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THE UNIVERSITY OF ALBERTA

THE PURIFICATION, MODIFICATION AND CHARACTERIZATION OF AN INTRACELLULAR EXONUCLEASE-PHOSPHATASE FROM *PSEUDOMONAS AERUGINOSA*

by

© LAWRENCE E. BRYAN

A THESIS

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for Acceptance, a thesis entitled "The Purification, Modification and Characterization of an Intracellular Exonuclease-Phosphatase from Pseudomonas aeruginosa" submitted by Lawrence E. Bryan in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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ABSTRACT

A deoxyribonuclease of Pseudomonas aeruginosa has been isolated and purified to a single enzymatically active major band on polyacrylamide gel electrophoresis and a second minor band containing a contaminant. Purification was carried out by DEAE cellulose chromatography, ammonium sulfate fractionation, pH 5.2 fractionation, hydroxylapatite, DEAE cellulose and G-75 Sephadex gel filtration. The enzyme underwent modification during early purification steps to produce a range of molecular forms. Binding of polynucleotide fragments, primarily DNA, was demonstrated to produce enzyme molecules which acted larger than the unassociated enzyme on gel filtration and sucrose density gradient centrifugation. Endogenous proteolytic activity was shown to produce a range of differently charged species and molecules of enzyme smaller than the unassociated and undegraded purified enzyme. The polynucleotide binding was eliminated by destruction of those fragments with endogenous DNase activity and the proteolysis by including phenylmethylsulfonylfluoride in purification steps up to and including the ammonium sulfate fractionation.

The DNase activity was labile throughout purification and required the inclusion of 30% glycerol in all purification steps. In addition, mercaptoethanol was necessary to obtain maximal activity, particularly with preparations partly or extensively purified.

The enzyme was shown to be an exonuclease-phosphatase with both activities properties of the same enzyme. The latter was demonstrated by sensitivity to the same inhibiting agents, similar cationic and pH requirements of activity, a constant activity ratio through several purification steps and by eluting both activities from the same fraction of a polyacrylamide gel.

Activity is initiated from the 3' terminus of DNA with the release of a phosphate if present and the subsequent release of 5' mononucleotides. Activity is not dependent on the presence of a 3' phosphoryl group.

Initial activity rates demonstrate a 3 to 4 preference for native DNA over heat denatured DNA. Total solubilization of native DNA can be produced. Maximal activity requires 0.0025 M Mg^{++} at pH 7.8 to 8.2 for the exonuclease and 0.001 M Mg^{++} at pH 7.5 for the phosphatase.

The exonuclease is inhibited by EDTA, PCMB, Zn^{++} and Yeast RNA.

The K_m using unmodified native or heat denatured DNA is 38.5 μg/ml. The inclusion of 0.07 M NaCl in the assay raises the K_m to 140 μg/ml. The V_{max} is reduced by heat denaturation of DNA and increased by an increase in DNA termini produced by micrococcal nuclease.

The molecular weight is estimated by gel filtration to be 42,500.

A second DNase was detected in crude materials in very low quantities. Initial characterization is suggestive that it is an endonuclease.

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LIST OF ABBREVIATIONS

DNA	- deoxyribonucleic acid
RNA	- ribonucleic acid
DNase	- deoxyribonuclease
RNase	- ribonuclease
ATP, CTP	- 5' triphosphates of adenosine and cytidine
dATP, dCTP, dGTP	- 5' triphosphates of deoxyadenosine, deoxycytidine and deoxyguanosine
TTP	- 5' triphosphate of thymidine
CDP, ADP	- 5' diphosphates of cytidine and adenosine
dCDP, dADP	- 5' diphosphates of deoxycytidine and deoxyadenosine
TCA	- trichloroacetic acid
UTCA	- trichloroacetic acid containing 0.25% uranyl acetate (weight to volume)
PCA	- perchloric acid
BSA	- bovine serum albumin
PCMB	- para-chloromercurobenzoate
ORD	- optical rotatory dispersion
EDTA	- ethylenediaminetetraacetate
Tris	- Tris(hydroxymethyl)aminomethane
DEAE	- Diethylaminoethyl
PEI	- Polyethyleneimine
OD	- Optical density
ΔOD	- Change in optical density
p.s.i.	- pounds per square inch
PMSF	- phenylmethylsulfonylfluoride
nm	- nanometer

mm	- millimeter
cm	- centimeter
M	- Molar
μM	- microMolar, micromole
nM	- nanoMolar, nanomole
mM	- milliMolar, millimole
ml	- millilitre
μl	- microlitre
l	- litre
μg	- microgram
mg	- milligram
g	- gram
v/v	- volume per volume
w/v	- weight per volume
$\bar{v}$	- partial specific volume
U	- units
V_e	- elution volume
V_t	- total volume
V_o	- void volume
K_{av}	- partition coefficient between liquid phase and gel phase

INTRODUCTION

The modification of enzymes during their purification is a subject which has received some recent consideration. Modification by several methods has been observed. Sine and Hass (1969) have described peptide bond hydrolysis at elevated pH values producing large electrostatic effects in muscle aldolase. The possibility that borate may act as a catalyst was considered. Funakoshi and Deutsch (1969) have also shown an effect of elevated pH in modifying human carbonic anhydrase to produce multiple electrophoretic and ion exchange species.

Limited proteolytic modification has been investigated in several yeast systems to a considerable extent. Lazarus et al (1966) and Schulze and Colowick (1969) have demonstrated that limited proteolysis is able to produce six forms of yeast hexokinase, all but two of which can be prevented by proteolytic inhibition. Juni and Heym (1968) have shown a remarkable destabilizing effect of proteolysis on yeast pyruvate decarboxylase. Sasaki (1966) has shown 51 amino acids are released from yeast phosphoglyceric acid mutase with no change in conformation as monitored by optical rotatory dispersion during modification of that enzyme, and that the crystalline form of the four derived enzymes and the parent were the same.

Price et al (1969) working with pancreatic DNase were able to prove that the low stability of extensively purified fractions was due to proteolytic contamination.

Bacterial systems have, in general, not been extensively examined for limited proteolytic modification. Hollis and Furano (1968) used phenylmethylsulfonylfluoride, an inhibitor of many serine esterases and thus many proteases, to produce a single factor from E. coli replacing the T_u, T_s and G factors required in protein synthesis. They were able to totally prevent the change of that factor into the G component and considerably retard the change to the T factors. These workers observed that aging of the E. coli extract at 0° produced an enhancement of this degradative process.

Klenow and Hennig (1970), during purification, separated molecules of DNA polymerase from E. coli in which the exonuclease activity was almost absent. These molecules were also produced by treatment of purified polymerase with the protease subtilisin.

In general, the significance of proteolytic modification in the preceding cases has been to produce multiple forms of an enzyme during purification and in some cases to decrease the stability. Pringle (1970), in addition, has demonstrated that fallacious estimations of the molecular weight due to endogenous proteolysis have occurred in the case of yeast hexokinase using the sodium dodecyl sulfate - gel electrophoresis method of estimation.

The role of substrate in modifying an enzyme during purification to produce more than a single species has been postulated by Kimhi and Littauer (1968). Those authors have suggested that two peaks of activity of polynucleotide phosphorylase obtained from DEAE Sephadex are due to association with oligonucleotides. The nature of that binding was not examined.

Another cause of enzyme heterogeneity during purification proposed by Reinhold et al (1968) is a variable content of polysaccharide in the glycoprotein porcine pancreatic ribonuclease. They found extreme polymorphism on ion exchange chromatography and considerable inconsistency in the number of species. Changes in stability were noted though these were not correlated by the authors with change in polysaccharide content. The origin of those changes was not clear.

Several bacterial systems have been examined for enzymes capable of degrading DNA. Such enzymes have been found both extracellularly and intracellularly. The enzymes detected have undergone all levels of characterization from rudimentary to extensive. *E. coli* represents the system most extensively studied with nine enzymes being so far described and extensively characterized. (Lehman *et al*, 1962a, b; Freidberg *et al*, 1969; Braun and Behrens, 1969; Oishi, 1969; Lehman, 1960; Lehman, 1962; Richardson and Kornberg, 1964; Jorgensen and Koerner, 1966; Klett and Cerami, 1968)

DNases have also been found in several other bacteria including *Micrococcus luteus* (*Micrococcus lysodiekticus*) (Strauss and Robbins, 1968; Kaplan *et al*, 1969; Anai *et al*, 1970a, b), *Bacillus subtilis* (Nishimura, 1960; Birboim, 1966; Nester and McCarthy, 1969), *Diplococcus pneumoniae* (Greenberg and Lacks, 1967), *Serratia marcescens* (Eaves and Jefferies, 1961; Nestle and Roberts, 1969a,b), Group A Streptococci (Wannamaker, 1958; Winter and Bernheimer, 1964), *Staphylococcus aureus* (*Micrococcus aureus*) (Cunningham *et al*, 1956), *Lactobacillus* sp. (Subatini and Hotchkiss, 1969), *Pseudomonas fluorescens* (Catlin and Cunningham, 1958) and *Pseudomonas aeruginosa* (Streitfield *et al*, 1962; Guschlbauer and Halleck, 1961). The DNases detected in these organisms have covered a wide range of characteristics.

The classification of DNases can be made on the basis of several criteria (Laskowski, 1967) which include a substrate specificity for DNA, RNA or both, preference for denatured or native DNA or an oligonucleotide as substrate, endo- or exonucleolytic mode of attack, the nature of the product, as well as other more restricted characteristics.

Many enzymes capable of attacking DNA have been shown to be able to hydrolyze RNA. Many extracellular enzymes in particular demonstrate this character. The extracellular nucleases of *Micrococcus aureus* (Cunningham *et al*, 1956), *Serratia marcescens* (Nestle and Roberts, 1969) and *Micrococcus sodonensis* (Berry and Campbell, 1967) are examples. Intracellular enzymes may also be capable of similar hydrolysis as is the case with the extramitochondrial enzyme of *Neurospora crassa* (Linn and Lehman, 1965).

A wide range of specificity for the secondary structure of DNA varies from the 10 to 40,000 to 1 preference of denatured DNA by exonuclease I of *E. coli* (Lehman, 1960) to a definite preference for native DNA by exonuclease III (Richardson, Kornberg and Lehman, 1964). Products released depend on whether hydrolysis occurs on the 3' or 5' side of the phosphorus atom of the phosphodiester bond. No enzyme is capable of both cleavages (Laskowski, 1967).

Thus, for example, exonuclease I of *E. coli* will release products with 5' phosphate (Lehman, 1960) and micrococcal nuclease products with 3' phosphate end groups (Cunningham *et al*, 1956).

Enzymes causing hydrolysis of internal phosphodiester bonds are endonucleases. Endonuclease I of *E. coli* (Lehman *et al*, 1962a, b) is such an enzyme, producing an average oligonucleotide length of 7 units at the termination of digestion.

Exonucleases are enzymes producing hydrolytic cleavages resulting in only mononucleotides. A few enzymes can act in both manners.

Micrococcal nuclease initially produces endonucleolytic cleavages followed by exonuclease attack of small polynucleotides (Sulkowski and Laskowski, 1968).

Exonucleases capable of only DNA hydrolysis exhibit a wide range of characteristics. Attack may initiate from the 5' or 3' terminus of the DNA. Lambda exonuclease initiates activity at the 5' end (Little, 1967b) and exonuclease III the 3' end (Richardson, Kornberg and Lehman, 1964). Products may be 3' mononucleotides as the nonbacterial exonuclease of spleen (Razzell and Khorana, 1961) or 5' mononucleotides as seen with many bacterial enzymes. Some exonucleases require specific groups for activity. Exonuclease I (Adler et al, 1958) of E. coli requires a 3' hydroxyl having no activity on 3' phosphate DNA.

At least five exonucleases occur in E. coli, exonuclease I (Lehman, 1960), exonuclease II and VI (Lehman, 1962; Klett and Cerami, 1968), exonuclease III (Richardson and Kornberg, 1964) and exonuclease IV (Jorgensen and Loerner, 1966). Another exonuclease dependent on ATP has been described by Oishi (1969) but a similar enzyme in M. luteus (Anai et al, 1970a) is an endonuclease. No other bacterial system has yet yielded this large a number of exonucleases.

Some exonucleases have a dual function such as the exonuclease activity of DNA polymerase (Richardson and Lehman, 1964; Klett, Cerami and Rich, 1968). Also some exonucleases have phosphatase activities such as exonuclease III (Richardson, 1964a).

In Pseudomonas aeruginosa the work of Streitfield et al (1962) demonstrated extracellular DNase activity on DNA embedded in agar plates. Guschlbauer and Halleck (1961) carried out $(NH_4)_2SO_4$ cuts of extracellular and intracellular extracts and found two DNases. The extracellular one detected was independent of cations. The intracellular enzyme had maximal activity with the chelating agent citrate and magnesium combined. A pH optimum of 6 was found for that enzyme.

The present study involves an examination of DNase activity in Pseudomonas aeruginosa with an evaluation of factors modifying the enzyme during purification and an extensive characterization of the principal deoxyribonuclease activity in that organism.

MATERIALS AND METHODS

I. Materials

All reagents used were of reagent grade and purchased from commercial sources.

DEAE cellulose, hydroxylapatite and Bio-gels were obtained from Bio-rad Laboratories and Sephadex from Pharmacia.

γ-labelled ATP was a gift of Dr. W. Bridger, Department of Biochemistry, University of Alberta. ^{3}H-TTP, purified *E. coli* DNA polymerase and polynucleotide kinase were the gifts of Dr. V. Paetkau, also of the Department of Biochemistry. Purified *Micrococcus sodonensis* enzyme was kindly provided by Miss Cecily Mills, Department of Microbiology, University of Alberta.

Pseudomonas aeruginosa MAC 264 was obtained from Dr. J. N. Campbell, Department of Microbiology.

Micrococcal nuclease was obtained from Miles Laboratory, and all other enzymes from Worthington Biochemicals.

II. Methods

A. Buffers

1. Glycerol buffer #1: Glycerol buffer contained 0.03 M Tris HCl pH 7.5, 0.01 M mercaptoethanol and 30% glycerol v/v.
2. Glycerol buffer #2: The buffer was identical to #1 except that Tris HCl was replaced by potassium phosphate 0.01 M pH 7.5.
3. Other buffers are as indicated.

B. Stability studies on crude enzyme preparations

The standard method used was storage in stoppered tubes of crude cell free extract at 4°, 22-25° or 37° in 0.05 M Tris HCl pH 7.5 and 0.01 M mercaptoethanol. Activity was examined at appropriate intervals. This procedure was modified as outlined in the Results, section I subsection C to examine the effects of various environmental changes. Addition of reagents was carried out at 0 to 4° and the initial assay was done before transfer to the storage temperature.

C. Growth and harvest conditions

1. Growth

Growth of Pseudomonas aeruginosa MAC 264 was in a complex medium modified from that devised by von Tigerstrom and Razzell (1968).

Tryptone...........................	0.1 %
$NH_4H_2PO_4$...........................	0.3 %
K_2HPO_4...........................	0.4 %
Yeast extract......................	0.1 %
$FeSO_4 \cdot 5H_2O$........................	5 p.p.m.
$MgSO_4 \cdot 7H_2O$........................	0.05 %
Glucose...........................	0.5 %

The $MgSO_4 \cdot 7H_2O$ and glucose were each prepared and sterilized separately. The rest of the mixture was adjusted to pH 7.4 with 5N KOH before sterilization.

Growth was carried out at 30° in two 10 litre New Brunswick Micro Ferm Fermenters at 4 to 8 litres of air flow a minute and stirring at 200 to 400 rpm. Small flask culture of cells was performed with 500 ml of medium in 2 litre erlenmeyer flasks on a New Brunswick Gyratory Shaker Model G-25 at 30°. Inocula were 5% by volume of 6 hour shaker grown cells in each case. Absorbancy at 600 nm was examined on 1.0 ml sterile samples at appropriate times.

The synthetic medium used in some experiments was modified from that of Norris and Campbell (1949).

$NH_4H_2PO_4$	3 g/litre
$FeSO_4 \cdot 7H_2O$	0.5 p.p.m.
K_2HPO_4	3 g/litre
$MgSO_4 \cdot 7H_2O$	500 mg/litre
Glucose	5 g/litre

$MgSO_4$, glucose and the remainder of the medium were sterilized separately. The pH of the medium was adjusted to 7.4 with 5 N KOH before sterilization.

2. Cell harvest

Cells in the late logarithmic phase of growth were harvested by continuous flow centrifugation in a Lourds Beta Fuge Model A-2 centrifuge using a 367 head at 12,000 rpm. The flow rate was 3 to 4 litres per hour. In small scale experiments, cells were collected by centrifugation for 10 minutes at 7,000 rpm in the G.S.A. head of the Sorvall RC2-B centrifuge.

3. Cell breakage

Cells were resuspended, broken, and centrifuged at 4° to produce the 100,000 x g supernatant within 12 hours of the termination of cell growth.

Resuspension of cells was 1 g wet weight in 5 ml of 0.03 M Tris HCl pH 7.5, 0.01 M mercaptoethanol and 0.001 M EDTA.

For large scale purifications, breakage was carried out in 300 ml volumes using the Biosonik III sonic oscillator with a 3/4 inch probe and maximal power of 300 watts for 6 minutes.

The Biosonik B.P.-I was also used in some experiments and early purification sequences. The probe used was 3/8 inch and the average power was 120 watts.

D. Purification

All procedures were carried out at 4° unless otherwise indicated.

1. 100,000 x g supernatant

Cultures after sonic oscillation were centrifuged in the Beckman Model L-2 Ultracentrifuge 30 head at 27,000 rpm for 90 minutes. The supernatant of this treatment is referred to as the 100,000 x g supernatant. To examine activity in the precipitate it was resuspended in 0.1 original volume of 0.03 M Tris HCl pH 7.5 and 0.01 M mercaptoethanol and samples assayed.

