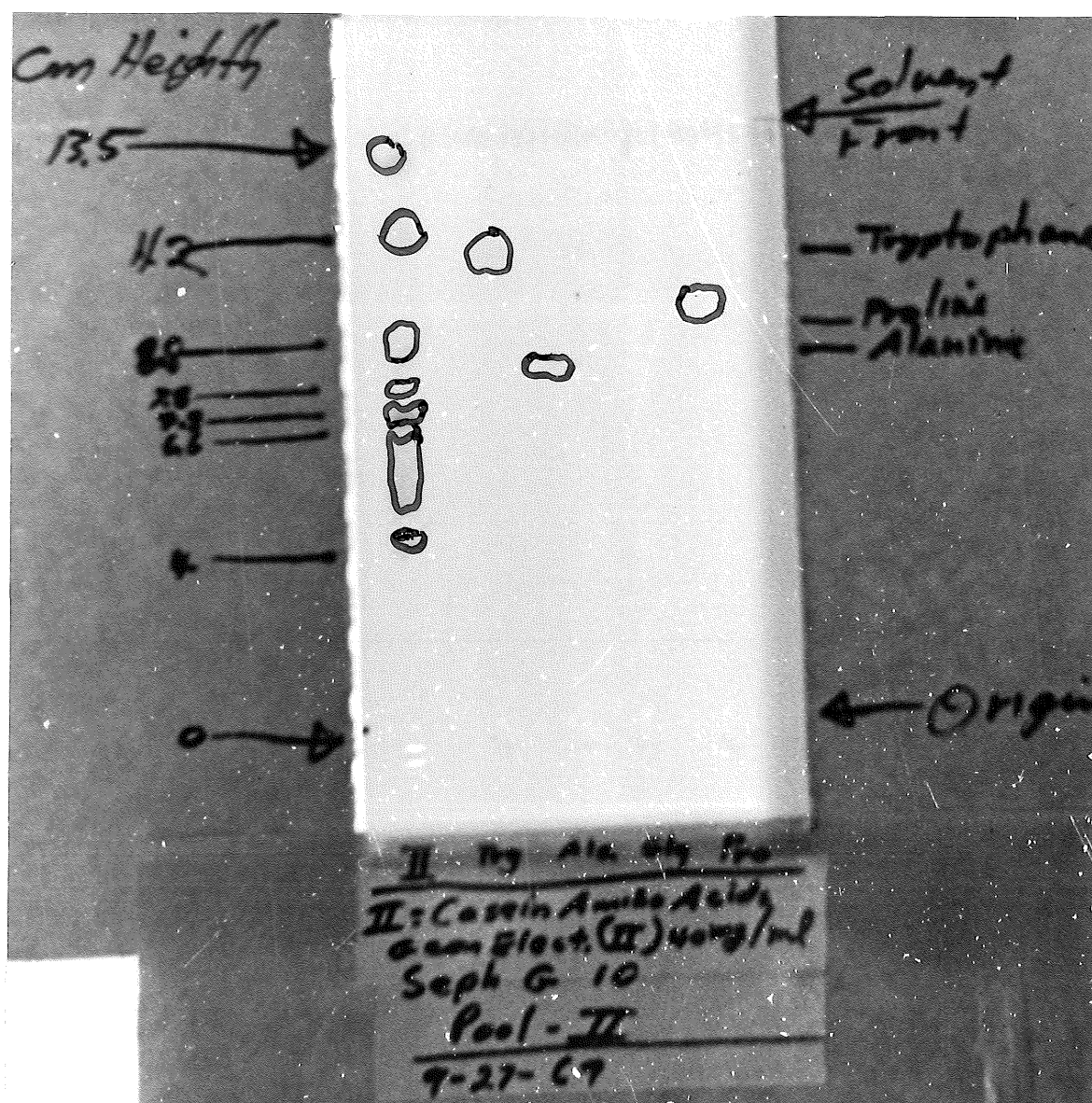
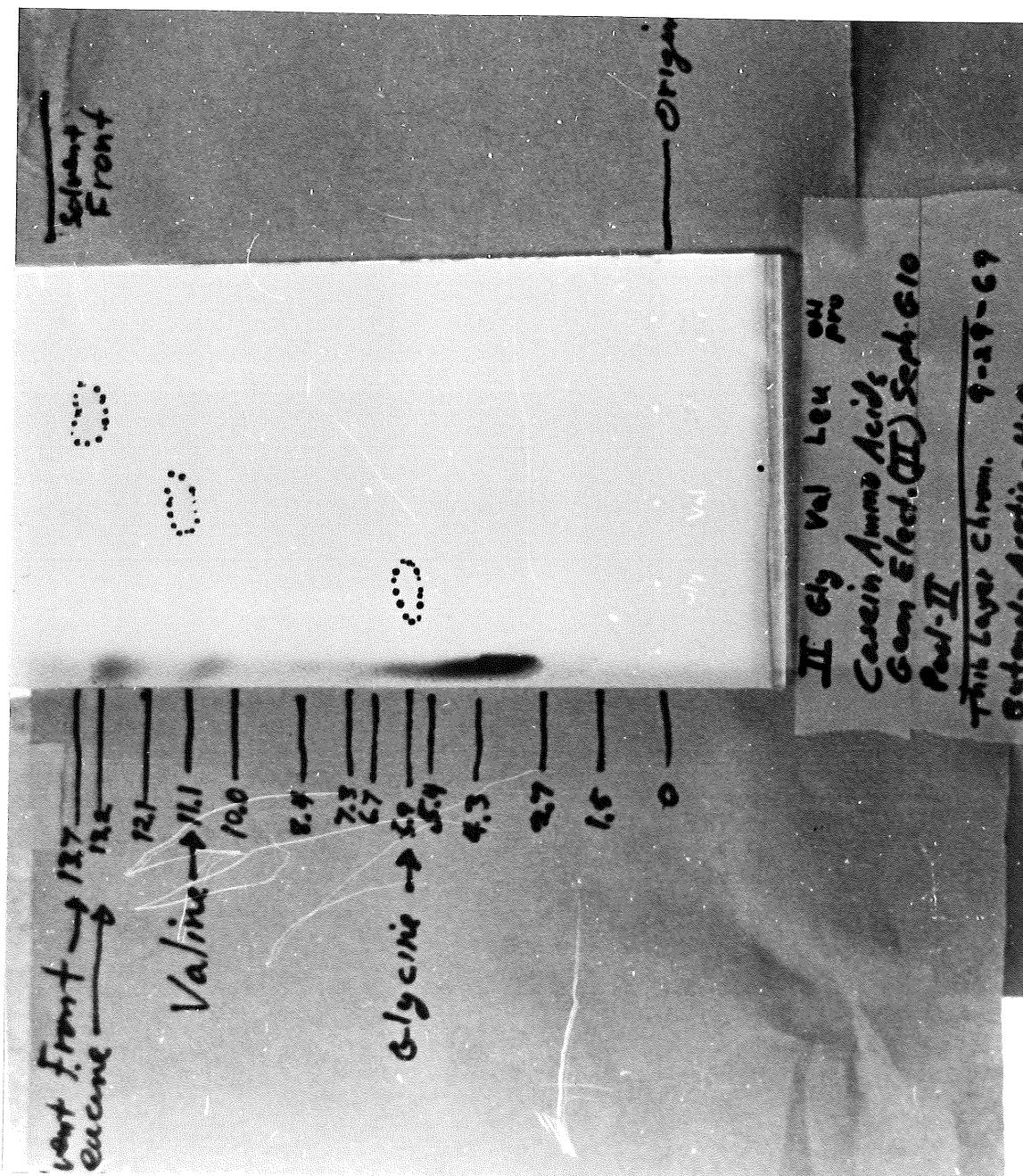


Figure 7. One dimensional thin layer chromatography of casamino acids, Geon electrophoresis II, sephadex G10 II. The solvent is butanol-acetic acid-water, (4:1:5).