2. Cell free extract

In some experiments, sonically broken cells were centrifuged for 10 minutes in the Sorvall RC2-B SS head at 10,000 rpm. The supernatant of this procedure is referred to as the cell free extract.

3. Activation

The 100,000 x g supernatant was adjusted to 30% glycerol, 0.03 M Tris HCl pH 7.5, 0.01 M mercaptoethanol and 0.002 M PMSF. $MgCl_2$ and pancreatic ribonuclease were added to provide final concentrations of 0.0025 M and 20 μg/ml respectively. That mixture was incubated at 37° for 90 minutes. Assays of exonuclease and acid soluble material absorbing at 260 nm were performed at 0 and 90 minutes to assure full activation. Acid solubility was determined on 100 μl samples added to 100 μl of cold 6% PCA. 50 μl of bovine serum albumin 10 mg/ml were added, the preparation mixed and left on ice for 5 minutes. It was centrifuged for 1 minute in the Beckman Microfuge. Absorbancy on 200 μl at 260 nm was determined. Proteolytic activity was assessed at 0 and 60 minutes using the benzoyl arginine ethyl ester assay. Trial batches of 1.0 ml were run on all large scale preparations to insure that 75% or more proteolytic inhibition was present by 60 minutes. In some cases up to 0.005 M PMSF was required to obtain proteolytic inhibition of that degree.

4. DEAE cellulose column #1

DEAE cellulose was placed in 5 times its volume of 1.0 M NaCl and 0.1 N NaOH for 60 or more minutes. It was filtered to dryness on a Buchner funnel and subsequently washed 5 times with 5 volumes of 0.03 M Tris HCl pH 7.5, 0.01 M mercaptoethanol and 0.005 M EDTA. Columns were packed in that buffer. After packing the columns were washed with 2 volumes of 0.03 M Tris HCl pH 7.5, 0.01 M mercaptoethanol, 30% glycerol and 0.005 M EDTA. In large scale purifications, the size of the column used was 2.5 x 60 cm, with a volume of 300 ml. The protein content added to the column as activated 100,000 x g supernatant was 2.1 g in a 100 ml volume. A wash volume of 100 ml of the equilibration buffer was used, followed by a gradient of 550 ml equilibration buffer and 550 ml of buffer with 0.5 M NaCl. Fractions were 20 ml and the flow rate was 30 to 40 ml per hour.

All tubes were assayed for exonuclease activity and absorbancy at 280 nm. Active tubes were combined and concentrated to 0.2 of the original volume of 100,000 x g supernatant in a 60 ml Amicon Ultrafiltration apparatus using a UM 10 filter at 40 p.s.i. of nitrogen.

DEAE cellulose and other gradients were confirmed by examining every third or fourth tube with a Radiometer conductivity meter.

5. pH 5.2 precipitation

5.0 M sodium acetate pH 5.2 was added to produce a final concentration of 0.05 M in the DEAE concentrate. Two N acetic acid was added to produce a pH of 5.2 in the concentrate. The procedure was carried out with stirring and constant pH monitoring on a Beckman expandomatic pH meter. When the pH reached 5.5, 10 minute stirring periods were allowed between acetic aicd additions. At pH 5.2 the mixture was stirred for 20 minutes and then centrifuged at 27,000 x g for 30 minutes in a Sorvall RC2-B centrifuge.

The pH of the supernatant was adjusted to pH 7.0 with 2.0 M Tris before the next procedure was carried out.

6. Ammonium sulfate supernatant

Solid ammonium sulfate was added to the pH 5.2 supernatant containing 12 mg/ml protein with stirring until 50% saturation in water (313 g/1). That preparation was centrifuged at 27,000 x g for 20 minutes. To the supernatant, a further 70 g/1 of ammonium sulfate was added to produce saturation (about 60% saturation in water). It was centrifuged as above and the supernatant dialyzed against 100 volumes of 30% glycerol, 0.01 M potassium phosphate pH 7.5 and 0.01 M mercaptoethanol. It was concentrated by ultrafiltration as in section 4 to 30 ml.

7. Hydroxylapatite chromatography

Hydroxylapatite was washed in 5 volumes of 0.2 M potassium phosphate pH 7.5 once. It was then washed in 5 volumes of 0.01 M potassium phosphate pH 7.5 five times and resuspended as a thin slurry in 0.01 M potassium phosphate and 0.01 M mercaptoethanol. Packing was carried out at the final flow rate of 4 ml per hour. Once packed, the 2.5 x 20 cm column was equilibrated with 1.5 volumes of glycerol buffer #2. 200 mg of protein from step 6 was added in a 15 ml volume. A wash volume of 80 ml of equilibration buffer was added, followed by a 0.01 M to 0.2 M phosphate gradient of 300 ml each. Fractions were 12.0 ml. Active fractions were combined and concentrated to about 3.0 ml by ultrafiltration.

8. DEAE cellulose column #2

A 1.5 x 25 cm column was pre-equilibrated with glycerol buffer #2. The 3.0 ml sample from (7.) containing 30 mg of protein was diluted to 18.0 ml in 30% glycerol and 0.01 M mercaptoethanol in water. That was applied and washed in with a volume equal to 1/3 the column volume. A gradient of 0.01 M to 0.2 M phosphate in glycerol buffer #2 each of 75 ml was used. Fractions of 1.0 ml were collected at a flow rate of 25 ml per hour. Active fractions were concentrated to 1.0 ml by ultrafiltration with an Amicon UM 10 filter.

9. G-75 Sephadex gel filtration

All Sephadex gels were prepared as recommended by Pharmacia and de-aerated by suction. All columns were packed in 0.05 M phosphate pH 7.5 and 0.01 M mercaptoethanol and then equilibrated with 30% glycerol in 0.05 M phosphate pH 7.5 and 0.01 M mercaptoethanol. Void volumes* were determined with dextran blue and total volumes with sodium chloride. Fractions were 3.0 ml eluted with the equilibration buffer. The G-75 column used throughout was 2.5 x 37.5 cm with a void volume of 66 ml and a total volume of 184 ml. The flow rate was 6.0 ml per hour.

E. Other purification methods

Streptomycin sulfate supernatant

Streptomycin sulfate at a concentration of 20 mg/ml was added to cell free extract to a final concentration of 3.33 mg/ml with stirring at 4°. Stirring was continued for 20 minutes and the preparation was centrifuged at 27,000 x g for 20 minutes. The precipitate was discarded. In each such procedure a trial precipitation was carried out on 1.0 ml and slight modifications made as required to maintain 90% of exonuclease activity in the supernatant.

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*void volumes and elution volumes are the volume to the height of the peak.

F. Preparation of DNA

1. Tritiated E. coli DNA

E. coli 5275, a mutant requiring 2 μg/ml of thymine, was grown in a synthetic medium modified from that of Davis and Mingioli (1950).

(1.)	K_2HPO_4	1.4 %
	KH_2PO_4	0.4 %
	$(NH_4)_2SO_4$	0.2 %
	L-arginine	50 mg/litre
	Thymine (unlabelled)	2 mg/litre
	^{3}H-thymine, specific activity = 5.4×10^7 cpm/litre	
(2.)	$MgSO_4 \cdot 7H_2O$	0.04%
(3.)	Glucose	0.4 %

The pH of (1.) was adjusted to pH 7.4 and (1.), (2.) and (3.) were sterilized separately. Growth was at 37° in shake cultures and was continued until late logarithmic phase. Uptake of ^{3}H-thymine was monitored by counting 50 μl samples of the supernatant of 200 μl samples centrifuged for 1 minute in the Beckman Microfuge Model 152. Radioactive counting was carried out in 5 ml of Bray's (Bray, 1960) scintillant in a Nuclear Chicago Mark I Liquid Scintillation Counter. Uptake of thymine was 30 to 60% depending on the age of the thymine preparation. Cells were recovered by centrifugation in the Sorvall RC2-B G.S.A. head at 7,000 rpm for 10 minutes. The DNA was isolated by the method of Marmur (Marmur, 1961) and was resuspended in 0.01 M NaCl at 250 μg/ml. The specific activity was adjusted with unlabelled *E. coli* DNA at the same concentration to 2.8×10^3 dpm per nM of nucleotide equivalent.

2. Unlabelled E. coli DNA

Preparation was identical to that for labelled E. coli DNA except that E. coli B was grown in the same medium with no added thymine.

3. ^{32}P-labelled E. coli DNA

^{32}P-labelled DNA was prepared by a modified method of Grossman (Grossman, 1967). The medium used was as follows.

$MgSO_4 \cdot 7H_2O$	1.00	mg/l
$MnSO_4 \cdot 7H_2O$	0.1	mg/l
$FeSO_4 \cdot 7H_2O$	0.05	mg/l
NH_4Cl	500	mg/l
NH_4NO_3	100	mg/l
Na_2SO_4	100	mg/l
2 M Tris HCl pH 7.4	50	ml/l
Bactopeptone	5	g/l
Glucose	2	g/l
^{32}P	10	mc
H_2O	to 1 litre	

The glucose, $MgSO_4 \cdot 7H_2O$ and the remainder of the medium were each sterilized separately. The Bactopeptone contained only sufficient phosphate to support growth to about 0.5 OD at 600 nm. Sterile 1 M phosphate buffer pH 7.5 was added to a concentration of 750 µg/l, which supported growth to 2.5 OD at 600 nm in late logarithmic phase. Uptake of ^{32}P was monitored exactly as for the uptake of tritium. Growth was stopped at 2.5 OD at 600 nm and cells were centrifuged as in (1.) and (2.) above. The DNA was isolated by the method of Marmur (Marmur, 1961). Uptake of ^{32}P was 94% and the specific activity was 5.8×10^4 dpm/nM of nucleotide equivalent.

4. Modified DNA

(a) 3' phosphoryl DNA

Labelled DNA was modified to produce 3' phosphoryl termini with micrococcal nuclease by the method of Richardson and Kornberg (1964a).

DNA......................	230 $\mu g/ml$
Glycine pH 9.3...........	0.05 M
$CaCl_2$.....................	0.01 M
Micrococcal nuclease......	20 $\mu g/ml$

The preparation was incubated at 37° until 25 to 30% of the label (^3H or ^{32}P) was solubilized. 50 μl samples were mixed with 50 μl of cold PCA in a microtube in an ice bath and 25 μl of 10 mg/ml BSA was added. The preparation was mixed and left 5 minutes. It was centrifuged for 1 minute in a Beckman Microfuge and 100 μl of the supernatant counted in Bray's scintillant in the Nuclear Chicago Mark I Liquid Scintillation Counter.

The preparation was cooled in ice at the termination of the digest and dialyzed twice against 1000 volumes of 1.0 M NaCl for 12 hours at 4°. A final dialysis was carried out against 0.01 M NaCl for 24 hours. Acid soluble background was less than ½ of 1% of the total cpm present. Estimations of DNA were based on the extinction at 260 nm with 20 OD's representing 1 mg/ml DNA.

(b) 5' phosphoryl DNA

1×10^{-3} $\mu g/ml$ of pancreatic DNase was used in a standard assay mix to produce 30% solubilization of the ^{32}P-DNA.

It was then transferred to 4° and 0.1% sodium dodecyl sulfate (w/v) added and dialyzed as in (a) above, and concentrated by pervaporation to 250 μg/ml DNA. No residual endonuclease activity remained. The DNA was used only for phosphatase assays.

(c) ^{32}P-labelled 5' phosphoryl termini of tritiated *E. coli* DNA

Standard ^{3}H *E. coli* DNA was treated as in (4) (a) above to produce new 3' phosphoryl termini and 5' OH groups. (Cunningham *et al*, 1956) It was mixed as follows:

DNA	35 μg/800 μl final vol.
Tris HCl pH 7.5	0.025 M
$MgCl_2$	0.01 M
Mercaptoethanol	0.017 M
Potassium phosphate pH 7.5	0.014 M
γ^{32}P-ATP (specific activity = 11,750 dpm/nMole)	0.044 M
Polynucleotide kinase	10 units

The incubation was carried out at 37° with new kinase added at 15, 30 and 45 minutes. At 60 minutes EDTA was added to 0.01 M and sodium dodecyl sulfate to 0.1% (w/v) to stop the reaction. It was twice dialyzed against 1000 volumes of 1.0 M NaCl for 12 hours, and finally against 1000 volumes of 0.01 M NaCl for 12 hours. The preparation was concentrated by pervaporation at 25° to produce a DNA concentration of 125 μg/ml. ^{32}P counts contained in 5 μg were 626 and 12% of the original γ^{32}P of ATP was recovered in the acid insoluble DNA. Background of acid soluble material was about 5%. The method used was modified from that of Richardson (Richardson, 1965).

(d) Denatured DNA

DNA at a concentration of 250 μg/ml was heated in a boiling water bath for 10 minutes and cooled rapidly in an ice water bath. The hyperchromicity associated was 37 to 39%.

G. Assays and analytical procedures

All readings of absorbancy were performed in 1 cm light path cuvettes.

1. Standard exonuclease assay

^{3}H-DNA was added to a final concentration of 50 μg/ml, Tris HCl pH 7.8 to 0.05 M, $MgCl_2$ to 0.0025 M, and mercaptoethanol to 0.01 M.

The preparation was maintained in an ice bath until the addition of sufficient enzyme (also on ice) to produce 6 to 32% solubilization was added. The preparation was stirred rapidly on a vortex mixer and transferred to a 37° water bath. The final volume was 100 μl and all assays were done in Beckman Microtubes. Timing was initiated 5 seconds after transfer to 37°. Each time value was an individual assay. Assays were stopped with 100 μl of cold 6% PCA and immediately transferred to an ice bath. 50 μl of 10 mg/ml BSA were added, the preparation was mixed and left on ice for at least 5 minutes. It was then centrifuged for 1 minute on a Beckman Microfuge and 200 μl were transferred to 5.0 ml of Bray's scintillant. 10 μl of 5 N KOH were added to restore neutrality. Any excess of KOH produced very high counts due to fluorescence. The preparation was counted at 4° in a Nuclear Chicago Mark I Liquid Scintillation Counter. One unit of activity is the production of 1 nM of acid soluble nucleotide equivalent of DNA in 12 minutes. Specific activity is the number of units per milligram of protein.

2. Phosphatase assay

The procedure was identical to that for the exonuclease assay, except that the final 200 µl sample was not transferred to Bray's scintillant. Instead, it was transferred to a new microtube and 25 µl of 25% Norit (v/v) and 25 µl of 5 mg/ml BSA, 0.025 M sodium pyrophosphate and 0.025 M sodium phosphate were added. The tube was thoroughly mixed for 1 minute on the maximal setting of a Genie Vortex Mixer (Fisher Scientific). After 5 minutes on ice, the mixture was centrifuged on the Microfuge for 1 minute and the supernatant was transferred quantitatively to a new tube and the above additions and mixing repeated. After a further 5 minutes on ice, the mix was again centrifuged and 200 µl of supernatant counted as for the exonuclease assay. The assay method was modified from that of Richardson (Richardson et al, 1964a, b). The unit is the production of 1 nM of phosphate in 12 minutes.

3. Exonuclease assays using ^{32}P-DNA

In the comparison of the ratio of exonuclease to phosphatase activity in various enzyme samples, ^{32}P-DNA was used for exonuclease activity also. The error due to inorganic phosphate release was compensated for by subtracting the value obtained in a phosphatase assay for each time period from the value obtained for the exonuclease. The answer resulted in a value for the acid soluble products containing no free inorganic phosphate.

4. Modified exonuclease assays

(a) Acid DNase

The assays are identical to (1) above except that the the buffer was 0.05 M acetate pH 5.2 and no cation was present. Instead EDTA at a concentration of 0.005 M was present. The assay procedure is modified from that of Bernardi (Bernardi, 1966).

(b) Acid or alkaline assays

The assays are identical to (1) above except at pH 5.2, 0.05 M acetate was the buffer and at pH 9.3, glycine 0.05 M was used.

(c) UTCA, TCA assays

The assays are identical to (1) above except that assays were terminated in 10% TCA or 10% TCA with 0.25% uranyl acetate (UTCA) (Helleiner, 1955).

(d) Micrococcal nuclease assays

A mix of 100 μl identical in composition to that given for the preparation of 3' P-DNA was used. The assays were treated otherwise as in (1) above.

(e) Hyperchromicity assay

The assay mix is identical to that of the radioactive assay except unlabelled E. coli DNA was used and the volume was doubled. The increment in absorbancy at 260 nm was monitored continuously with a Gilford 2000 recorder and a Beckman DU monochromator. 200 μl samples of the assay mixture less the enzyme were placed in quartz microcuvettes in the cuvette holder of the spectrophotometer. The heating chamber was adjusted to 37° by a circulating water pump and samples were incubated for 5 minutes prior to enzyme addition. Readings were carried out for 5 minutes after the enzyme addition and the absorbancy change after correction for a blank with no DNA present and a blank with no enzyme present was calculated from the linear portion of the slope. Activity is expressed as change in OD_{260}/minute. The assay is used only qualitatively to determine activity.

5. Micrococcus sodonensis nuclease assay

This enzyme was assayed for DNase activity and 5' nucleotidase activity as described by Berry and Campbell (1967).

6. Phosphodiesterase assays

(a) Phosphodiesterase I was assayed according to Razzell (unpublished method) using p-nitrophenylthymidine 5' phosphate at 0.5 mM concentration in 0.05 M Tris HCl pH 7.5 or 9.3 and $MgCl_2$ at 0.005 M. The 0.12 ml assay was incubated at 37° and terminated with 0.25 ml of 0.3 N NaOH. The absorbancy at 400 nm was determined relative to a blank containing no enzyme.