Figure 8. One dimensional thin layer chromatography of casamino acids, Geon electrophoresis II, sephadex G10 II. The solvent is butanol-acetic acid-water, (4:1:5).







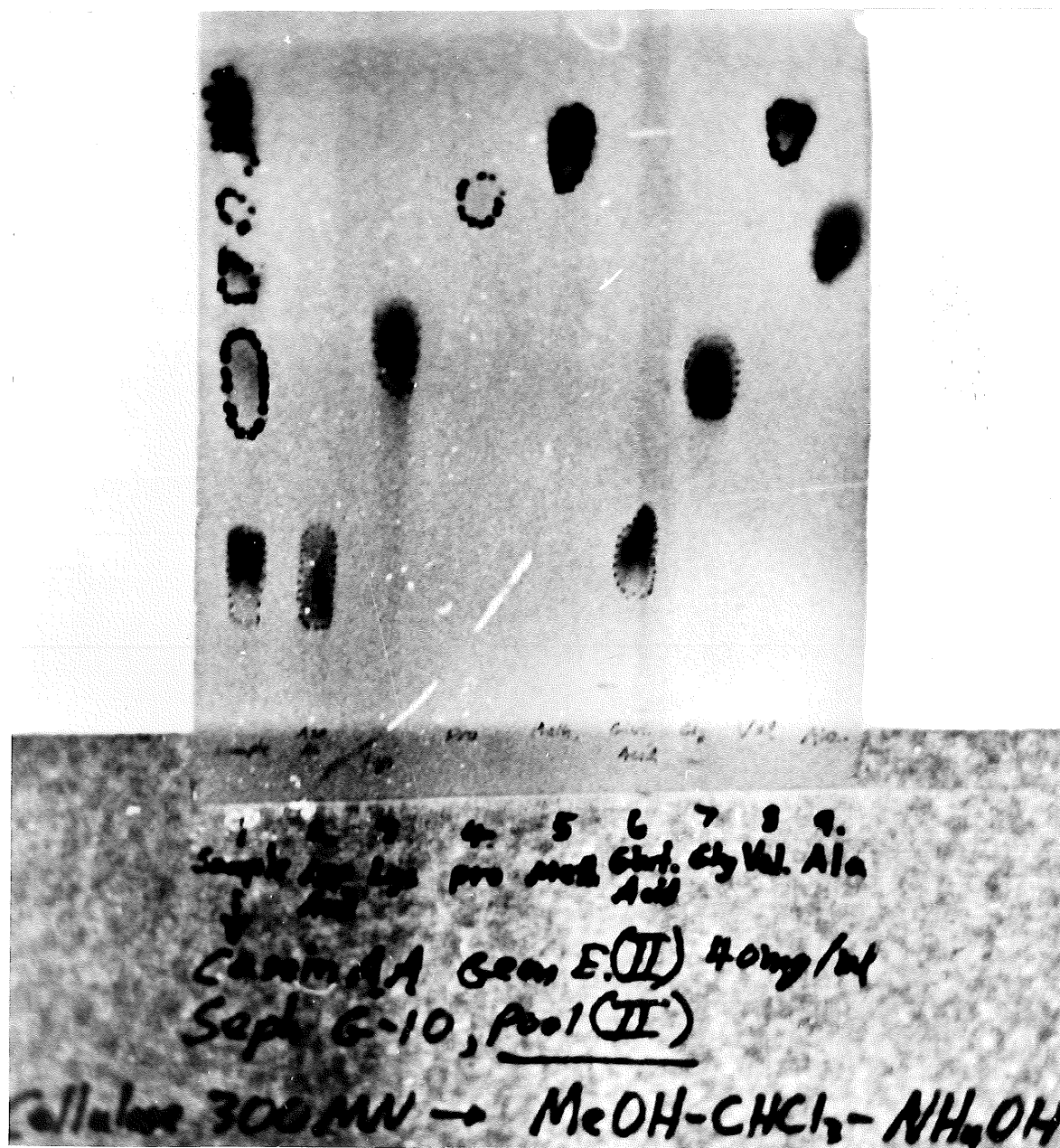


Figure 9. One dimensional thin layer chromatography of casamino acids, Geon electrophoresis II, sephadex G10 II. Solvent is methanol-chloroform-ammonia, (2:2:1).