(b) Phosphodiesterase II was assayed by the method of Razzell (unpublished method). Final volume was 0.12 ml containing 0.1 ml of stock containing $(NH_4)_2$acetate pH 5.9 0.25 M, EDTA 0.001 M or $MgCl_2$ 0.0025 M, p-nitrophenylthymidine 3' phosphate 0.001 M and Tween 80 0.05% (v/v). Enzyme was present up to 30 μl per assay. Incubation was carried out at 37° for 6 hours and terminated by 0.25 ml of 0.3 N NaOH. Absorbancy at 400 nm relative to a blank assay with no enzyme was determined. For assays at pH 7.5, Tris HCl at a final concentration of 0.05 M was used.

7. Phosphomonoesterase assays

(a) Hydrolysis of p-nitrophenylphosphate

The assay was modified from that of Garen and Leventhal (1960). It contained p-nitrophenylphosphate 0.001 M, Tris HCl pH 8.0 0.075 M, $MgCl_2$ 0.0025 M and enzyme up to 50 μl with a final volume of 0.2 ml. The incubation at 37° was maintained until color change occurred. The assay was transferred to ice and the absorbancy at 410 nm relative to a blank containing no enzyme was determined. The molar extinction coefficient for p-nitrophenol at 410 nm is 1.62×10^4 and a unit is the production of 1 μM of that material in one minute.

(b) 5' nucleotide assay

The assay contained 5' ribo- or deoxyribonucleotide at a concentration of 0.002 M, Tris HCl pH 7.8 0.05 M, $MgCl_2$ 0.0025 M and mercaptoethanol 0.01 M in a final volume of 100 μl. Enzyme was present up to 20 μl per assay. Incubations were at 37° for up to 2 hours and were terminated by an equal volume of 6% cold PCA and 50 μl of 10 mg/ml BSA. After 5 minutes in ice, the mix was centrifuged for 1 minute in a Beckman Microfuge. 100 μl of the supernatant were examined at 0 and 120 minutes for phosphate content by the Ames-Dubin method. A unit is the hydrolysis of 1 μM of substrate per minute.

8. RNase assay

RNase assays contained yeast RNA 1 mg/ml, Tris HCl pH 7.8 0.1 M, EDTA 0.005 M and mercaptoethanol 0.01 M. In some cases, EDTA was replaced by 0.0025 M $MgCl_2$. Final volume was 100 μl containing up to 50 μl of enzyme. Incubation was at 37° and was terminated with an equal volume of cold 6% PCA and 50 μl of 10 mg/ml BSA. After 5 minutes in ice, the preparation was centrifuged in the Beckman Microfuge for 1 minute. Absorbancy on 200 μl samples was determined at 260 nm. One unit is the production of 1 OD_{260} unit of acid soluble material per minute.

9. DNA polymerase

DNA polymerase activity was determined by an assay similar to that of Richardson, Schildkraut, Aposhian and Kornberg (1964). It contained 0.067 M glycine pH 9.2 or 0.075 M phosphate pH 7.5, 0.0067 M $MgCl_2$, 0.001 M mercaptoethanol, 0.0002 M dATP, dGTP, dCTP, and ^{3}H-TTP (specific activity of ^{3}H-TTP was 15,000 dpm/nM) and 1.0 OD_{260}/ml of activated calf thymus DNA. The calf thymus DNA was prepared by the method of Aposhian and Kornberg (1962). Final volume was 250 µl and enzyme up to 20 µl was added. Incubation was at 37° with 50 µl samples removed at 10, 20, 30 and 40 minutes to Whatman 1 MM filter discs previously treated with 50 µl of 0.01 M EDTA and a 1:10 dilution of saturated sodium pyrophosphate. After sampling, the filters were left 60 seconds at room temperature and then placed into 5% cold TCA. The discs were washed 3 times for 15 minutes at 4° in 5% TCA and twice in ethanol for 10 minutes at room temperature. The filters were counted in 10 ml of Omnifluor (14.4 g per 3.8 l) scintillant in the Nuclear Chicago liquid scintillation system used throughout. One unit is 10 nM of total nucleotide incorporation into acid insoluble product in 30 minutes.

10. Proteolysis assays

(a) Casein hydrolysis

A method similar to that of Kunitz (Kunitz, 1947; Rick, 1963) was used to measure casein hydrolysis. The assay mix contained 1.0 ml of 1.0% casein in 0.1 M phosphate buffer pH 7.6, enzyme up to 200 µl and phosphate buffer 0.1 M pH 7.6 to a final volume of 2.0 ml. The assays were incubated at 37° for 20 minutes at which time 3.0 ml of 5% TCA was added. The mixture was allowed to stand at room temperature for 30 minutes and then centrifuged in the Sorvall RC2-B centrifuge at 8,000 rpm in the SS head for 7 minutes. Absorbancy at 280 nm was determined on 1.0 ml of the supernatant.

Blanks were prepared by adding an identical volume of the sample to be assayed to 3.0 ml of 5% TCA followed immediately by sufficient phosphate buffer to produce a final volume of 5.0 ml. These were then treated at room temperature and centrifuged as indicated above.

In some cases samples were first adjusted to pH 3.0 with 1 N HCl and allowed to remain at room temperature for 5 minutes. The samples were then treated as normal samples. The incubation pH in the assay was not detectably changed by this treatment.

(b) Benzoyl 1-arginine ethyl ester hydrolysis

The assay contained 0.95 ml of benzoyl 1-arginine ethyl ester (0.343 mg/ml in 0.05 M Tris HCl pH 8.0), up to 0.05 ml of sample and sufficient of the Tris buffer to give a 1.0 ml final volume. A blank containing only substrate and a blank containing an identical sample in Tris buffer only were also used.

The mixtures minus the sample were incubated at 37° for 1 minute and the samples were added. The change in absorbancy at 254 nm for 5 minutes was recorded on a Model 2000 Gilford recorder using a Beckman DU monochromator. The values obtained for the blanks were summed and subtracted from the assay. The molar extinction coefficient at 254 nm for benzoyl l-arginine is 1.15×10^3. A unit is the release of 1 µM of benzoyl l-arginine in one minute (Schwert and Takenaka, 1955; Rick, 1963).

(c) Ninhydrin assay

The ninhydrin assay was used in the detection of proteolysis of endogenous protein. Cell free extract was incubated at 37° in the presence and absence of 0.002 M PMSF. Up to 20 µl samples obtained at zero and after 90 minutes incubation were added to the ninhydrin assay. That assay contained 200 µl of a solution of ninhydrin prepared as follows: 20 g of ninhydrin was dissolved in 500 ml of methyl cellosolve and mixed with 0.8 g $SnCl_2 \cdot 2H_2O$ dissolved in 500 ml 0.1 M citrate buffer pH 5.0. The sample and the ninhydrin solution were heated in a boiling water bath for 20 minutes. 1.0 ml of water and n-propanol (1:1 by volume) was added and absorbancy at 570 nm determined. Standard preparations of 0.25 to 2.0 nM leucine were used to prepare a standard curve. Results were obtained in leucine equivalents (Spies, 1957).

11. Protein

Protein determinations were by the method of Lowry (Lowry, 1951) and by the absorbancy ratio at 280/260. Except for the Sephadex G-75 stage, all protein evaluations were by both methods.

12. Inorganic phosphate determination

The method used was that of Ames and Dubin (1960).

13. Diphenylamine procedure for deoxyribose

The diphenylamine reagent was used to detect deoxyribose spectophotometrically by a micromethod (Steele _et al_, 1949).

Deoxyribose of pyrimidine nucleosides or nucleotides is not measured in this procedure (Ashwell, 1957).

14. Orcinol procedure for pentoses

The Bial method modified by Mejbaum (1939) and Horecker et al (1951) was used to detect pentoses. Compensation for glucose was by the method of Dische et al (1953).

H. Other methods

1. Polyacrylamide gel electrophoresis

The method of Davis (1964) was used to perform polyacrylamide gel electrophoresis. Analytical electrophoresis was performed at room temperature with a current of 2 ma per tube for 110 minutes. Staining was with 1% Amido Schwartz for 1 hour. Electrophoresis performed for recovery of activity was at 4° with all buffers precooled to 4°. Sample gels were not used. In their place 0.2 ml of 40% sucrose was added to a tube as the first step in gel formation. After polymerization, the sucrose was removed. Samples in 30% glycerol were then applied into the tubes after they had been placed in the apparatus containing the buffers. At the termination of the electrophoresis, gels were placed on glass overlying plotting paper and sliced into 15 sections. Each section was placed in 200 µl of 30% glycerol, 0.025 M potassium phosphate pH 7.5 and 0.01 M mercaptoethanol at 4°. 50 µl samples were assayed in the exonuclease and phosphatase assays after 24 hours of elution.

Electrophoresis was carried out at pH 8.3 in all cases except one G-75 sample which was run at pH 4.0 using acetate buffer.

2. DEAE cellulose chromatography in 7 M urea

DEAE cellulose was prepared as for purification. 1 x 22 cm columns were packed and equilibrated with 0.02 M Tris HCl pH 7.8 and 7 M urea. Samples were diluted 1:1 with the same urea buffer and applied to the column, followed by 10 ml of that buffer as a wash. Gradients of 500 ml urea buffer and 500 ml of 0.4 M NaCl in the same buffer were run. Fractions of 4.5 ml at a flow rate of 25 ml/hour were collected. Samples were examined for absorbancy at 260 nm and where applicable 200 μl samples were counted in Bray's scintillant (Tomlinson and Tener, 1962; 1963).

3. Micrococcus sodonensis incubations to determine the nature of the phosphoryl linkage in mononucleotide products

A large scale digest with P. aeruginosa exonuclease was set up containing E. coli DNA 0.5 mg/ml, Tris HCl pH 7.8 0.05 M, $MgCl_2$ 0.0025 M, 0.01 M mercaptoethanol, E. coli ^{3}H-DNA 12.5 μg/ml and 28 units of enzyme per ml. 0.75 ml final volume was allowed to incubate at 37° until 40% solubilization had occurred. 50 μl of 60% PCA and 250 μl of 10 mg/ml BSA were added at the termination of the incubation. After sitting for 5 minutes in ice the preparation was spun in 350 μl volumes in microtubes in the Beckman Microfuge for 1 minute. The supernatant was neutralized with 5 N KOH and the insoluble perchlorates centrifuged off as above. The supernatant was concentrated by pervaporation to 100 μl containing 15.2 OD/ml at 260 nm. An identical procedure was repeated with a 1.5 ml final volume preparation and was concentrated to 33 OD_{260} units/ml in 100 μl.

Micrococcus sodonensis (Berry and Campbell, 1967b) enzyme digestion mixtures of 25 μl were set up with Tris HCl pH 8.8 0.036 M, $MgCl_2$ 0.0144 M, $MnCl_2$ 0.002 M, mercaptoethanol 0.01 M and EDTA 0.001 M. Substrate for controls was present as dCMP at a concentration of 2 μM/ml. For analysis of products, the amount of substrate was based on 11×10^3 OD_{260} as an average molar extinction coefficient for the four deoxyribonucleotides in equal concentrations. The final substrate concentration was 1.08 μM/ml and 2.35 μM/ml using that figure.

The enzyme present was 0.1 units and the incubation time 120 minutes. One unit releases 1 μM of phosphate per hour.

4. Sucrose density gradients

4.8 ml gradients were prepared at 25° by adding 2.4 ml of 20% sucrose in 0.05 M Tris HCl pH 7.5 and 0.01 M mercaptoethanol and 2.4 ml of 5% sucrose in the same buffer to each side of a 5.0 ml gradient mixer. Stirring was maintained on the side closest to the outlet throughout. A flow rate of 4.8 ml per hour was maintained by a Buchler polystaltic pump. Gradients were chilled one hour or more up to four hours prior to use. Centrifugation was in the Beckman Model L-2 Ultracentrifuge SW 39 head at 35,000 rpm for 10 or 16 hours. At the termination of the run, tubes were placed at 4° and sampled to 24 tubes by puncturing the gradient tube bottom and collecting 15 drops per tube. Samples were periodically checked for linearity of gradients by using a refractometer and by using alkaline phosphatase as a marker in one gradient tube.

5. Chromatography

(a) Paper

Descending paper chromatography for 7 hours using saturated ammonium sulfate 79 parts, 0.1 M ammonium acetate pH 6.2 19 parts and ethanol 2 parts according to Greenberg and Lacks (1967) was used.

Spots were located by UV for standards and by ^{32}P radioactivity using the Nuclear Chicago Actigraph III Model 1002 for labelled products.

(b) PEI cellulose

PEI cellulose sheets obtained from J. T. Baker Chemical Co. were used according to Randerath and Randerath (1967). Sheets were prewashed in 1.0 M NaCl once for 1 minute and rinsed in distilled water 5 or more times. Sheets were run by ascending chromatography in 1 N acetic acid until the solvent had risen 4 cm. They were then transferred to 0.3 M LiCl for about 2 hours. Sheets were removed, dried and spots located by UV light.

6. Molecular weight determinations by gel filtration

The method of Andrews (1964) was used to estimate molecular weights by gel filtration. The G-75 column used was that used in purification. The G-200 column prepared in an identical manner was 2.5 x 35.0 cm with a void volume of 63 ml and a total volume of 166 ml. In both cases fractions were 1.0 ml and the flow rate 6 ml per hour.

The gels were eluted with 0.05 M potassium phosphate, 0.01 M mercaptoethanol and 30% glycerol.

The concentration of protein solutions used was 4 mg/ml except for the exonuclease which was 0.25 mg/ml and sample volumes were 0.5 ml.

Molecular weights for the standards were as follows:

cytochrome c...............	12,270	(Sigma Biochemicals)
soybean trypsin inhibitor...	21,500	(Wu and Scheraga, 1962)
pepsin.....................	35,000	(Bovery and Yanari, 1960)
ovalbumin..................	46,000	(Warner, 1954)

The standards were detected by the absorbancy peaks produced at 280 nm.

7. Ultracentrifugal studies

The Beckman Model E Ultracentrifuge utilizing Rayleigh interference optics was used to perform sedimentation equilibrium studies. The buffer used was 0.05 M potassium phosphate pH 7.5 and 0.01 M mercaptoethanol. The procedure was carried out on tubes from the Sephadex G-75 step selected to contain no detectable RNase activity. These were dialyzed against the above buffer to eliminate glycerol. Protein concentration was 0.30 mg/ml and rotor speeds were 22,000, 30,000 and 36,000. The $\overline{V}$ was assumed to be 0.73. The method used was that of meniscus depletion (Yphantis, 1964; Chervanka, 1969).

RESULTS

I. Modification and Purification of an Exonuclease of Pseudomonas aeruginosa

A. Enzyme production and cell growth

1. Kinetics of growth and enzyme production in small flask cultures

(a) The growth rate and yield of cells at the termination of the logarithmic phase of growth for the complex and synthetic media are seen in Table I.

(b) Kinetics of cell growth and enzyme production for the two media are seen in Fig. 1. Specific activity is seen to decline as growth progresses.

(c) The total yield of activity before and after activation of cell free extract is seen in Table II.

2. The effect on growth rate, cell yield, and specific activity of scaling up cell production is seen in Table III. A noticeable decline in specific activity and an increase in the final cell density as indicated by absorbancy at 600 nm is evident.

3. The decision to harvest cells in late lagarithmic growth phase in spite of declining specific activity and from fermentor batches in spite of the lower specific activity was justified by the increased total enzyme yield associated with the larger cell yield obtained.

B. Activity distribution in cells disrupted by sonic oscillation

Less than 1% of the total activity present was found in the resuspended precipitate of the 100,000 x g (see Materials and Methods) preparation. Exceptions to that statement occurred only in circumstances where cell breakage was known to be incomplete.

TABLE I

CELL GROWTH OF PSEUDOMONAS AERUGINOSA

IN COMPLEX AND SYNTHETIC MEDIA

Medium	Doubling Time (Minutes)	Absorbancy at 600 nm at termination of logarithmic growth phase	Wet Weight of cells per litre
A. Complex	54	1.55	2.6 g
B. Synthetic	127	1.4	2.4 g

TABLE II

TOTAL YIELD OF EXONUCLEASE ACTIVITY IN CELL FREE EXTRACTS OF PSEUDOMONAS AERUGINOSA DURING GROWTH IN COMPLEX AND SYNTHETIC MEDIUM

Medium	Absorbancy at 600 nm	Total Activity (units)	
		Before Activation	After Activation
Complex	0.9	1345	4788
	1.55	2314	6926
	1.75	2640	7989
	2.2	2915	9081
Synthetic	0.45	820	2340
	0.9	1610	4750
	1.4	2410	6950
	1.75	2625	7850

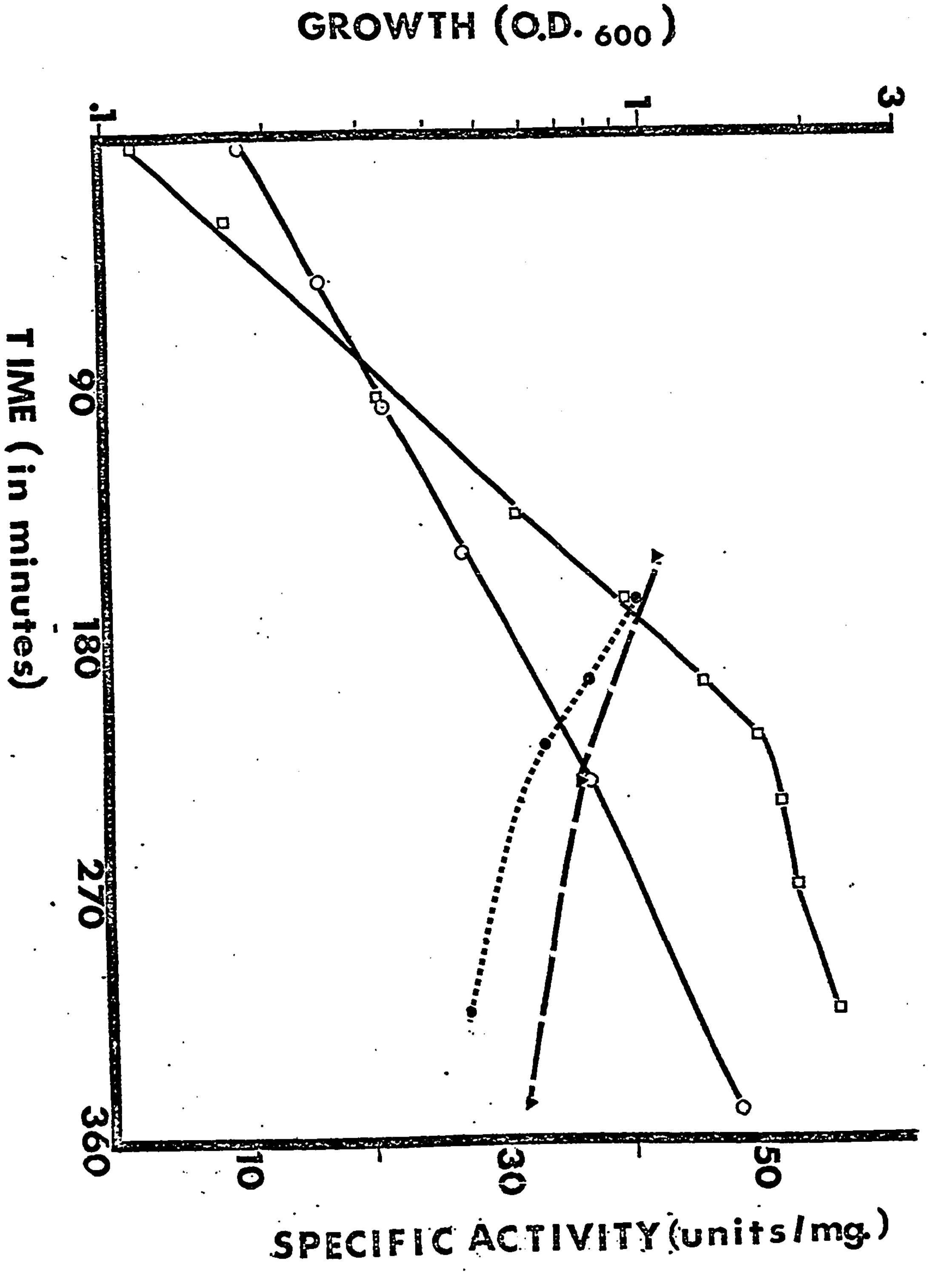

GROWTH (O.D. 600)
.1
1
3
TIME (in minutes)
90
180
270
360
10
30
50
SPECIFIC ACTIVITY (units/mg.)

FIGURE 1

KINETICS OF CELL GROWTH AND DNASE PRODUCTION
IN PSEUDOMONAS AERUGINOSA

The procedures for growth, cell preparation and assays are given in Materials and Methods. Specific activities are post-activation. Protein determination for specific activities was by the method of Lowry.

□————□ Absorbance at 600 nm - complex medium
●----------● Specific activity - complex medium
○————○ Absorbance at 600 nm - synthetic medium
▲----------▲ Specific activity - synthetic medium

TABLE III

CELL GROWTH AND EXONUCLEASE YIELD IN CULTURES OF PSEUDOMONAS AERUGINOSA GROWN IN SMALL FLASKS AND FERMENTORS

Type of Growth	Doubling Time (Minutes)	Average Absorbancy at 600 nm at time of yield	Specific* Activity (units/mg)
Small flask	54	1.55	26 - 40
Fermentor	50 - 60	1.8 - 2.2	15 - 25

*Post-activation

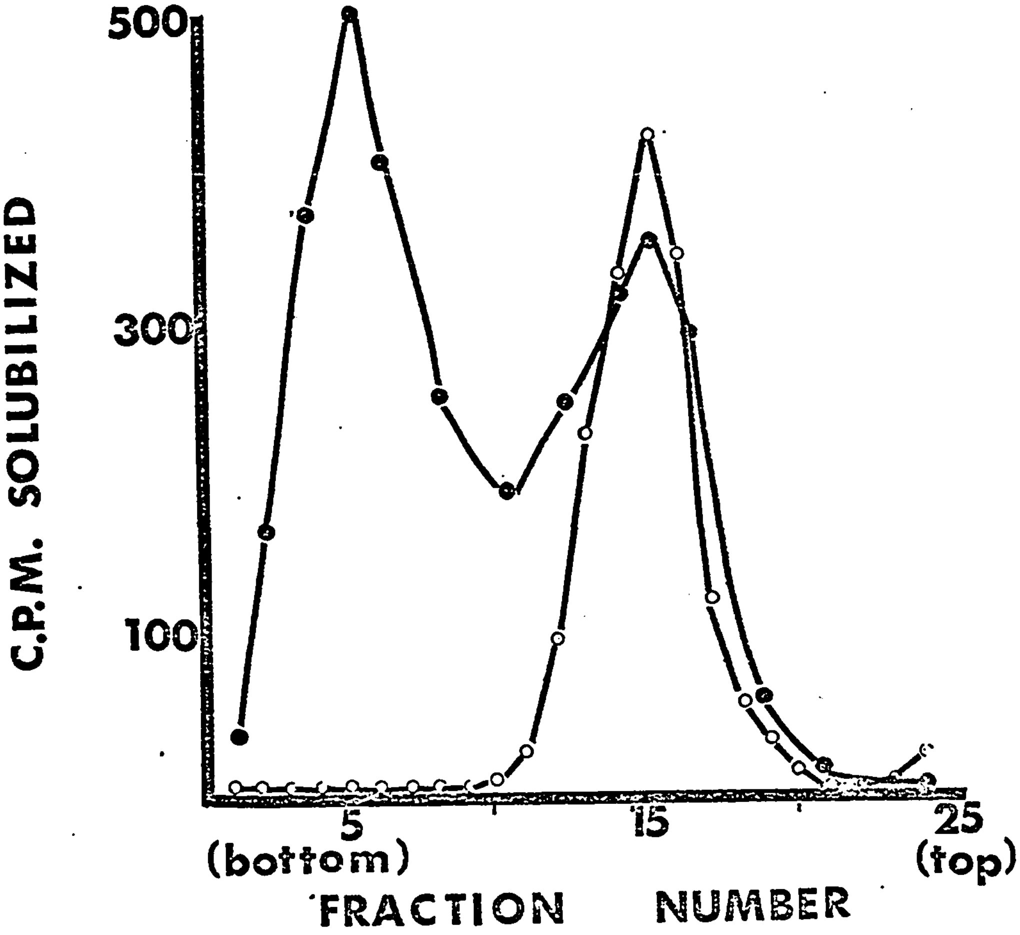

500
300
100
C.P.M. SOLUBILIZED
5
(bottom)
15
25
(top)
FRACTION NUMBER

FIGURE 2

SUCROSE DENSITY GRADIENTS OF CELL FREE EXTRACTS PREPARED BY DIFFERENT METHODS OF CELL BREAKAGE

200 µl samples of cell free extract prepared as given in Materials and Methods with the exceptions noted below were applied to 4.8 ml gradients. Gradient preparation and centrifugation preparations are given in Materials and Methods. The duration of centrifugation was 16 hours.

○——○ cells were broken in 50 ml volumes using the Biosonik III with a 3/4 inch probe for 5 minutes at 350 watts.[*] 25 µl volumes were assayed.

●——● cells were broken in 50 ml volumes using the BP-I sonic oscillator with a 3/8 inch probe for 5 minutes at 120 watts.[**] 50 µl volumes were assayed.

[*] Produces greater than 95% cell breakage

[**] Produces 50 - 70% cell breakage

The activity profile obtained from a sucrose density gradient of the 100,000 x g supernatant is seen in Fig. 2. The peak tube, under the conditions used, was consistently number 15 or 16 in repeated experiments. The contour of the activity profile is not fully symmetrical, suggesting some heterogeneity of molecular size of the active fraction.

C. Stabilization of activity in crude preparations

The exonuclease is a labile protein and acts so throughout the purification. In crude cell free extract preparations, the loss of 80% of activity (for method, see Materials and Methods) or more occurred in a period dependent on the conditions of storage.

(i) 4° - 14 days
(ii) 22-25° - 60-72 hours
(iii) 37° - 10 hours
(iv) -20° - stable indefinitely

In order to be able to recover activity from purification procedures it was first necessary to establish conditions for maximum stability of the enzyme. Stability studies were carried out under the basic conditions given in Materials and Methods modified as noted below.

(i) pH: pH values from 5.5 to 10.0 using sodium acetate-acetic acid, Tris maleate, Tris HCl and glycine buffers were tested. At pH 7.5 to 8.0 stability was as noted at 37° in (iii) and at pH 6.5, 7.0, 7.5, 8.0 and 8.5 it was approximately 90% of that value.

(ii) ionic strength: A reduction in the survival activity in 4 or 10 hours at 37° was seen with ionic strength much over 0.05 M Tris HCl. At 0.1 M, survival was reduced by 30 to 40% and at 0.2 M by 50%. Similar results were seen using 0.05 M and 0.15 M NaCl in 0.05 M Tris HCl.

(iii) cations: Mn^{++}, Mg^{++}, Co^{++}, Ni^{++}, Cu^{++}, Cd^{++}, Fe^{++}, Fe^{+++} and Zn^{++} were added at 0.001 M or 0.005 M to crude cell free extract but failed to improve stability at 37°. Fig. 3 demonstrates the destabilizing effect of 0.005 M Mg^{++} relative to a control preparation. Other cations had similar or more marked destabilizing effects.

(iv) anions: No effect of changing the anions from chloride to acetate, phosphate, sulfate or pyrophosphate was observed.

(v) Thymine, cytosine, guanine or adenine as bases, as ribo- or deoxyribonucleosides, or as ribo- or deoxyribonucleotides did not change the stability at 37° when present at a concentration of 0.05 µM/ml.

(vi) *E. coli* DNA did improve stability. Fig. 3 demonstrates that at a final concentration of 0.2 mg/ml in crude cell free extract, 20% activity was still present after 24 hours incubation at 37°. The addition of 0.005 Mg^{++} and 0.2 mg/ml DNA was considerably less effective as is shown in Fig. 3. If tritiated DNA with a specific activity of 2800 dpm/nM was incubated under identical conditions it was found that 75% was rendered acid soluble in 60 minutes in the presence of Mg^{++} and 25% in the absence. Acid solubility was determined as in the standard exonuclease assay.

The diminished stabilizing effect of DNA in the presence of Mg^{++} is thus likely related to its more rapid destruction presumably due to the endogenous exonuclease activity present.

Another observation can be made from Fig. 3. The first samples assayed after the addition of the DNA at 0° to the cell free extract show almost no activity. Thus added unlabelled DNA appears to inhibit nuclease activity as detected by using the standard exonuclease assay which depends on the release of ^3H-labelled acid soluble products. If instead the hyperchromic assay is used, at zero time activity in a control and with 0.2 mg DNA added are similar (change of 0.14 OD_{260}/ml/min). Thus the added DNA appears to inhibit the radioactive assay only because the enzyme is bound to unlabelled DNA which must be first hydrolyzed. The enzyme and DNA apparently form a stable complex which is carried over from the stability preparation to the assay. The same DNA-enzyme complex is likely the source of the stabilization of activity shown by the substrate.

(vii) reducing agents: Na bisulfite at concentrations up to 0.12 M had no detectable effect. The effect of mercaptoethanol was deceptive. In crude prep-

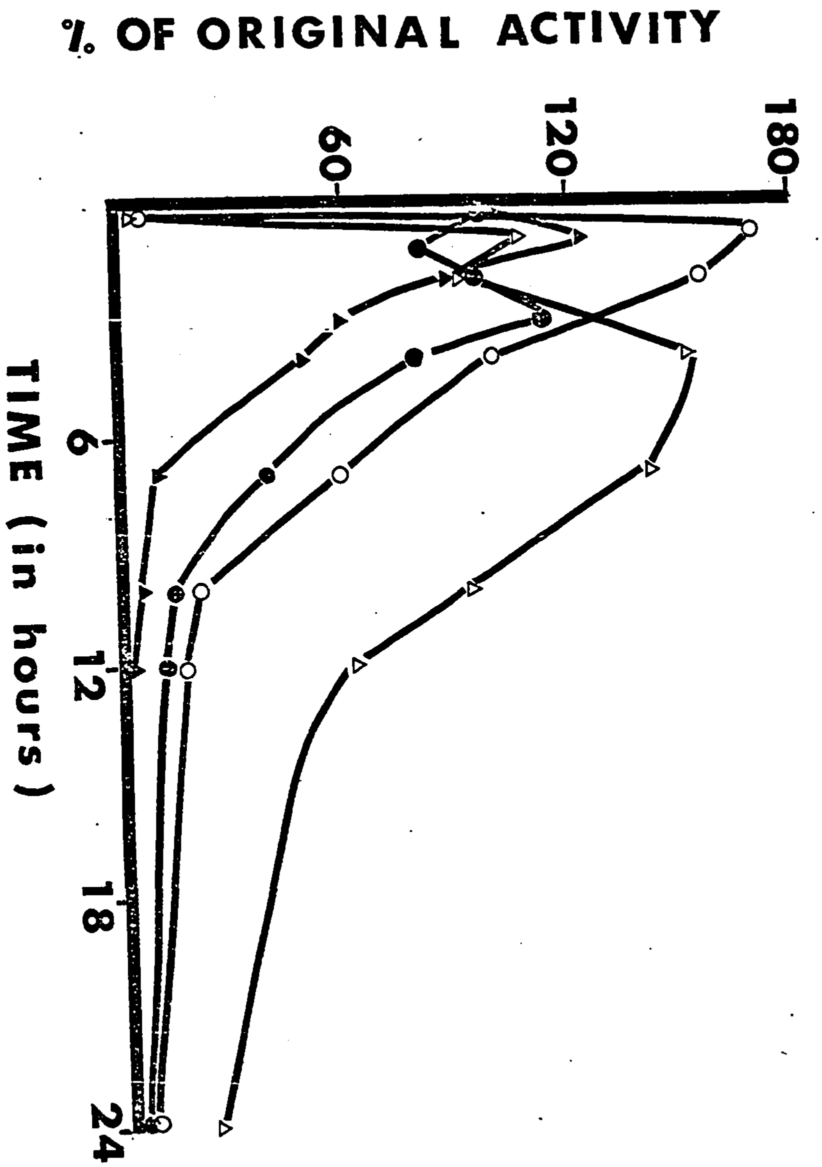

% OF ORIGINAL ACTIVITY
180
120
60
TIME (in hours)
6
12
18
24

FIGURE 3

STABILITY OF CRUDE CELL EXTRACT
OF *PSEUDOMONAS AERUGINOSA*
UNDER SEVERAL CONDITIONS

Stability of preparations was examined at 37° as noted in Materials and Methods. 10 μl volumes were assayed at the noted times.

●——————● Control

▲——————▲ Control plus 0.005 M $MgCl_2$

○——————○ Control plus *E. coli* DNA 0.2 mg/ml and 0.005 M $MgCl_2$

△——————△ Control plus *E. coli* DNA 0.2 mg/ml arations it did not appear to produce stabilization at a concentration up to 0.1 M whereas in purified preparations its presence was mandatory (see section II, subsection A).

(viii) organic solvents: The addition of ethylene glycol from 0.5 to 20% (v/v), ethanol 10 to 40% (v/v), propanol 10 to 20% (v/v), acetone 10 to 20% (v/v) and dioxane at 10% (v/v) were not effective.

The addition of glycerol, however, was very effective. Fig. 4 shows the stabilizing effect of several concentrations of glycerol. 30% glycerol was selected for use since it produces stabilization nearly as effectively as 40% glycerol, but retains a lower viscosity. Viscosity is significant because of the slowing effect on column operation at 4°. Again the effect of Mg^{++} is also seen in the glycerol preparation.

D. The number of DNases present in cell preparations

Knowledge of the number of endogenous DNases present in the cell preparations is essential. The most obvious effect is that if two or more are present, it is necessary to use a selective assay in crude preparations to study a given DNase. In order to examine this question, two approaches were used.