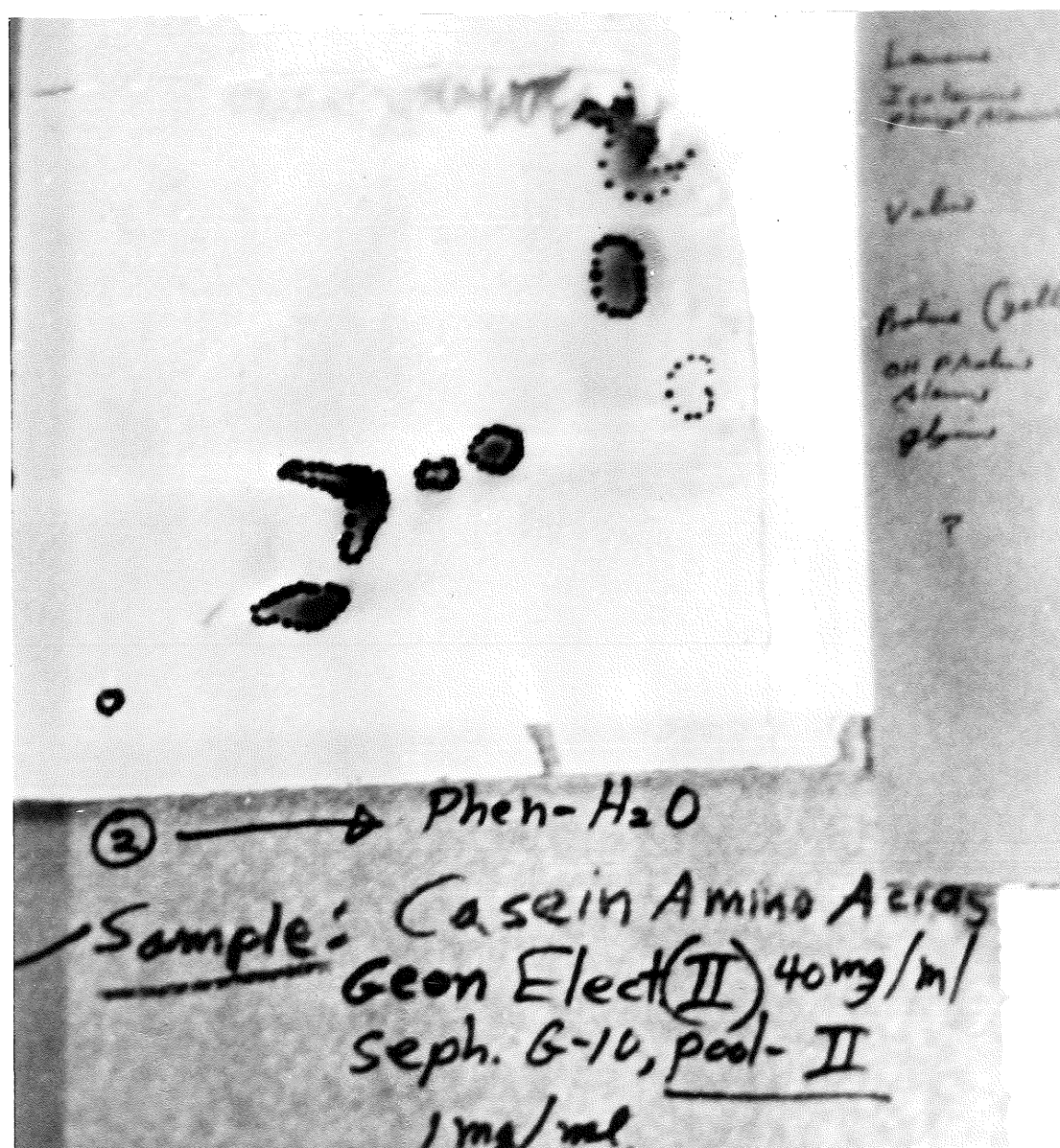
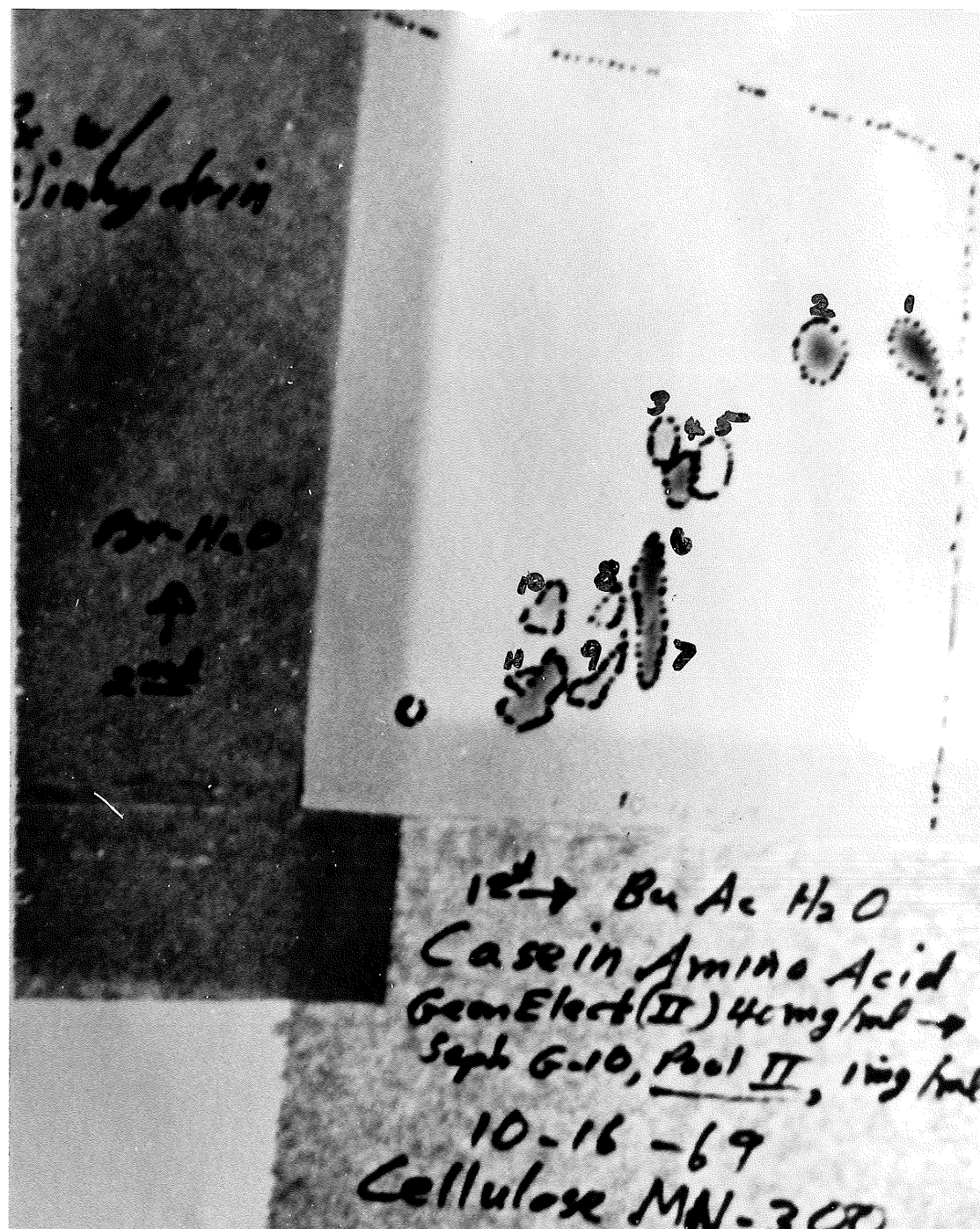


Figure 10. Two dimensional thin layer chromatography of casamino acids, Geon electrophoresis II, sephadex G10 II. The solvents are (1) butanol-acetic acid-water, (4:1:5); (2) phenol-water, (4:1).



**Figure 11.** Two dimensional thin layer chromatography of casamino acids, Geon electrophoresis II, sephadex G10 II. The solvents are (1) methanol-chloroform-ammonia, (4:1:5); (2) pyridine-water, (4:1). The spot numbers represent the following amino acids: (1) leucine, (2) isoleucine, (3) methionine, (4) valine, (5) phenylalanine, (6) alanine, (7) proline, (8) serine, (9) glycine and asparagine, (10) histidine, (11) cysteine and lysine.