(a) The examination of the activity profiles obtained from an early purification step. DEAE cellulose chromatography is the first purification step used. Fig. 5 represents the results of a DEAE cellulose column carried out in a manner identical to the standard purification

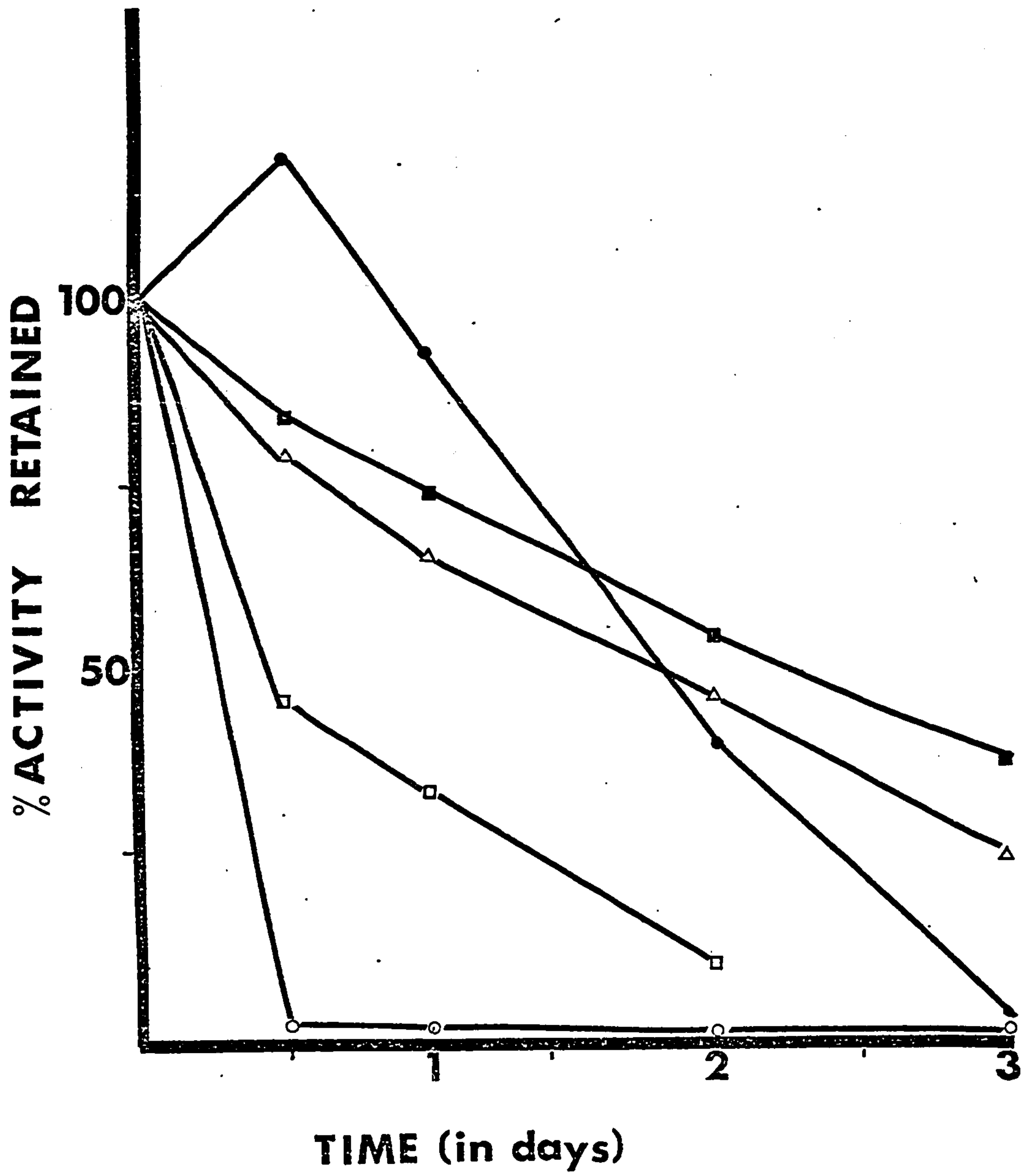

100
50
% ACTIVITY RETAINED
1
2
3
TIME (in days)

FIGURE 4

THE EFFECT OF GLYCEROL ON STABILITY OF CRUDE EXONUCLEASE PREPARATIONS FROM PSEUDOMONAS AERUGINOSA

Estimation of stability at 37° was carried out as in Materials and Methods. Glycerol was added to the cell free extract preparations while still at 4° in a volume sufficient to produce the given concentration percentages (v/v).

●————● 40% glycerol + 0.005 M $MgCl_2$

■————■ 40% glycerol

△————△ 30% glycerol

□————□ 20% glycerol

○————○ Control (no glycerol)

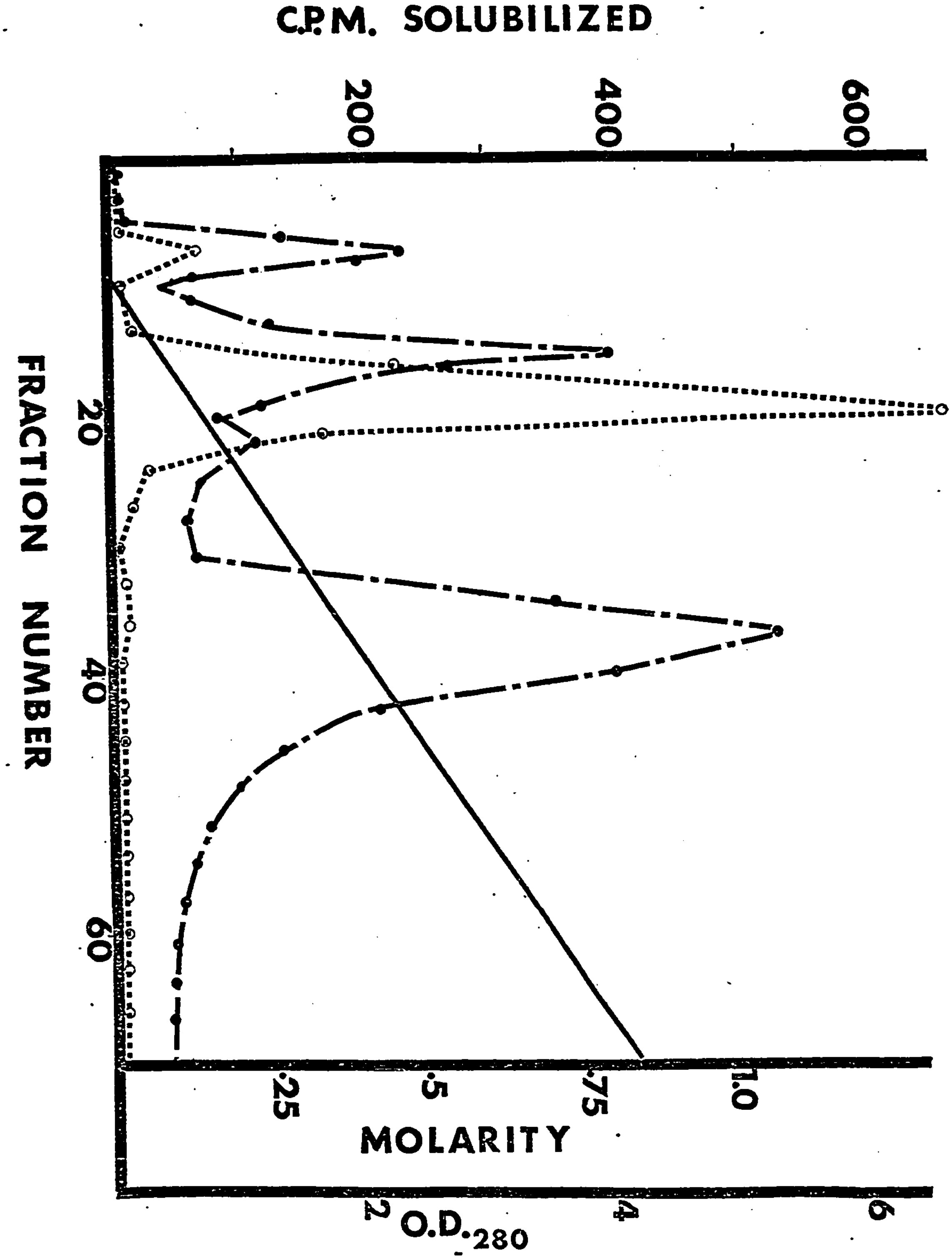

C.P.M. SOLUBILIZED
200
400
600
FRACTION NUMBER
20
40
60
·25
·5
·75
1.0
MOLARITY
2
4
6
O.D.280

FIGURE 5

DEAE CELLULOSE CHROMATOGRAPHY OF UNACTIVATED 100,000 x g SUPERNATANT OBTAINED FROM PSEUDOMONAS AERUGINOSA

14 ml of 100,000 x g supernatant containing 250 mg of protein was adjusted to 30% glycerol and 0.01 M mercaptoethanol to give a final volume of 20.1 ml. It was applied to a 1.5 x 24 cm column of DEAE cellulose followed by 10 ml of glycerol buffer #1 (see Materials and Methods). The enzyme was eluted with a gradient consisting of 300 ml of glycerol buffer #1 and 300 ml of 1.0 M NaCl in that buffer. Fractions were 8.0 ml collected at a flow rate of 0.5 ml per minute.

●—— — ——● Absorbance (OD) at 280 nm

O------------O Exonuclease activity

———————— Molarity (NaCl)

technique (see section I, subsection E) with the following modifications.

(i) the eluting gradient was up to 1.0 M NaCl.

(ii) no activation step was carried out (for a detailed reference to activation see Results section I, subsection G - 1 - iv). The higher gradient was used to detect more acidic DNases if present. Activation was not carried out because of the possiblity of destroying a labile enzyme(s) at the temperature used (37°).

From Fig. 5 it can be seen that there are only two peaks of activity present, the larger of these accounting for 98 to 99% of the total activity. Table IV compares the activities of the two peaks using several different assay conditions. There are sufficient differences to indicate that the minor peak is distinct and may be an endonuclease.

(b) The second method compares some characteristics of activity obtained in crude extracts with those obtained during the sequential purification steps. Table V illustrates clearly that for a broad range of conditions, the activity of purified enzyme closely resembles that of the crude extract. This almost identical pattern argues strongly that the exonuclease is the only significant DNase present in terms of quantitative activity.

E. Purification

The final purification sequence is summarized in the flow sheet of Table VI. A tabular summary of the purification results is seen in Table VII. The total purification of activity is 325-fold and the total reduction in protein is 6,200-fold. Final recovery is about 6% if based on the initial pre-activated activity.

TABLE IV

A COMPARISON OF DNASE ACTIVITY FROM TWO PEAKS OF A DEAE CELLULOSE COLUMN (FIG. 5) USING SEVERAL PARAMETERS OF ACTIVITY

Activity Parameter	Peak I	Peak II
Ratio of activity obtained* with UTCA to TCA	0.55 - 0.76	0.9 - 1.0
Ratio of activity obtained with denatured to native DNA	1.28	0.25 - 0.35
Activity with 0.005 M EDTA in assay	0.28	0.0

---

*See Results, section II, subsection D.

TABLE V

A COMPARISON OF PARAMETERS OF DNASE ACTIVITY IN THE CRUDE CELL FREE EXTRACT AND FINAL PURIFIED EXONUCLEASE (G-75 SEPHADEX)

Conditions of Assay	Activity Relative to the Conditions of the Standard Exonuclease Assay	
	Cell Free Extract	G-75
Mg^{++} 0.025 M	1.0	1.0
Ca^{++} 0.025 M	0.1 - 0.2	0.1 - 0.2
EDTA 0.025 M	0.0 - 0.2	0.0
pH optimum	7.8 - 8.2	7.8 - 8.2
UTCA/TCA	0.85 - 0.95	0.9 - 1.0
pH 9.0	0.65	0.65
pH 5.2, 0.0025 M EDTA, no Mg^{++}	0.0	0.0
Stabilizing effect of 30% glycerol	marked	marked
Effect of 0.1 M NaCl in assay	20 - 30% inhibition	20 - 30% inhibition

TABLE VI

A SUMMARY OF PURIFICATION (FLOW SHEET)

Harvested cells

Resuspend at 1 g wet weight per 5 ml of glycerol buffer
(see Materials and Methods) with 0.001 M EDTA

Cell disruption in Biosonik III, 3/4 inch probe, 350 watts,
300 ml volume, 4° for 6 minutes

SUPERNATANT = Cell free extract

Beckman Model L-2 Ultracentrifuge 100,000 x g x 90 minutes
(SUPERNATANT = 100,000 x g supernatant)

Glycerol 30% by volume, PMSF 0.002 to 0.005 M, Mg^{++} 0.0025 M,
RNase 20 μg/ml, 37° x 90 minutes (ACTIVATION STEP)

DEAE cellulose column #1

Isoelectric precipitation at pH 5.2. SUPERNATANT contains activity.
Adjust to pH 7.5

Ammonium sulfate saturation. SUPERNATANT contains activity.

Dialysis vs. 100 volumes of glycerol buffer.
Concentration by Ultrafiltration, Amicon UM 10 Filters.

Hydroxylapatite column. Concentration of active fractions
by ultrafiltration. Diluted 1:5 in 30% glycerol in H_2O
containing 0.01 M mercaptoethanol

DEAE cellulose column #2

Concentration of active fractions to 1.0 ml by ultrafiltration

G-75 Sephadex column

TABLE VII

PURIFICATION OF *PSEUDOMONAS AERUGINOSA* EXONUCLEASE

Preparation	Total Protein (mg)	Total Activity (units)	Specific Activity (units/mg protein)	Recovery (%)
Cell free extract	2300			
pre-activation		12,900	5.6	100
post-activation		41,280	18	320
DEAE cellulose	910	52,800	66	409
Isoelectric precipitation	770	53,000	69	410
Ammonium sulfate	405	26,400	65	203
Hydroxylapatite	59.5	8,000	135	62
DEAE cellulose	2.0	2,900	1450	22.5
Sephadex G-75	0.37	760	1810	5.9

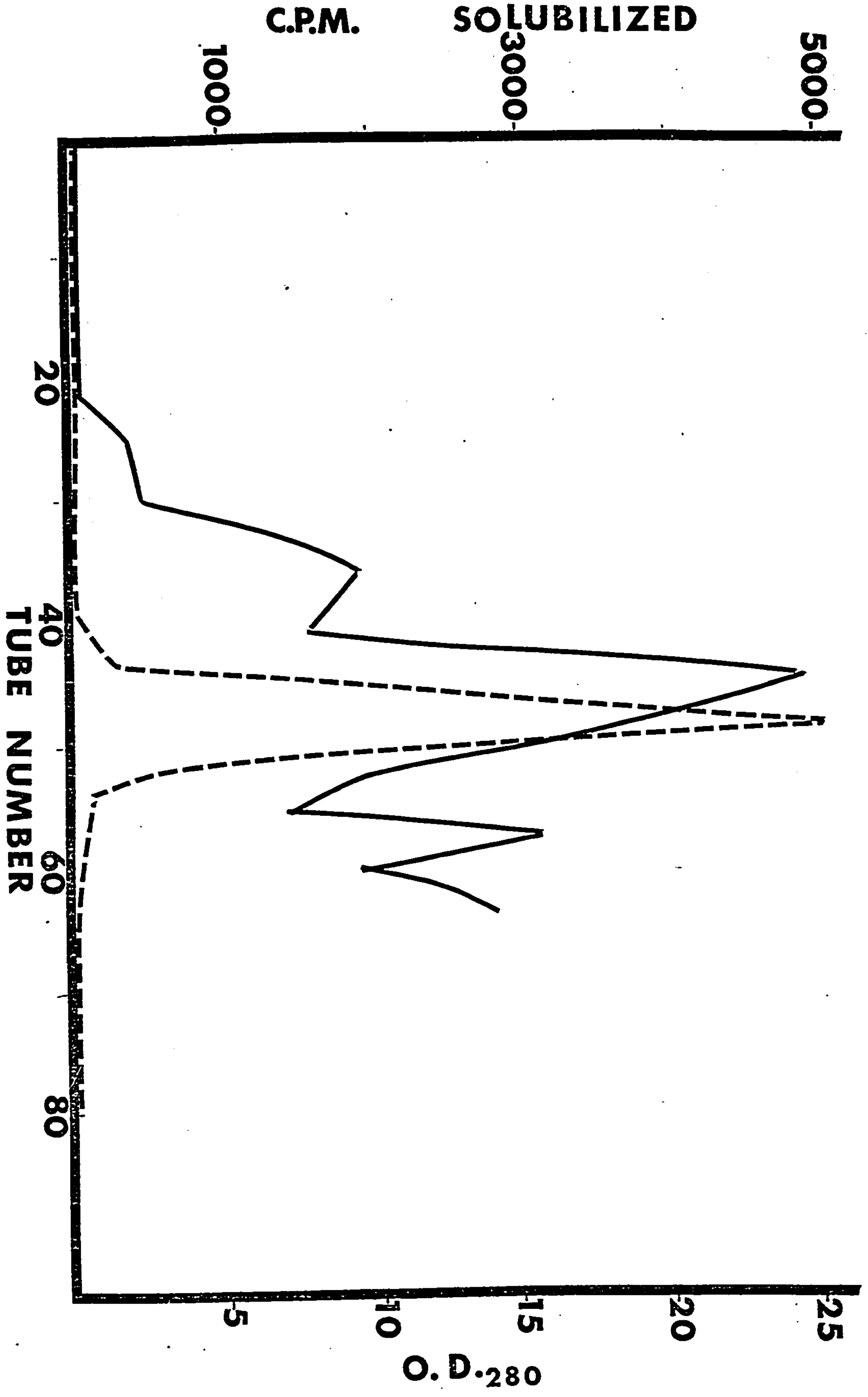

C.P.M. SOLUBILIZED
1000
3000
5000
20
40
60
80
TUBE NUMBER
5
10
15
20
25
O.D.280

FIGURE 6

DEAE CELLULOSE CHROMATOGRAPHY OF ACTIVATED
100,000 x g SUPERNATANT OF PSEUDOMONAS AERUGINOSA

Column specifications and operation are given in Materials and Methods. All tubes were examined for exonuclease activity and for absorbancy at 280 nm.

- - - - - - - - - -   Exonuclease activity

————————   Absorbance (OD) at 280 nm

The elution of activity as a single symmetrical peak at 0.07 M NaCl from DEAE cellulose is seen in Fig. 6. The small initial peak seen in Fig. 5 is not seen in this preparation. It is presumed that the 37° activation procedure preliminary to DEAE cellulose destroys that activity.

Ammonium sulfate saturation in 30% glycerol occurs at the quantity of ammonium sulfate required to produce 60% saturation in water. Reference to Table VII demonstrates that this step produces no net purification. No activity can be detected in the resuspended precipitate before or after dialysis. The step cannot be replaced by a variety of combinations of other procedures in that lower specific activities are obtained in those cases.

The pH 5.2 isoelectric precipitation produces a small net purification with no apparent activity loss.

Chromatography on hydroxylapatite is an effective procedure. In Fig. 7, activity is seen to elute at 0.05 M potassium phosphate on the downslope of the second of two large OD_{280} peaks. Decreasing the steepness of the 0.01 M to 0.2 M phosphate gradient does not improve the specific activity obtained. However, if very small protein loads are used, the net purification is increased (see section I, subsection F - 3 on Purity).