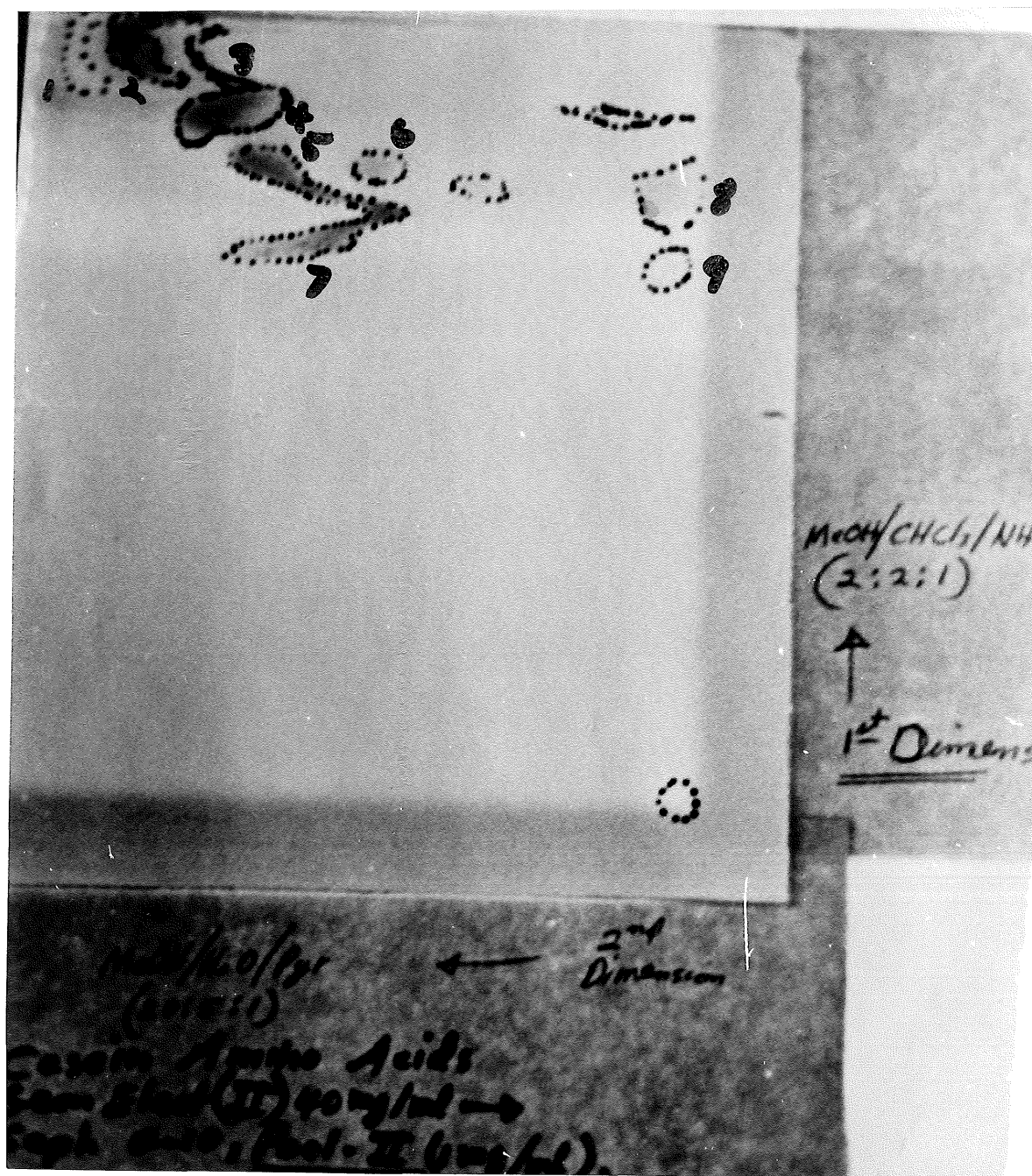


Figure 12. Two dimensional thin layer chromatography of casamino acids, Geon electrophoresis II, sephadex G10, pool II. The solvents are (1) methanol-chloroform-ammonia, (2:2:1); (2) methanol-water-pyridine- (20:5:1). The spot numbers represent the following amino acids: 1) valine, 2) isoleucine and leucine, 3) phenylalanine, 4) proline, 5) alanine, 6) glycine, 7) serine, 8) arginine, 9) lysine.

as seen in Table VII, none of these significantly contributed to protease production.

#### Amino Acid Combinations Tested as Substrate For Ps-1C Protease

Production: The amino acids: alanine, phenylalanine, leucine, isoleucine, proline, hydroxyproline, valine and glycine were employed in various combinations in order to determine how many, and which ones served as nutritional requirements to the bacterium for enzyme production. The step wise determination of the three required amino acids; phenylalanine, isoleucine, and valine is seen in Tables VIII, IX, X, and XI.

The minimal concentration for maximum protease production for these amino acids were: 2.0 mg/ml for valine, 1.0 to 0.5 mg/ml for isoleucine, and 0.5 mg/ml for phenylalanine. See Tables XII, XIII, and XIV.

Inhibition by excess amino acid was tested, and it was determined that isoleucine in excess concentrations, i.e. greater than 1.0 mg/ml, did inhibit protease production, as seen in Table XV.

#### Divalent Metal Ion Requirement

The divalent metal ions tested as nutritional requirements for enzyme production at various concentrations were magnesium, calcium, cobalt, copper, and zinc. Only magnesium ion allowed good enzyme production, of which the maximum enzyme production was at the magnesium concentration of  $10^{-2}$  M, see Figure 13.

#### Growth Curves

Growth Curve of Pseudomonas aeruginosa Ps-1C as Correlated to Optical Density at Wavelength 660 nm. There was no measurable lag phase in this experiment, however the data as plotted in Figure 14. does

TABLE VII  
SUBSTRATE TESTS OF GENERAL  
AMINO ACID GROUPS FOR  
ENZYME PRODUCTION

Flask set number	Amino acids and groups tested	Optical density at wavelength 420 nm
I	Monamino and monocarboxyl: alanine, valine, leucine, iso- leucine, serine, threonine, cysteine, and methionine	0.000
II	Dicarboxylic: glutamic acid, aspartic acid	0.015
III	Basic: lysine, histidine, asparagine	0.010
IV	Aromatics: phenylalanine, tyrosine	0.012
V	Heterocyclics: histidine, tryptophane, proline, hydroxyproline	0.012
VI	None - control	0.000

Ten ml of each 0.01% amino acid to be tested was added to a flask and then made up to 100 ml aliquots with Hershey's M-9 medium with 0.01% dextrose. The culture flasks were inoculated from a starter culture, incubated for 14 hours on a shaker at 37° centigrade, and assayed for proteolytic activity.

TABLE VIII  
AMINO ACIDS COMBINATIONS--I TESTED AS  
NUTRITIONAL REQUIREMENTS FOR  
PS-1C PROTEASE PRODUCTION

Optical density	0.010	0.150	0.105	0.028	0.087	0.190	0.150
Flask numbers	1	2	3	4	5	6	7
Amino acids tested	none	ala	ala	-----	ala	-----	150 mg
	con- trol	Ph-A	Ph-A	-----	-----	Ph-A	of crude
		leu	-----	leu	leu	-----	casamino
		IsoL	-----	IsoL	-----	IsoL	acids
		pro	-----	pro	pro	-----	
		OH-P	-----	OH-P	-----	OH-P	
		val	val	-----	val	val	
		gly	gly	-----	gly	gly	

Each amino acid tested was added in 150 mg amounts to 100 ml aliquots of Hershey's M-9 medium with 0.05% dextrose. The test flasks were inoculated from a starter culture, incubated for 14 hours at 37° C on a shaker, and assayed for proteolytic activity. The optical density readings are at wavelength 420 nm, and the abbreviations used are as follows: ala=alanine; Ph-A=phenyl-alanine; leu=leucine; pro=proline; OH-P=hydroxyproline; val=valine; gly=glycine. These abbreviations will be used throughout the following tables.

TABLE IX

AMINO ACID COMBINATIONS-II TESTED AS  
NUTRITIONAL REQUIREMENTS FOR  
PS-1C PROTEASE PRODUCTION

Optical density	0.100	0.253	0.034	0.031	0.028	0.022	0.050	0.058
Flask numbers	1	2	3	4	5	6	7	8
Amino acids tested	Ph-A	Ph-A	Ph-A	Ph-A	Ph-A	---	---	---
	IsoL	IsoL	IsoL	IsoL	IsoL	IsoL	IsoL	IsoL
	OH-P	OH-P	OH-P	OH-P	OH-P	OH-P	OH-P	OH-P
	val	val	----	----	----	----	val	val
	gly	----	gly	----	----	gly	----	gly
	----	----	----	----	pro	pro	pro	pro
	----	----	----	----	----	leu	leu	leu

Each amino acid tested was added in 150 mg amounts to 50 ml aliquots of Hershey's M-9 medium with 0.05% dextrose. The test flasks were inoculated from a starter culture, incubated for 14 hours at 37° C on a shaker, and assayed for proteolytic activity. The optical density readings are at wavelength 420 nm.