The second DEAE cellulose column activity profile is seen in Fig. 8. The activity elutes at 0.06 M phosphate with a very small OD_{280} peak. It is always a single peak.

The final purification step is G-75 Sephadex gel filtration. In repeated experiments, activity has eluted in a consistent set of fractions as seen in Fig. 9. Absorbance at 280 nm is so low that detection is prone to considerable error.

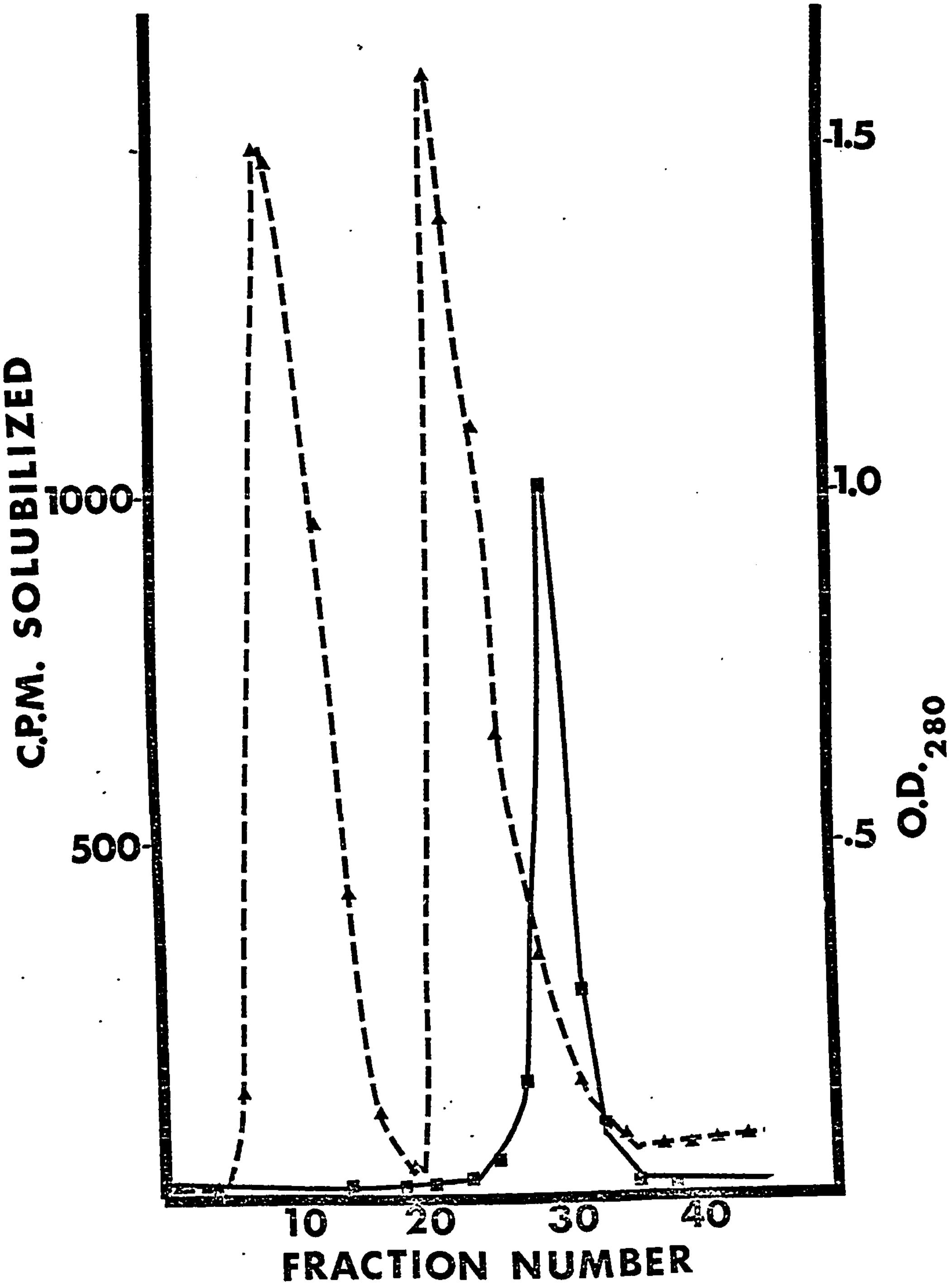

C.P.M. SOLUBILIZED
1000
500
O.D.$_{280}$
1.5
1.0
.5
10
20
30
40
FRACTION NUMBER

FIGURE 7

HYDROXYLAPATITE CHROMATOGRAPHY
OF pH 5.2 SUPERNATANT

The column specifications are given in Materials and Methods.

▲----------▲ Absorbance (OD) at 280 nm

■———————■ Exonuclease activity

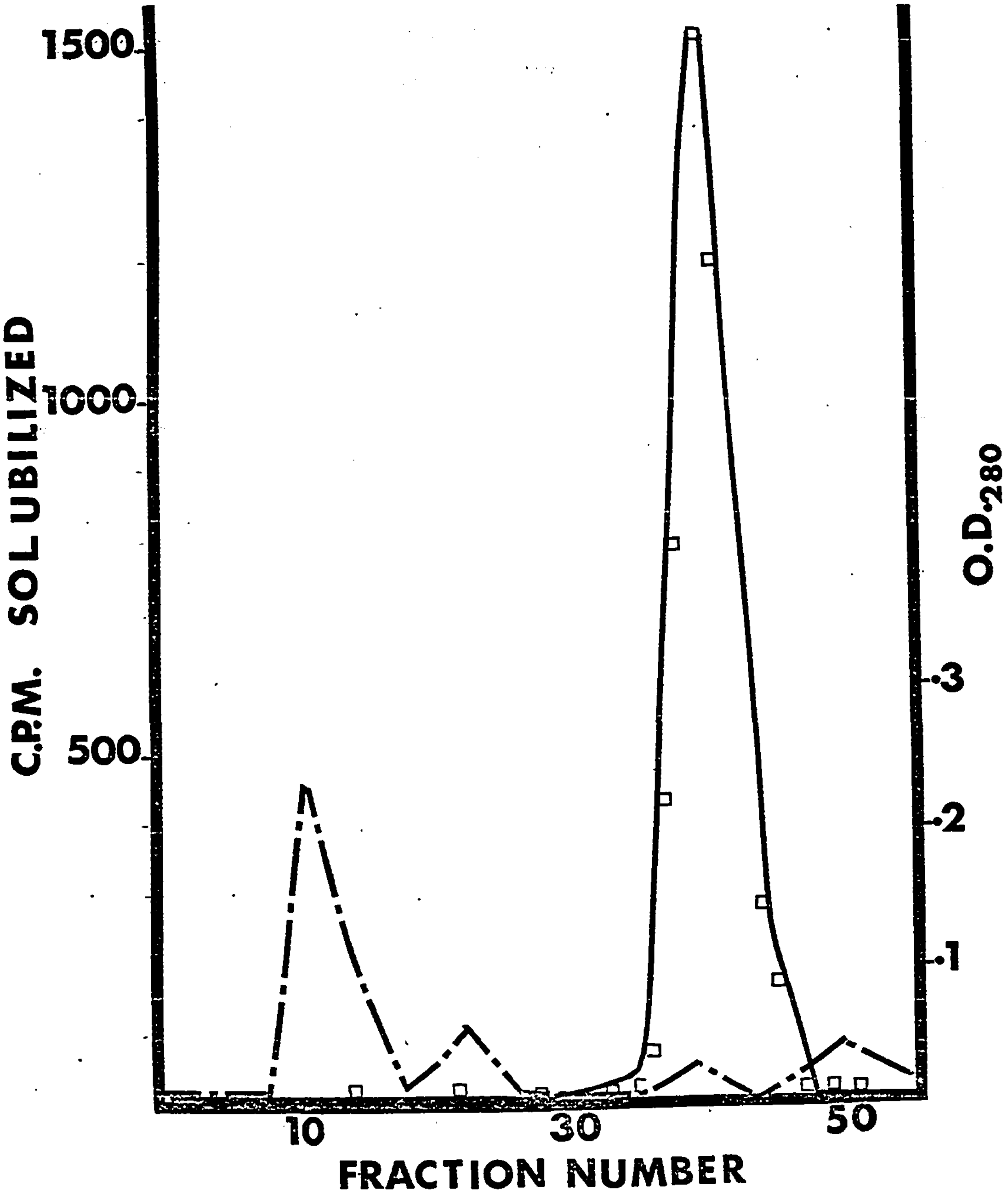

C.P.M. SOLUBILIZED
1500
1000
500
O.D.$_{280}$
.3
.2
.1
10
30
50
FRACTION NUMBER

FIGURE 8

DEAE CELLULOSE CHROMATOGRAPHY OF

ACTIVE FRACTIONS FROM HYDROXYLAPATITE CHROMATOGRAPHY

Column specifications are given in Materials and Methods. All tubes were examined for absorbancy at 280 nm.

□————□	Exonuclease activity
----------	Absorbance (OD) at 280 nm

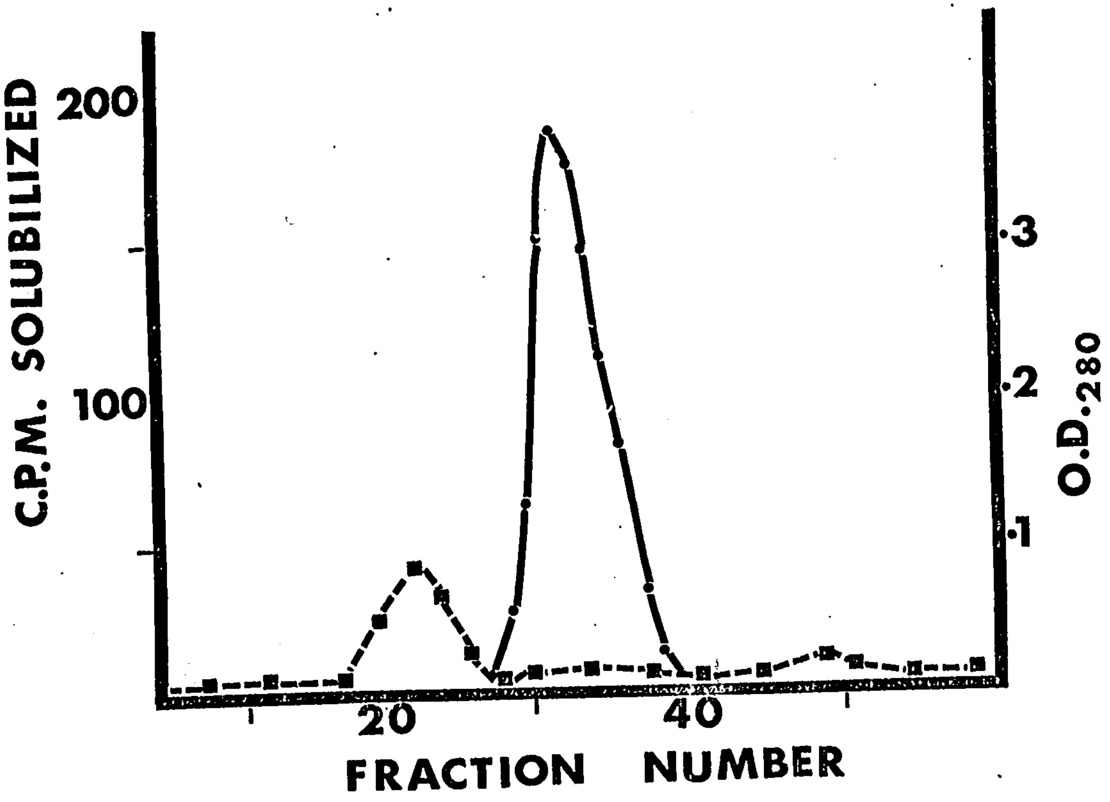

C.P.M. SOLUBILIZED
200
100
·3
·2
·1
O.D.$_{280}$
20
40
FRACTION NUMBER

FIGURE 9

G-75 SEPHADEX GEL FILTRATION OF
ACTIVE FRACTIONS FROM THE DEAE CELLULOSE COLUMN #2

The 1.0 ml concentrate containing 1.0 mg of protein obtained from the second DEAE cellulose column in the purification sequence was applied to a G-75 Sephadex column. The column specifications and operation are given in the Materials and Methods.

●————● exonuclease activity

■—— ——■ absorbance (OD) at 280 nm

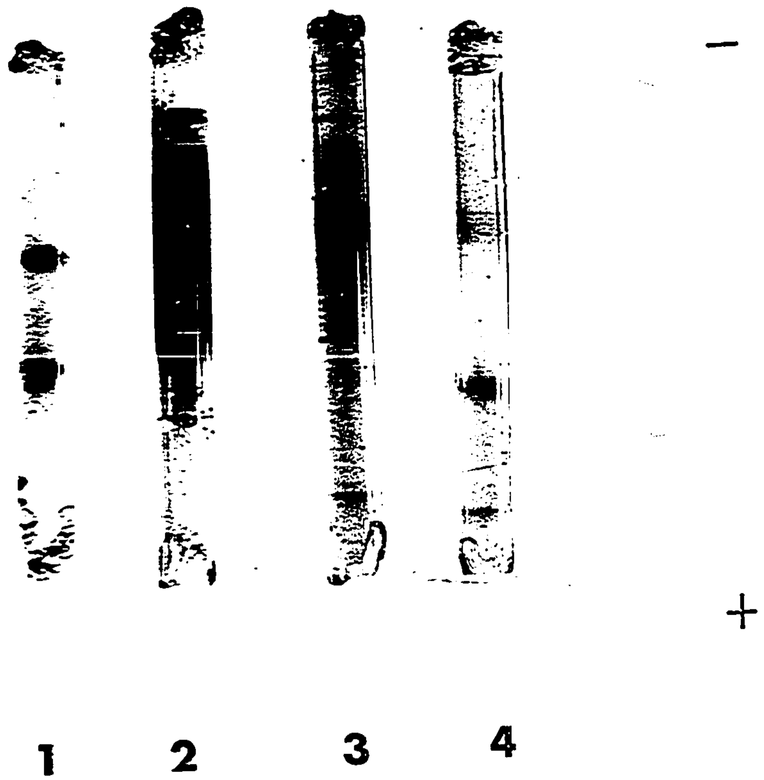

−
+
1
2
3
4

FIGURE 10

POLYACRYLAMIDE GEL ELECTROPHORESIS OF PREPARATIONS FROM THE PURIFICATION SEQUENCE

Electrophoresis was carried out as given in Materials and Methods at pH 8.3. Protein used on each gel was (1) 40 μg, (2) 200 μg, (3) 40 μg and (4) 20 μg. Due to the faintness of bands, high contrast film was used which results in the bands of (2) being visible only as a broad protein containing area.

(1) Hydroxylapatite preparation obtained from a small scale purification (initial protein in cell free extract stage was 200 mg).

The remainder of samples were obtained during standard large-scale purification.

(2) Hydroxylapatite

(3) DEAE cellulose column #2

(4) G-75 Sephadex gel filtration

F. Purity

The effectiveness of a purification sequence was found to depend on the size of the batch and maintainence of full activity. Fig. 10 shows the results of polyacrylamide gel electrophoresis for the last three purification steps done on a large scale. The final step is contaminated by a single minor band when gels are run at pH 8.3. No additional bands were seen at pH 4.0. DNase activity eluted from such gels occurs only at the position of the most prominent band. If purification is done on a small scale, the hydroxylapatite concentrates are much more highly purified than on a large scale basis. That may be observed by comparing (1) and (2) in Fig. 10.

The contaminating protein is a ribonuclease which has been shown to be a distinct enzyme. This evidence is presented in section II, subsection G - 1. The elution of RNase activity from a G-75 column is seen in Fig. 11 to be widespread, occurring both in the void volume and the late internal volume. Both of these peaks behave identically in the parameters examined in section II, subsection D indicating they are the same enzyme. The possibility of association or electrostatic retardation on the gel is probable for the RNase on the G-75 column. It clearly demonstrates the difficulty in purifying out the contaminating RNase. Tubes containing no RNase can, however, be selected from the G-75 elution fractions. The content of RNase in the final exonuclease concentrate ranges from undetectable (one case) to 40 units per mg protein.

The possibility that the contaminant is the pancreatic ribonuclease added at the time of the activation detailed in section I subsection G - iv is unlikely for two reasons. The first is that on initial DEAE cellulose columns pancreatic ribonuclease and the exonuclease are eluted separately. The second is that in a purification trial with no added ribonuclease, endogenous activity was detected up to and including the G-75 Sephadex step.

The maintainence of full activity is dependent on several factors but the well defined effect of mercaptoethanol is very important and is examined in section II subsection A.

G. Modification of Pseudomonas aeruginosa exonuclease

The purification scheme presented in section I subsection E is the scheme devised only after solution of several problems. One of these was the lability of the enzyme and is considered in section C. The second was that the enzyme could be present in a modified form during purification. As a result, purification was essentially impossible until the reasons for such modification could be determined. The manifestations of this process were profound in that activity fractionated heterogeneously at some stage in every purification scheme and did so to a major degree. For example, Fig. 21 can be examined in order to see the effect shown on DEAE cellulose chromatography. The results of this were:

(i) activity was widespread in purification procedures and fractionation was, thus, poor.

(ii) definition of what species represented the enzyme was difficult.

(iii) the possibility that several distinct enzymes were present was quite probable.

(iv) the heterogeneity was inconsistent in degree and reproduction of procedures was not possible.

(v) evidence indicating large and small derivatives of the exonuclease was detected.

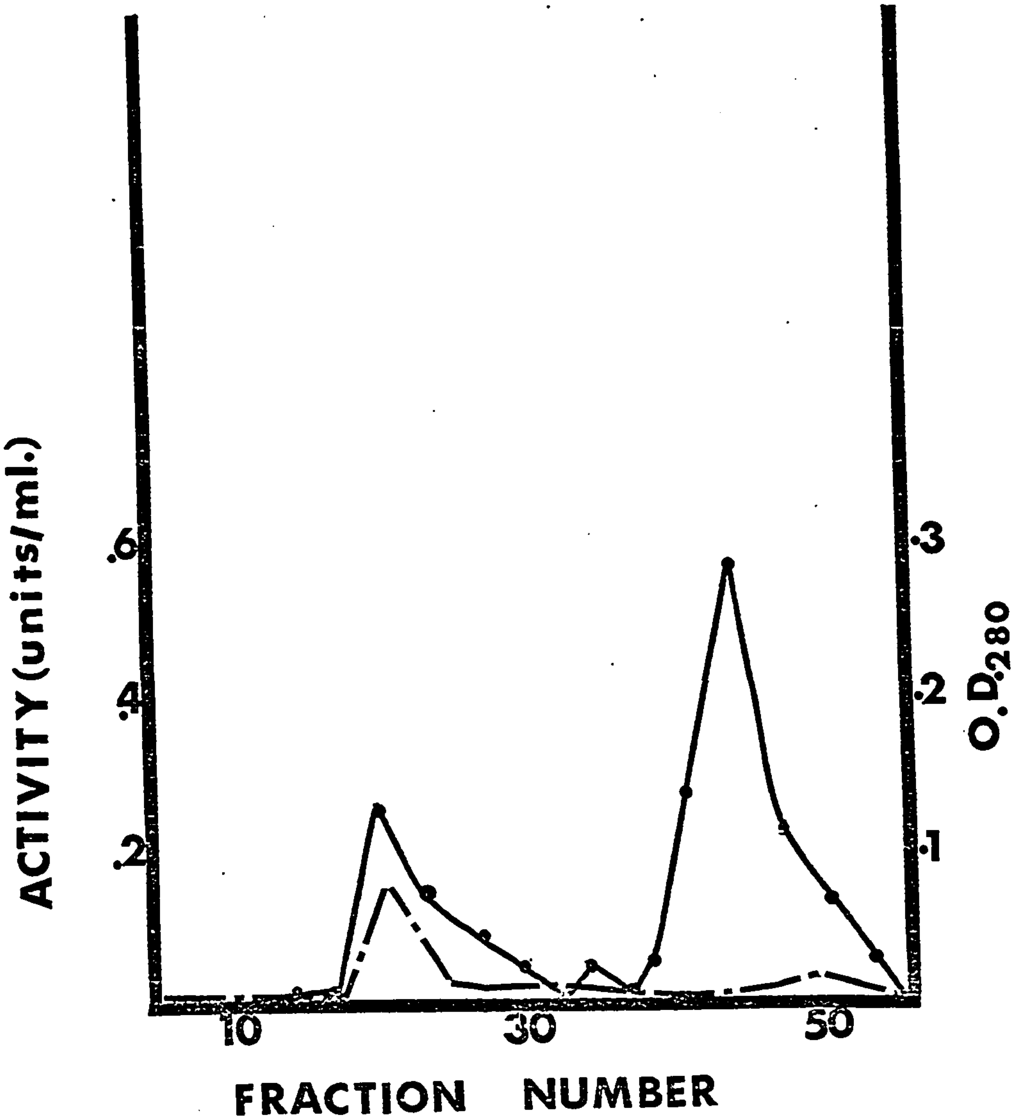

ACTIVITY (units/ml.)
.6
.4
.2
.3
.2
.1
O.D.$_{280}$
10
30
50
FRACTION NUMBER

FIGURE 11

G-75 SEPHADEX GEL FILTRATION

OF CONTAMINATING RNASE ACTIVITY

Conditions identical to that of Fig. 9 were used. All tubes were examined for absorbancy at 280 nm and as indicated for RNase activity. The latter activity was detected as outlined in the Materials and Methods, using the assay containing EDTA. The peak of absorbancy at 280 nm represents the void volume of the column.

●——● RNase activity

—- — Absorbance (OD) at 280 nm

It cannot be overemphasized how serious a problem modification was for it effectively prevented progress on purification for a year of intense effort.

In order to orientate the reader into the solution of this problem, a brief introduction to the plan of the results which follow seems indicated. In the case of polynucleotide binding, evidence for associated forms of the enzyme is presented in gel filtration and sucrose density data. The nature of the association is next examined by demonstrating that processes removing or destroying polynucleotides affected that complex and finally that a partly stable complex of DNA and purified enzyme can be reformed.

Proteolytic modification was considered by demonstrating the presence and inhibition of proteolytic activity, the presence of apparently degraded forms of the enzyme (gel filtration, ultrafiltration), ion exchange polymorphism and the elimination of such with proteolytic inhibition.

1. Polynucleotide binding

(a) Sucrose density gradients of ultrasonic treated material

The following experiments are intended to examine the effect on the types of enzyme sedimentation forms produced by varied effectiveness of sonic oscillation.

Fig. 2 demonstrates the effect of the use of two distinct energy sources and probes. The 350 watt sonic oscillator produces more effective sonication based on cell breakage and coincidentally produces a single enzyme. Under the less effective conditions of the BP-I sonic oscillator, a fast sedimenting form is also present.

The effect of a longer period of sonic disruption under

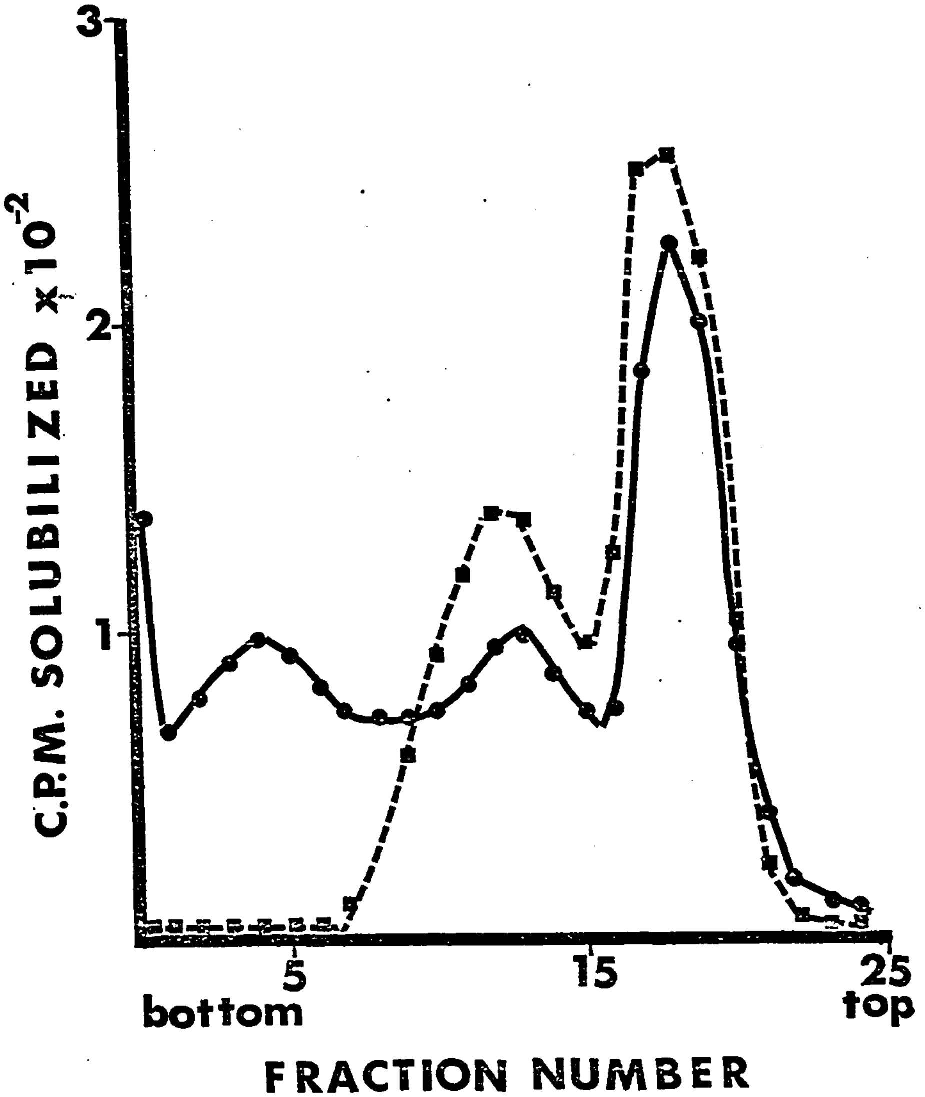

3
2
1
C.P.M. SOLUBILIZED x10^{-2}
5
15
25
bottom
top
FRACTION NUMBER

FIGURE 12

SUCROSE DENSITY GRADIENTS OF CELL FREE EXTRACTS OF PSEUDOMONAS AERUGINOSA PREPARED BY DIFFERENT PERIODS OF SONIC OSCILLATION

Sucrose density gradients were prepared and centrifuged as in Materials and Methods for 10 hours. Cell free extracts were prepared as given in Materials and Methods with the exceptions noted below. 200 μl of each extract was applied to the gradient.

●———————●	Cells were broken in 50 ml volumes in the Biosonik BP-I Ultrasonic device with 3/8 inch probe for 1 minute at 120 watts.
■----------■	Cells were broken as above but for 5 minutes.

otherwise identical conditions is seen in Fig. 12. Two fast sedimenting forms are eliminated and the amount of activity in the slower sedimenting forms is increased.

The results of these experiments indicated the enzyme can exist in some form of an association and the degree of that association can be influenced by the effectiveness of sonic oscillation.

(b) Gel filtration studies

The size distribution of activity in the cell free extract and at early purification stages was explored using Sephadex G-100 and G-200 gel filtration.

Fig. 13 demonstrates the presence of large molecular weight enzyme on a G-200 column. The cell free extract used was prepared by breaking whole cells in the Biosonik BP-I sonic oscillator for 5 minutes in 50 ml volumes (suspended as given in Materials and Methods), and centrifuging them at 27,000 x g for 10 minutes. The supernatant was applied as indicated in the text of the figure. In this and repeated experiments, a minimum of 80% of the total activity eluted near the void volume.

A G-100 column was used to fractionate activity after some purification had been carried out. In this case, the cells were broken as described for the G-200 column and centrifuged for 90 minutes at 100,000 x g. Streptomycin sulfate was added and the supernatant dialyzed against 100 volumes of pH 5.2 acetate buffer at 4° for 16 hours. After concentration of the supernatant by ultrafiltration, it was applied to the column. The result is seen in Fig. 14.

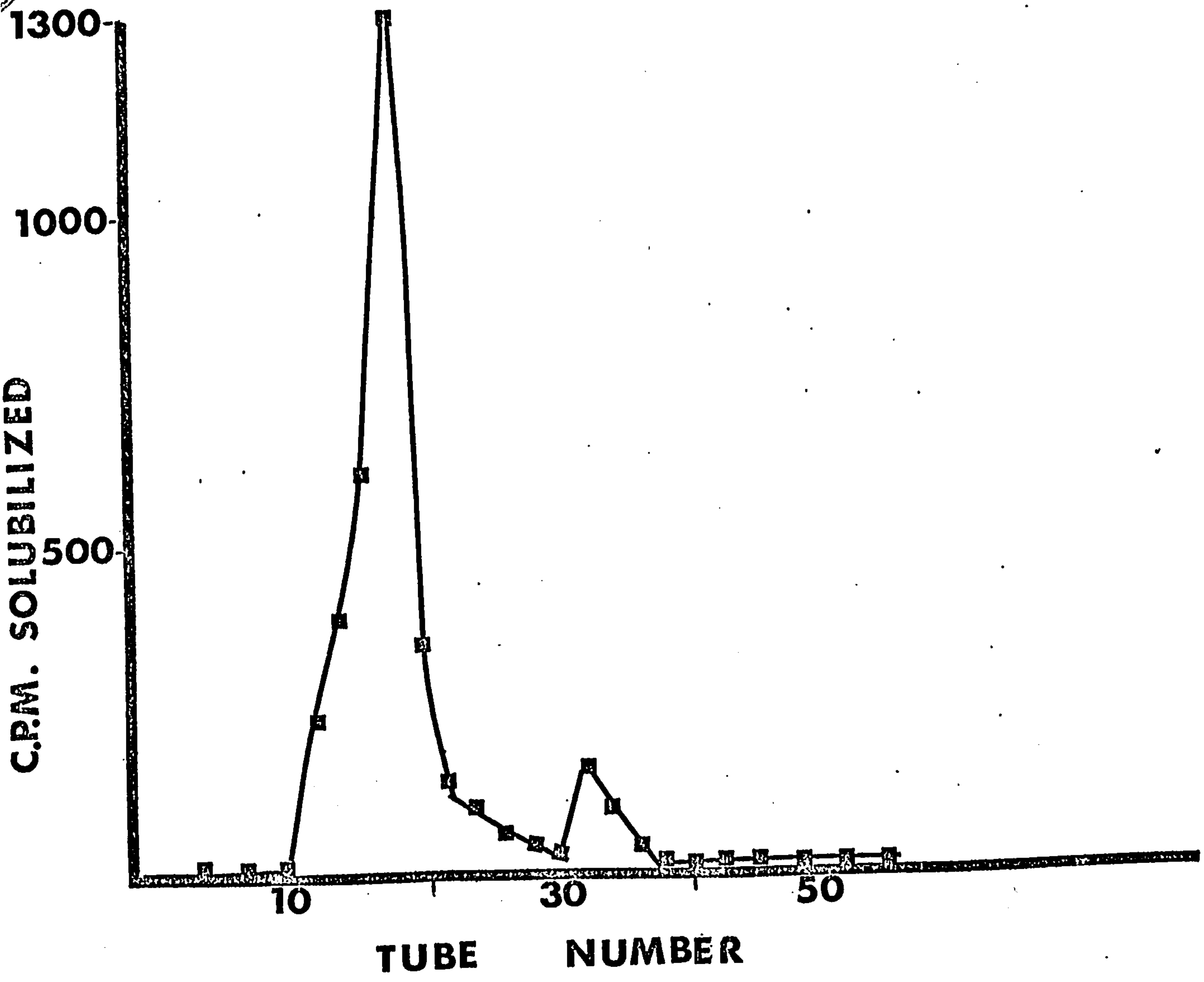

1300
1000
500
C.P.M. SOLUBILIZED
10
30
50
TUBE NUMBER

FIGURE 13

G-200 SEPHADEX GEL FILTRATION OF CELL FREE EXTRACT OF

PSEUDOMONAS AERUGINOSA

4.0 ml of cell free extract prepared as in the text of Results section I subsection G - 1 - b containing 76 mg of protein was applied to a 2.5 x 37 cm G-200 column. Equilibration and elution was with 0.1 M Tris HCl pH 7.5 and 0.01 M mercaptoethanol buffer. Fractions of 4.0 ml were collected at a flow rate of 6.0 ml/hour. The peak of the void volume is tube 15.

■———■ Exonuclease activity

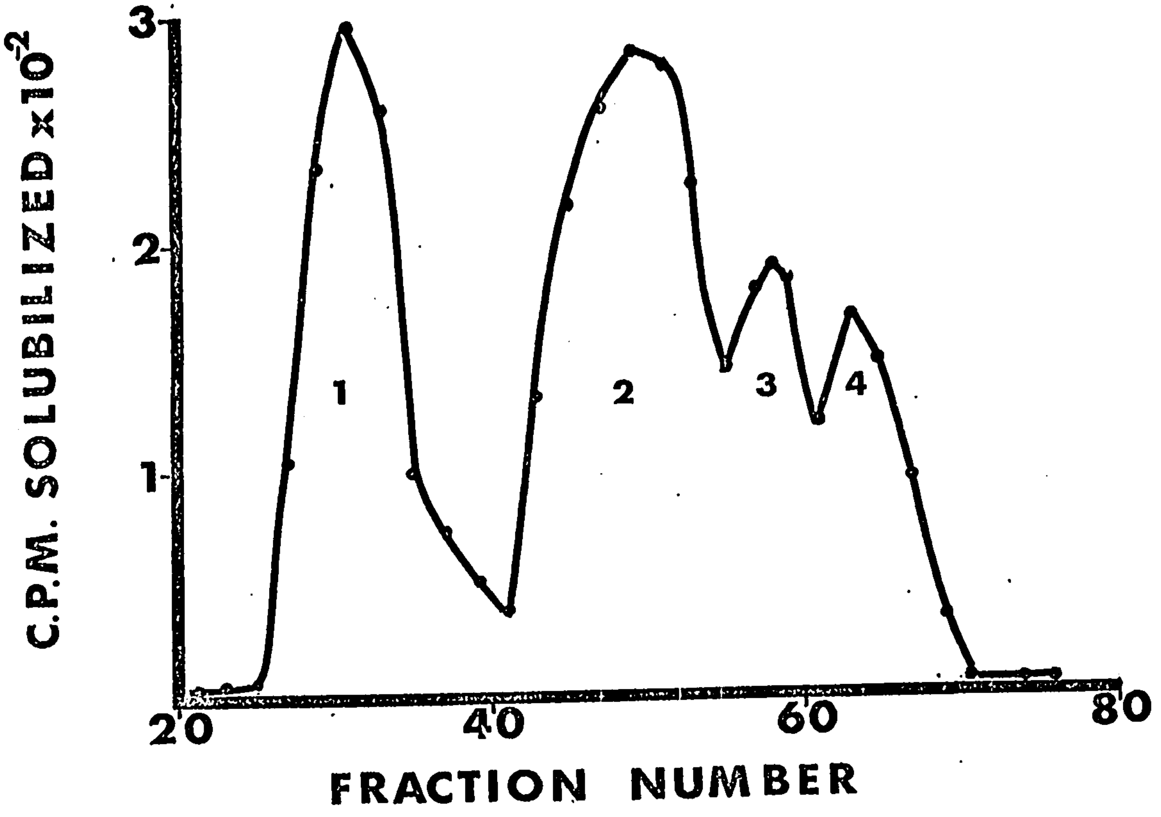

C.P.M. SOLUBILIZED x10^{-2}
3
2
1
20
40
60
80
FRACTION NUMBER
1
2
3
4

FIGURE 14

G-100 SEPHADEX GEL FILTRATION OF A
PARTIALLY PURIFIED PREPARATION OF
PSEUDOMONAS AERUGINOSA EXONUCLEASE

The enzyme preparation was prepared as in the text of Results section I subsection G - 1 - b. 2.0 ml of that containing 360 mg of protein was applied to a 2.5 x 30 cm column of Sephadex G-100. Fractions of 2.0 ml were collected at a flow of 8 ml/hour. The indicated tubes were assayed in the standard exonuclease assay. The buffer used throughout was 0.1 M Tris HCl pH 7.5, 0.01 M mercaptoethanol and 30% glycerol.