TABLE X  
AMINO ACID COMBINATIONS-III TESTED AS  
NUTRITIONAL REQUIREMENTS FOR  
PS-1C PROTEASE PRODUCTION

Optical density	0.323	0.040	0.485	0.191	0.400	0.113	0.013
Flask numbers	1	2	3	4	5	6	7
Amino acids tested	Ph-A	Ph-A	Ph-A	Ph-A	----	600 mg	none
	IsoL	IsoL	IsoL	-----	IsoL	crude	control
	OH-P	OH-P	-----	OH-P	OH-P	casamino	
	val	-----	val	val	val	acids	

Each amino acid tested was added in 150 mg amounts to 100 ml aliquots of Hershey's M-9 medium with 0.05% dextrose. The test flasks were inoculated from a starter culture, incubated for 14 hours at 37° C on a shaker, and assayed for proteolytic activity. The optical density readings are at wavelength 420 nm, and are the average of two experiments.

TABLE XI  
AMINO ACID COMBINATIONS-IV TESTED AS  
NUTRITIONAL REQUIREMENTS FOR  
PS-1C PROTEASE PRODUCTION

Optical density	0.166	0.236	0.023	0.172	0.261	0.345	0.221	0.002
Flask numbers	1	2	3	4	5	6	7	8
Amino acids tested	IsoL val OH-P -----	IsoL val -----	IsoL ----- OH-P -----	----- val OH-P -----	IsoL val OH-P ----- Ph-A	IsoL val ----- Ph-A	IsoL val OH-P -----	None Control

Each amino acid tested was added in 150 mg amounts to 100 ml aliquots of Hershey's M-9 medium with 0.05% dextrose. The test flasks were inoculated from a starter culture, incubated for 14 hours at 37° C on a shaker, and assayed for proteolytic activity. The optical density readings are at wavelength 420 nm, and are the average of two experiments.

TABLE XII  
 ISOLEUCINE CONCENTRATION REQUIREMENT  
 FOR PS-1C PROTEASE PRODUCTION

Amino acid concentration in mg/ml for:			Optical dens- at wavelength 420 nm
Phenylalanine	Isoleucine	Valine	
0.1	2.5	2.0	0.061
0.1	2.0	2.0	0.052
0.1	1.5	2.0	0.061
0.1	1.0	2.0	0.104
0.1	0.5	2.0	0.143
0.1	none	2.0	0.100
none	none	none	0.053

The culture medium consisted of 10 ml aliquots of Hershey's M-9 medium with 0.05% dextrose. The test tubes were inoculated from a starter culture, incubated at 37° on a shaker, and assayed for proteolytic activity. The optical density readings are the average of two experiments.

TABLE XIII  
 PHENYLALANINE CONCENTRATION REQUIREMENT  
 FOR PS-1C PROTEASE PRODUCTION

Amino acid concentration in mg/ml for:			Optical density at wavelength 420 nm
Phenylalanine	Isoleucine	Valine	
1.0	1.0	2.0	0.084
0.5	1.0	2.0	0.087
0.1	1.0	2.0	0.056
0.05	1.0	2.0	0.054
none	1.0	2.0	0.049
none	none	none	0.018

The culture medium consisted of 10 ml aliquots of Hershey's M-9 medium with 0.05% dextrose. The test tubes were inoculated from a starter culture, incubated at 37° C on a shaker, and assayed for proteolytic activity. The optical density readings are the average of two experiments.

TABLE XIV  
VALINE CONCENTRATION REQUIREMENT  
FOR PS-1C PROTEASE PRODUCTION

Amino acid concentration in mg/ml for:			Optical density at wavelength 420 nm
Phenylalanine	Isoleucine	Valine	
0.1	2.0	2.0	0.108
0.1	2.0	1.5	0.078
0.1	2.0	1.0	0.077
0.1	2.0	0.5	0.041
0.1	2.0	0.1	0.009
0.1	2.0	none	0.007
none	none	none	0.003

The culture medium consisted of 10 ml aliquots of Hershey's M-9 medium with 0.05% dextrose. The test tubes were inoculated from a starter culture, incubated at 37° C on a shaker, and assayed for proteolytic activity. The optical density readings are the average of two experiments.

TABLE XV  
TESTS FOR INHIBITION BY EXCESS  
REQUIRED AMINO ACID

Tube set number	Amino acid concentrations in mg/ml:			Comment	Optical density
	Isoleucine	Valine	Phenylalanine		
I	1.0	2.0	0.1	all are minimal	0.065
II	2.0	2.0	0.1	excess isoleucine	0.043
III	1.0	2.0	2.0	Excess phenylalanine	0.144
IV	2.0	2.0	2.0	excess isoleucine, phenylalanine	0.055
V	none	none	none	control	0.053

The culture medium consisted of 10 ml aliquots of Hershey's M-9 medium with 0.05% dextrose. The optical density readings are the average of two experiments, and are at wavelength 420 nm.

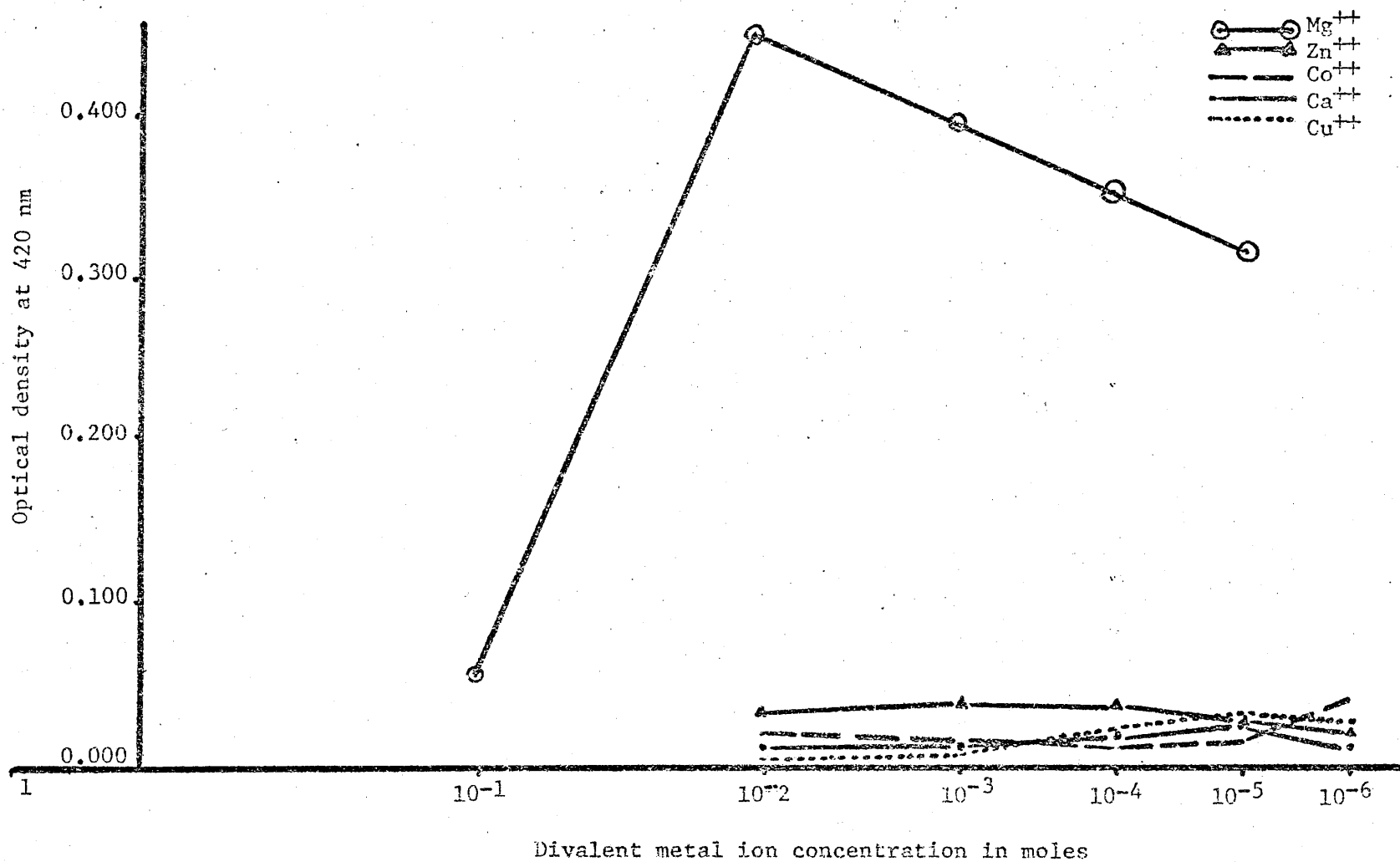


Figure 13. Comparison of metal ion concentration requirement for Ps-lC protease production.

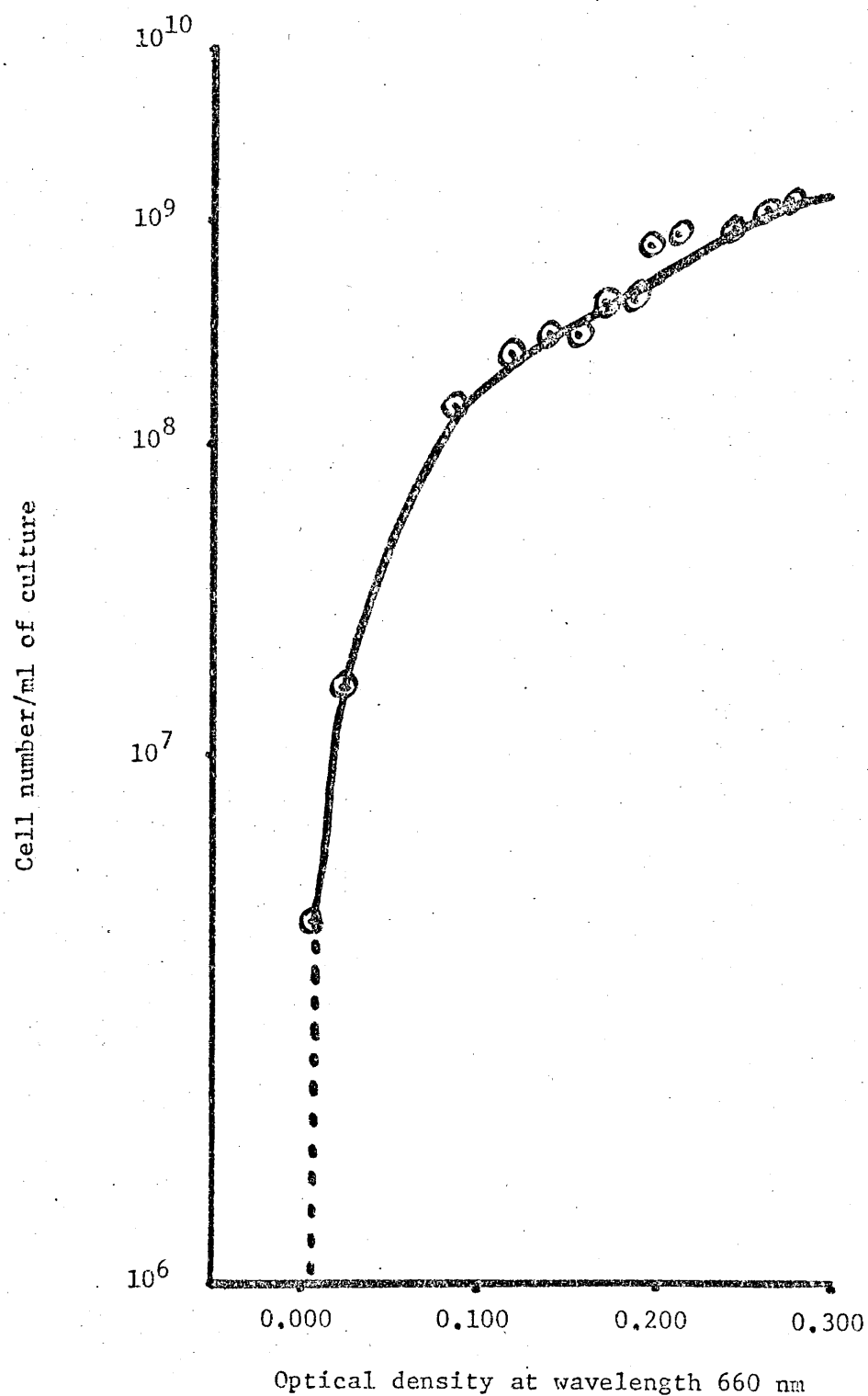


Figure 14. Growth verses optical density at wavelength 660 nm, for Pseudomonas aeruginosa, Ps-1C in nutrient broth-yeast extract.



give adequate information for determining the number of viable cells per ml of starter culture at time of inoculation into test medias. All dilutions of the plate counts at zero time were confluent in growth, and therefore no calculations for cell number was possible. However most of the remaining data was consistent.

Growth and Protease Production Versus Time. Protease production began in mid-log phase of growth and continued throughout log phase and into stationary phase. The enzyme activity leveled off somewhat after 18 hours of growth, but again increased slightly after 20 hours. See Figure 15.

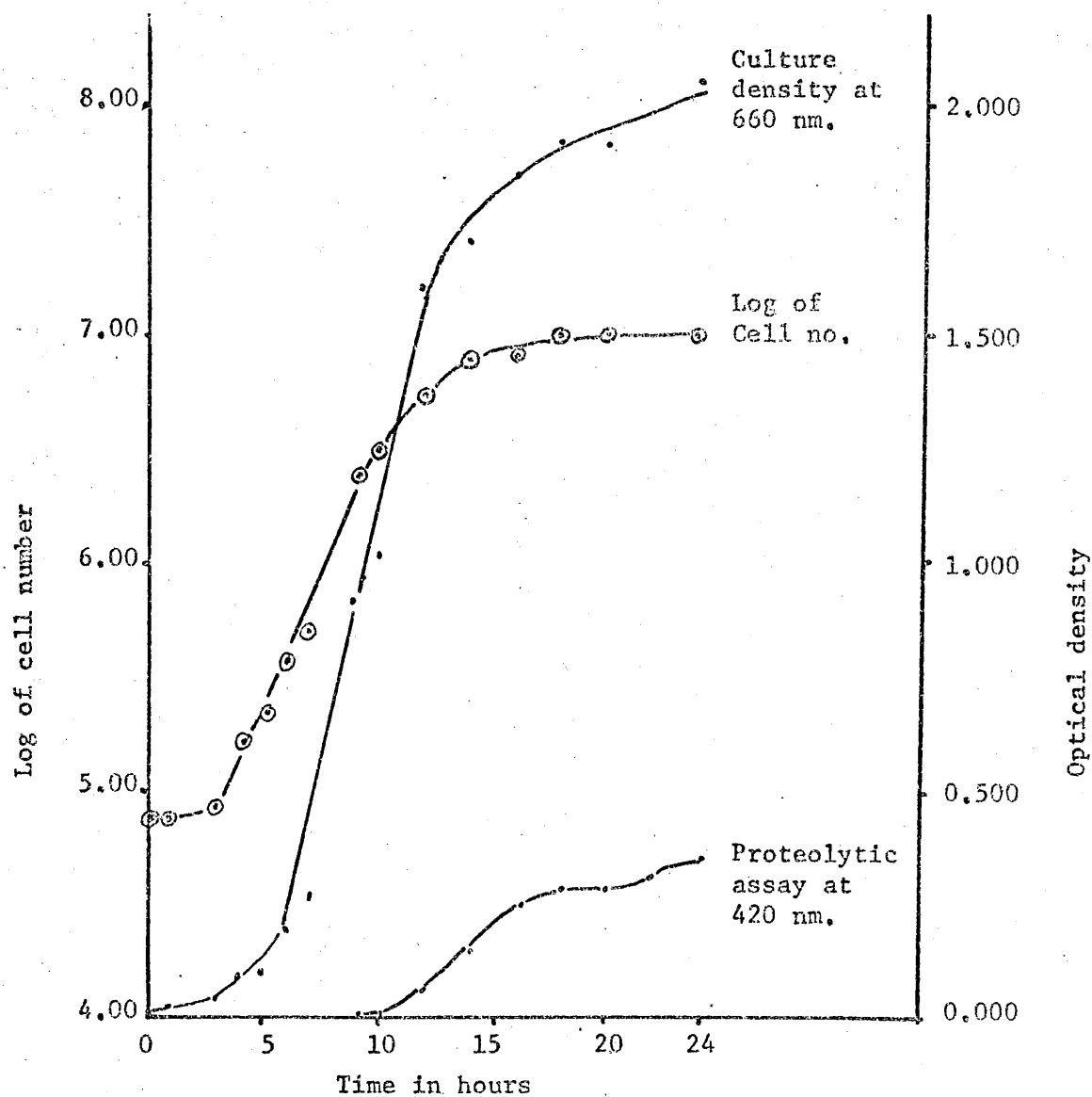


Figure 15. Growth and protease production versus time in *Pseudomonas aeruginosa*, Ps-1C.

## DISCUSSION

### Glucose Inhibition of Protease Production

At concentrations greater than 0.05%, glucose, a rapidly metabolized carbohydrate, apparently inhibits or represses enzyme production, but not necessarily growth. Other investigators have had similar results; Litchfield and Prescott (1970), Keen and Williams (1967) using sucrose and Pseudomonas lachrymans. Norton and Sokatch (1966) also noted that when Pseudomonas aeruginosa was grown on glucose, that the inducible enzyme which catalyzes D-valine oxidation was somehow inhibited.

### Protease Peptone Analysis

Earlier work in the laboratory of Dr. E. Fisher, Jr. (1969) (Unpublished results) indicated that protease peptone allowed the best enzyme production out of five different undefined medias tested. However the only significant thing obtained from the separation techniques applied to protease peptone was that this media probably contained a large number of amino acids which may have contributed to enzyme production, Figure 4.

### Amino Acid Analysis

Various investigators have noted that specific amino acids are required for enzyme production in many different organisms: (Litchfield and Prescott, 1970; Hammel and Zimmerman, 1966; Norton and Sokatch, 1966) The results in this paper indicate that Pseudomonas aeruginosa Ps-1C does in fact require the presence of the three amino acids listed in their respective concentrations for maximum protease production in Hershey's M-9 media. However, the results also indicate, in Tables X, XI, and XII,

that there may be some competition between isoleucine and hydroxyproline, although when tested individually isoleucine does allow greater proteolytic activity to take place than does hydroxyproline. The effect of hydroxyproline cannot be ignored since it is an important constituent of collagen. Although in structure isoleucine and hydroxyproline appear to be very dissimilar,  $\alpha$ -ketoglutarate is a common constituent to the biosynthesis of both amino acids, and this in some way may be involved with competition between them, as a nutritional requirement for induction of Ps-1C protease.

Although isoleucine appears to be required for enzyme production, it also acts as an inhibitor at concentrations greater than 1.0 mg/ml. Sashital and Zimmerman (1968) also indicated that isoleucine and four other amino acids specifically and individually repressed the formation of the induced extracellular protease of Streptococcus faecalis var. Liquefaciens.

McDonald and Chambers (1966) in studying the regulation of proteinase formation in a Micrococcus sp. concluded that because extracellular proteinase formation is induced by amino acids and suppressed by various carbon sources, and because the organism can utilize amino acids as carbon sources for growth, therefore the function of extracellular proteinase of this organism is to ensure a supply of carbon for growth rather than a supply of amino acids for protein synthesis. It has already been documented that Pseudomonas aeruginosa can utilize amino acids e.g. valine, as carbon source and nitrogen source. This study may indicate that the three amino acids presence does induce enzyme production, or at least allows the nutritional requirements for it to occur; and enzyme production is suppressed when concentrations of

dextrose are greater than 0.05%. However it seems to me that the function of the enzyme could encompass both aspects: to ensure a supply of carbon for growth, and to provide a supply of amino acids for protein synthesis.

#### Divalent Metal Ion Requirement

Previous experiments by Fisher (1960) indicated that chelating agents greatly reduced enzyme activity by *Pseudomonas aeruginosa* Ps-1C. Other proteinases of *Pseudomonas aeruginosa* have been shown to require divalent ions, (Moriyama, 1963; Marvin, et. al., 1969). Therefore it was no surprise to determine that  $Mg^{++}$  was required for Ps-1C protease production, while calcium, cobalt, copper, and zinc inhibited production of enzyme as well as growth in some cases, Figure 13.

Proteinases from other organisms also require divalent metal ions such as  $Zn^{++}$  required for protease secretion by *Streptococcus faecalis* (Casas and Zimmerman, 1969), and the  $Mg^{++}$  required for the peptidases of *E. coli*, *Proteus*, *Pseudomonas fluorescens*, and *Pseudomonas tumefaciens*, (Berger, Johnson, and Peterson, 1938 a,b), and the  $Ca^{++}$  requirement for the clostridial collagenase (Gallop, Seifter, and Meilman, 1957; Mandl, Keller, and Manahan, 1964).

#### Enzyme Production Versus Growth

The results of Figure 15 show that the enzyme production begins in mid-log phase of growth and continues into stationary phase, when the organism is grown in M-9 medium with 0.05% dextrose and supplemented with the amino acids, valine, isoleucine, and phenylalanine.

The fact that enzyme is liberated during logarithmic phase of

of growth is evidence that the enzyme is extracellular (Pollock, 1962).

Both plots in Figure 15, i.e. log of cell number, and the culture optical density at 660 nm, as related to the proteolytic assay and against time, substantiate that protease production reaches its maximum activity during stationary phase.

## SUMMARY

1. The organism requires good aeration for enzyme production.
2. The required amino acids and their respective concentrations needed for enzyme production are as follows: 0.5 mg/ml phenylalanine, 0.05 to 1.0 mg/ml isoleucine, and 2.0 mg/ml valine. Isoleucine at concentrations greater than 1.0 mg/ml tend to inhibit active enzyme synthesis.
3. Magnesium at a concentration of 0.01 M fulfills the divalent ion concentration for maximum enzyme formation.
4. Enzyme production is limited when the dextrose concentration is greater than 0.05%, although growth does not appear to be affected.
5. Enzyme production begins during log phase of growth, and peaks during stationary phase.

## REFERENCES

- Bender, J.L., and F.J. Kezdy. 1965. Mechanism of action of proteolytic enzymes. Annual Review of Biochemistry. 34:49.
- Berger, J., M.J. Johnson, and W.H. Peterson, 1938,a. The proteolytic enzymes of bacteria. I. The Peptidases of Leuconostoc mesenteroides. Journal of Biological Chemistry. 124:395.
- Berger, J., M.J. Johnson, W.H. Peterson. 1938,b. The proteolytic enzymes of bacteria. II. The peptidases of some common bacteria. Journal of Bacteriology. 36:521.
- Bidwell, E. 1948. The  $\kappa$ -toxin (collagenase) of Clostridium welchii. Biochemical Journal. 44:28.
- Bidwell, E. 1950. Proteolytic enzymes of Clostridium welchii. Biochemical Journal. 46:589.
- Bidwell, E., W.E. van Heyningen, P.A. Charlwood. 1948. The biochemistry of gas gangrene toxins: The  $\kappa$ -toxin (collagenase) of Clostridium welchii. Biochemical Journal. 42:140.
- Casas, I.A., and L.N. Zimmermann. 1969. Dependence of protease secretion by Streptococcus faecalis var. liquefaciens on arginine and its possible relation to the site of synthesis. Journal of Bacteriology. 97:307.
- Conn, E.E., and P.K. Stumpf. 1964. Outlines of Biochemistry. John Wiley & Sons, Inc., New York. Pp 58-74.
- Eagon, R.G. 1968. Advanced General Microbiology Laboratory Methods. Burgess Publishing Co., Minneapolis, Minn. Pp 25-32.
- Evans, D.G. 1948. The production by certain species of Clostridium of enzymes disintegrating hide powder. Journal of General Microbiology. 1:378.
- Fisher, E. Jr. 1960. Some properties of a proteinase obtained from Pseudomonas aeruginosa. Bacteriological Proceedings. 60:60.
- Fisher, E. Jr., and J.H. Allen. 1958. Corneal ulcers produced by cell free extracts of Pseudomonas aeruginosa. American Journal of Ophthalmology. 46:21.
- Gallop, P.M., S. Seifter, E. Meilman. 1957. Studies on collagen. I. The partial purification, assay, and mode of activation of bacterial collagenase. Journal of Biological Chemistry. 227-891.



- Jennison, M.W. 1945. Bacterial collagenase. Journal of Bacteriology. 50:369.
- Kay, W.W. and A.F. Gronlund. 1969. Influence of carbon or nitrogen starvation on amino acid transport in Pseudomonas aeruginosa. Journal of Bacteriology. 100:276.
- Keen, N.T. and P.H. Williams. 1967. Effect of nutritional factors on extracellular protease production by Pseudomonas lachrymans. Canadian Journal of Microbiology. 13:863.
- Kocholaty, W. and L.E. Krejci. 1948. The activation mechanism and physicochemical properties of Clostridium histolyticum proteinase. Archives of Biochemistry. 18:1.
- Litchfield, C.D. and J.M. Prescott. 1970. Regulation of proteolytic enzyme production by Aeromonas proteolytica. I. Extracellular endopeptidases. Canadian Journal of Microbiology. 16:17.
- Mandl, I., S. Keller, J. Manahan. 1964. Multiplicity of Clostridium histolyticum collagenase. Biochemistry. 3:1737.
- Marvin, L., W.R. Hearn, J.W. Zyskind, D.J. Tipper, J.L. Strominger. 1969. Specificity of a bacteriolytic enzyme from Pseudomonas aeruginosa. Journal of Bacteriology. 100:254.
- Maschmann, E. 1938. Uber bacterien proteasen IX. Die anaerobiase der gasbranderreger. Biochemische Zeitschrift. 297-284.
- McDonald, I.J., A.K. Chambers. 1966. Regulation of proteinase formation in a Micrococcus. Canadian Journal of Microbiology. 12:1175.
- Moriyama, K. 1963. Pseudomonas aeruginosa proteinase. I. Purification and general properties. Biochimica et Biophysica Acta. 73:113.
- Narayanan, E.K., P. Devi, P.S. Menon. 1953. Enzymes of Vibrio cholerae with possible role in pathogenesis. Indian Journal of Medical Research. 41:295.
- Norton, J.E. and J.R. Sokatch. 1966. Oxidation of D- and L-valine by enzymes of Pseudomonas aeruginosa. Journal of Bacteriology. 92:116.
- Pataki, G. 1968. Techniques of Thin-layer Chromatography in Amino Acid and Peptide Chemistry. Ann Arbor Science Publishers, Inc. Ann Arbor, Michigan. Pp 65-84.
- Pollock, M.R. 1962. Exoenzymes, p. 121-170. In I.C. Gunsalus and R.Y. Stanier (ed.), The Bacteria, vol. IV. Academic Press, New York.
- Sashital, K.S., and L.N. Zimmerman. 1968. Catabolite repression and feedback inhibition of protease in Streptococcus faecalis var.

- liquefaciens. Canadian Journal of Microbiology. 14:1265.
- Schoellmann, G., and E. Fisher, Jr. 1966. A collagenase from Pseudomonas aeruginosa. Biochimica et Biophysica Acta. 122:557.
- Weifter, S., P.M. Gallop, L.R. Klein, and E. Meilman. 1959. Studies on collagen. II. Properties of purified collagenase and its inhibition. Journal of Biological Chemistry. 235:285.
- Smith, I. 1958. Chromatographic Techniques. William Heinemann Medical Books, Ltd. London. Pp. 6-35, 59-91.
- Sokatch, J.R. 1966. Alanine and aspartate formation during growth on valine C<sup>14</sup> by Pseudomonas aeruginosa. Journal of Bacteriology. 92:72.
- Somkuti, G.A. and F.J. Babel. 1967. Conditions influencing the synthesis of acid protease by Mucor pusillus Lindt. Applied Microbiology. 15:1309.
- Thimann, K.V. 1963. The Life of Bacteria. The Macmillan Co. New York. Pp. 293-305.
- Tomarelli, R.M., J. Charney, and M.C. Harding. 1949. The use of azoalbumin as a substrate in the colorimetric determination of peptic and tryptic activity. Journal of Laboratory and Clinical Medicine. 34:428.