The elution profile contains 4 peaks. Peak 2 eluted at an identical position to purified enzyme obtained from G-75 Sephadex when chromatographed on Sephadex G-100. Therefore, peak 2 is felt to be enzyme free of digestion and degradation. Peaks 3 and 4 are further discussed in section I subsection G - 2 - a. Peak 1 thus represents a form of the enzyme which is larger than the free enzyme.

The results demonstrated in the G-200 and G-100 columns are further evidence indicating some form of molecular association of the enzyme can occur with a structure not defined by these experiments.

(c) Streptomycin sulfate treatment

Evidence from parts (a) and (b) of this section suggest strongly that the exonuclease is present in some form of association which was considered possibly to be with polynucleotides. To explore this problem, streptomycin sulfate, a polyvalent cation, was used to precipitate nucleic acids. Table VIII demonstrates that as streptomycin sulfate is added an initial increase followed by a loss of activity occurs. The loss is due to precipitation, as 20 to 40% of the activity can subsequently be eluted from the precipitate. The activation documented here is not quantitatively reproducible on all occasions. In some cases, precipitation results before activation occurs. Indeed, for each different batch of cell free extract, the amount of streptomycin sulfate required to precipitate the enzyme had to be established in a trial procedure.

The activation has been produced to that extent in only

TABLE VIII

THE EFFECT OF THE ADDITION OF STREPTOMYCIN SULFATE TO CELL FREE EXTRACT OF *PSEUDOMONAS AERUGINOSA*

Final concentration of Streptomycin sulfate	Activity in Supernatant (units/ml)	OD 280/260 of Supernatant
3.33 mg/ml	133	0.50
4.15 mg/ml	465.5	0.53
5.0 mg/ml	20.0	0.65
5.8 mg/ml	0	0.66

one other manner. That is by the inclusion of Mg^{++} (or Mn^{++}) in the cell free extract and incubation at 37°. Such treatment apparently activates the exonuclease. Thus precipitation of nucleic acids or enzymatic destruction of DNA have in common the ability to produce an increase in the total activity yield.

The precipitation of activity with streptomycin sulfate also supports the hypothesis of intimate association of enzyme and nucleic acid. If streptomycin sulfate is added to the enzyme at later stages of purification as seen in Table IX, it is not a precipitant of the enzyme unless DNA is also re-added, in which case the activity precipitates.

(d) Activation

The activity obtained in crude cell extracts can be considerably increased by incubation under proper conditions. To define these conditions and explore the nature of this phenomenon, incubation at 37° with a variety of additives was explored. Fig. 15 demonstrates several points:

(i) The maximal rate of activation and total increase in activity is dependent on Mg^{++} (identical experiments have shown Mn^{++} will replace Mg^{++} though the rate is slower) and is independent of the presence of pancreatic RNase.

(ii) EDTA abolishes activation demonstrating a need for divalent cations.

The production of acid soluble products absorbing at 260 nm and resulting from the same incubation as Fig. 15 is seen in Fig. 16. This result demonstrates the pancreatic RNase was active under the experimental conditions and therefore the failure of that enzyme to produce activation cannot be attributed to a loss of activity.

The nature of the acid soluble products absorbing at 260 nm is primarily accounted for by a parallel increase in acid soluble ribose and deoxyribose as shown in Table X.

In a separate experiment Ca^{++} was used at a concentration of 0.01 M and EDTA at 0.005 M but no activation resulted. In fact a decline of about 40% occurred in the 90 minute incubation.

Table XI is a similar experiment in which micrococcal nuclease (Cunningham *et al*, 1956; Reddi, 1958, 1959), an enzyme attacking both RNA and DNA, was used in the presence of Ca^{++} and EDTA at 0.01 M and 0.005 M respectively. The micrococcal enzyme produces no activity in the assays for the exonuclease since it has an absolute requirement for Ca^{++}. Controls with a considerable excess of the nuclease were used, however, to give zero values for the assay. These values were identical with normal zero values. The results show no activation using micrococcal nuclease although an increase in acid soluble OD_{260} occurred. Thus, activation is produced by creating conditions in the incubation which allow the endogenous exonuclease to carry out rapid DNA hydrolysis (see section II, subsection 3 for cation requirements) but not by using pancreatic Ribonuclease or micrococcal nuclease. The activation thus appears to require the destruction of DNA fragments bound

TABLE IX

THE EFFECT OF THE ADDITION OF STREPTOMYCIN SULFATE TO PREPARATIONS OF *PSEUDOMONAS AERUGINOSA* EXONUCLEASE AT DIFFERENT STAGES OF PURIFICATION

Preparation	Protein (mg/ml)	Final concentration Streptomycin sulfate (mg/ml)	Activity in Supernatant
Hydroxylapatite	4.8		
(i) alone		5.8	100%
(ii) + 0.2 mg/ml *E. coli* DNA		4.5	10%
G-75	0.8	7.0	100%

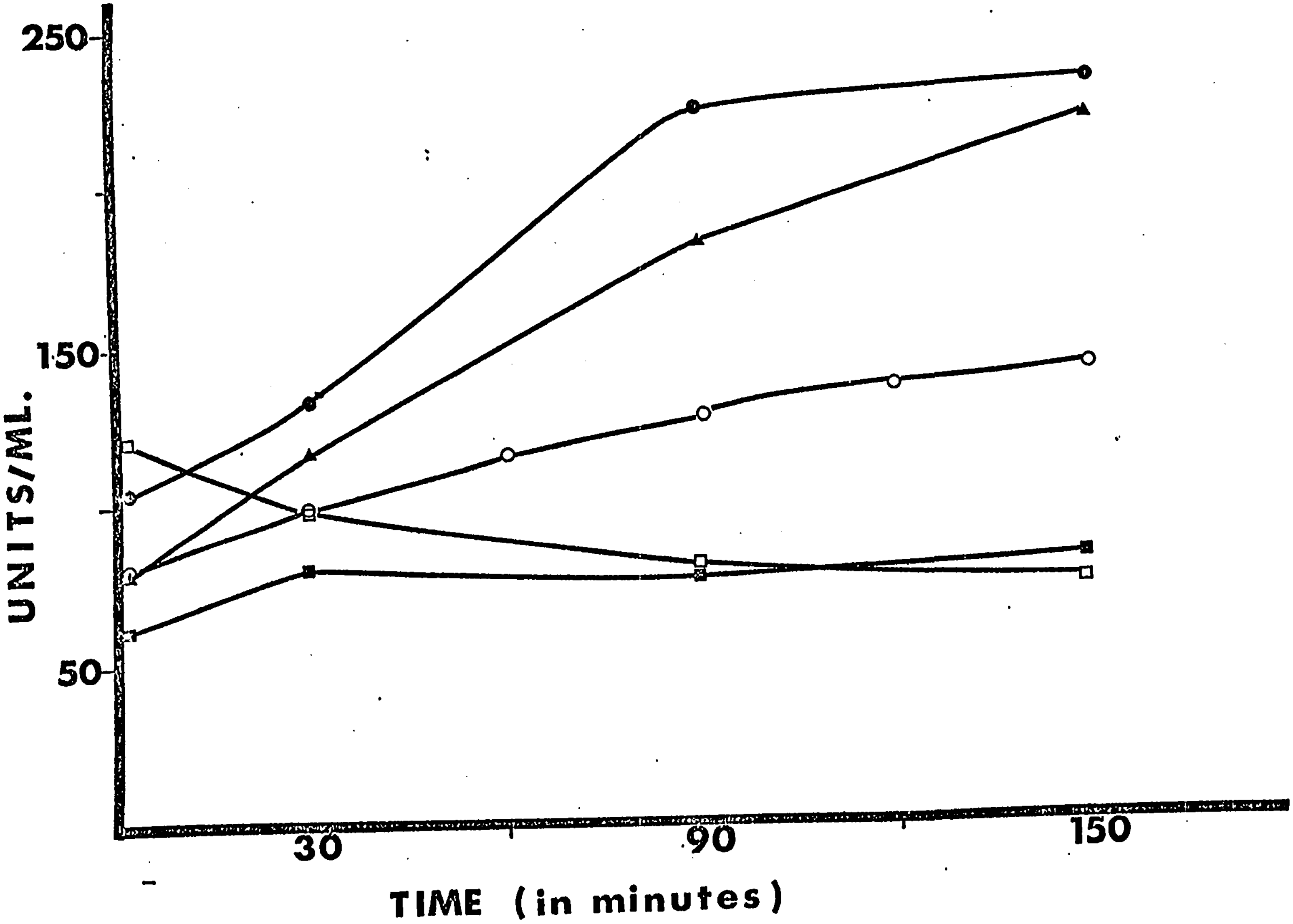

250
150
50
UNITS/ML.
30
90
150
TIME (in minutes)

FIGURE 15

ACTIVATION OF CELL FREE EXTRACT OF *PSEUDOMONAS AERUGINOSA*

Cell free extract was adjusted to 30% glycerol and 0.002 M PMSF. Materials were added at the concentrations given below to 5.0 ml volumes of cell free extract, the tubes were stoppered and zero time assays were performed. Tubes were incubated at 37° and 0.25 ml samples removed to ice at the times indicated for subsequent assay.

●————● Mg^{++} 0.005 M

▲————▲ Mg^{++} 0.005 M, pancreatic RNase 20 μg/ml

○————○ No additives

□————□ EDTA 0.005 M

■————■ EDTA 0.005 M, pancreatic RNase 20 μg/ml

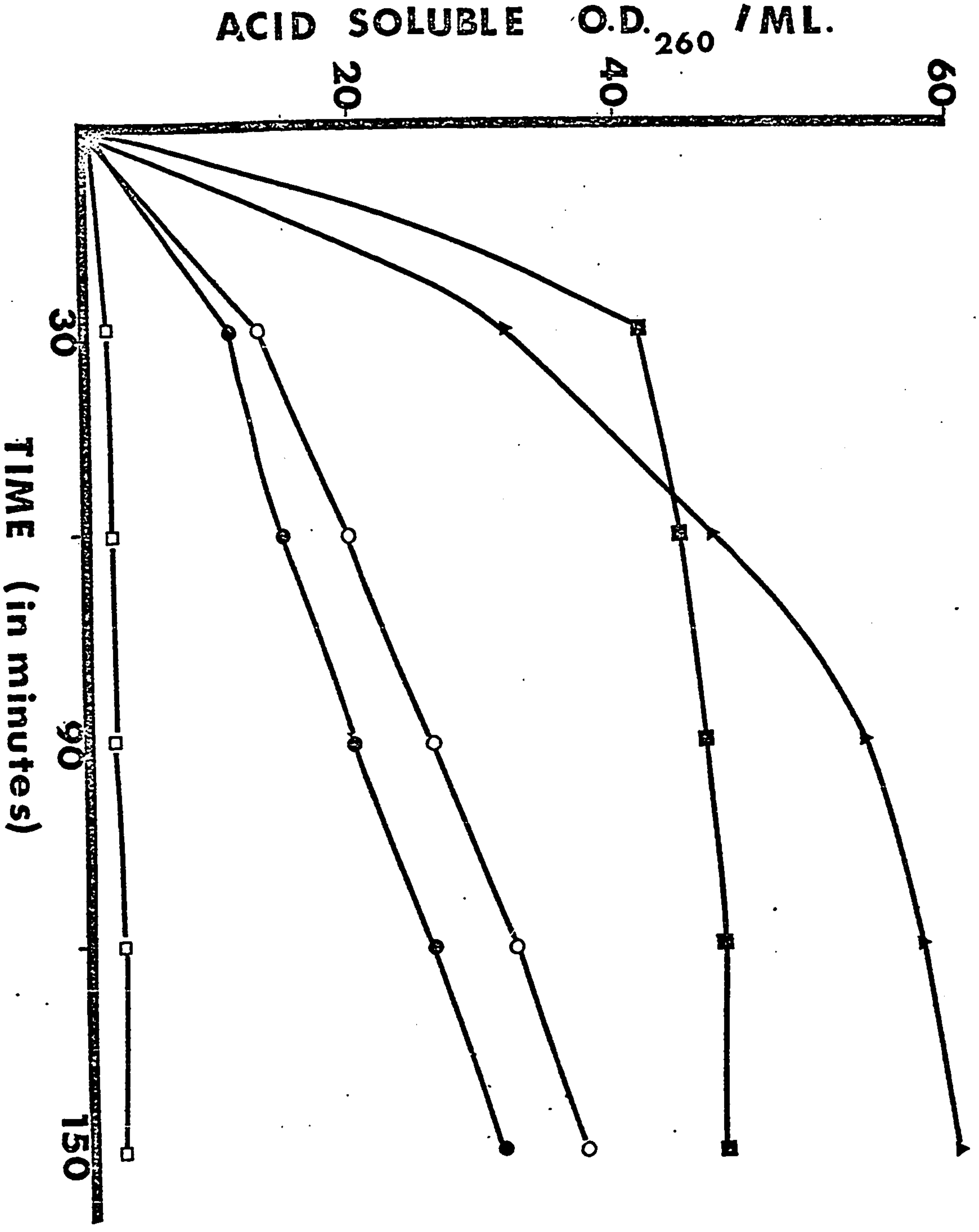

ACID SOLUBLE O.D.260 /ML.
20
40
60
TIME (in minutes)
30
90
150

FIGURE 16

ACID SOLUBLE PRODUCTS ABSORBING AT 260 nm RESULTING FROM THE ACTIVATION OF CELL FREE EXTRACT OF *PSEUDOMONAS AERUGINOSA*

The experiment was that of Fig. 15. 100 μl samples were removed at the indicated times to Beckman microtubes kept on ice. 100 μl of cold 6% PCA were added and 50 μl of 10 mg/ml bovine serum albumin. After shaking, the preparation was left on ice for 5 minutes and then centrifuged for 1 minute in a Beckman Microfuge. 200 μl samples were examined on the Gilford 2000 spectrophotometer for absorbancy (OD).

●——●	Mg^{++} 0.005 M
▲——▲	Mg^{++} 0.005 M, pancreatic RNase 20 μg/ml
○——○	No additives
□——□	EDTA 0.005 M
■——■	EDTA 0.005 M, pancreatic RNase 20 μg/ml

TABLE X

THE PRODUCTION OF ACID SOLUBLE RIBOSE AND DEOXYRIBOSE IN THE ACTIVATION INCUBATION OF PSEUDOMONAS AERUGINOSA CELL FREE EXTRACT

Preparation	Change in acid soluble products	
	Ribose* 150 minutes at 37°	Deoxyribose* 150 minutes at 37°
EDTA	0.025 mg/ml	0.0**
Mg^{++}	0.03 mg/ml	0.26 mg/ml
No additives	0.085 mg/ml	0.16 mg/ml
Pancreatic RNase + EDTA	0.28 mg/ml	0.0
Pancreatic RNase + Mg^{++}	0.21 mg/ml	0.20 mg/ml

*No correction for the failure to release pyrimidine ribose or deoxyribose has been made.

**0.0 indicates no change detectable.

TABLE XI

THE FAILURE OF MICROCOCCAL NUCLEASE TO PRODUCE ACTIVATION OF THE EXONUCLEASE

Preparation	Time at 37° (minutes)	Activity (units/ml)	Acid Soluble[*] Change in OD_{260}/ml
1. Control (Cell free extract with Ca^{++} 0.01 M and EDTA 0.005 M)	0	98.5	----
	180	58.5	4.8
2. Control + micrococcal nuclease (100 µg/ml)	0	91.5	----
	180	58.5	54.5

[*] Determined as in Fig. 16.

to the exonuclease. The presence of such fragments prevents destruction of those enzyme molecules in assays until the carried-over unlabelled fragment is destroyed and hydrolysis of labelled DNA commenced. The activation could more aptly be termed the destruction of competing and bound substrate.

In order to test the assumption that the activation is limited to the exonuclease and that no other enzyme is being activated, activity was monitored throughout the incubation using six sets of conditions. The conditions outlined in the legend of Fig. 17 retain a fixed relationship to the standard assay throughout the 37° incubation period. The conclusion is that a single enzyme is being activated. The alternatives that multiple enzymes are activating at identical rates or that multiple enzymes have identical activities under all of the test conditions are unlikely.

(e) The formation of a partially stable complex of DNA and the most purified exonuclease preparation

Results presented in parts (a) to (d) of this section have shown that the enzyme appears as an associated form and that association with DNA in crude cell extracts occurs.

It seems most probable that the DNA association is responsible for the behavior of the enzyme on the gel filtration and sucrose density gradient experiments.

Two further results suggest strongly that this is true:

(i) Crude extracts activated under the conditions of Fig. 15 chromatograph as a single peak on G-75 Sephadex identical to that of Fig. 9 provided care

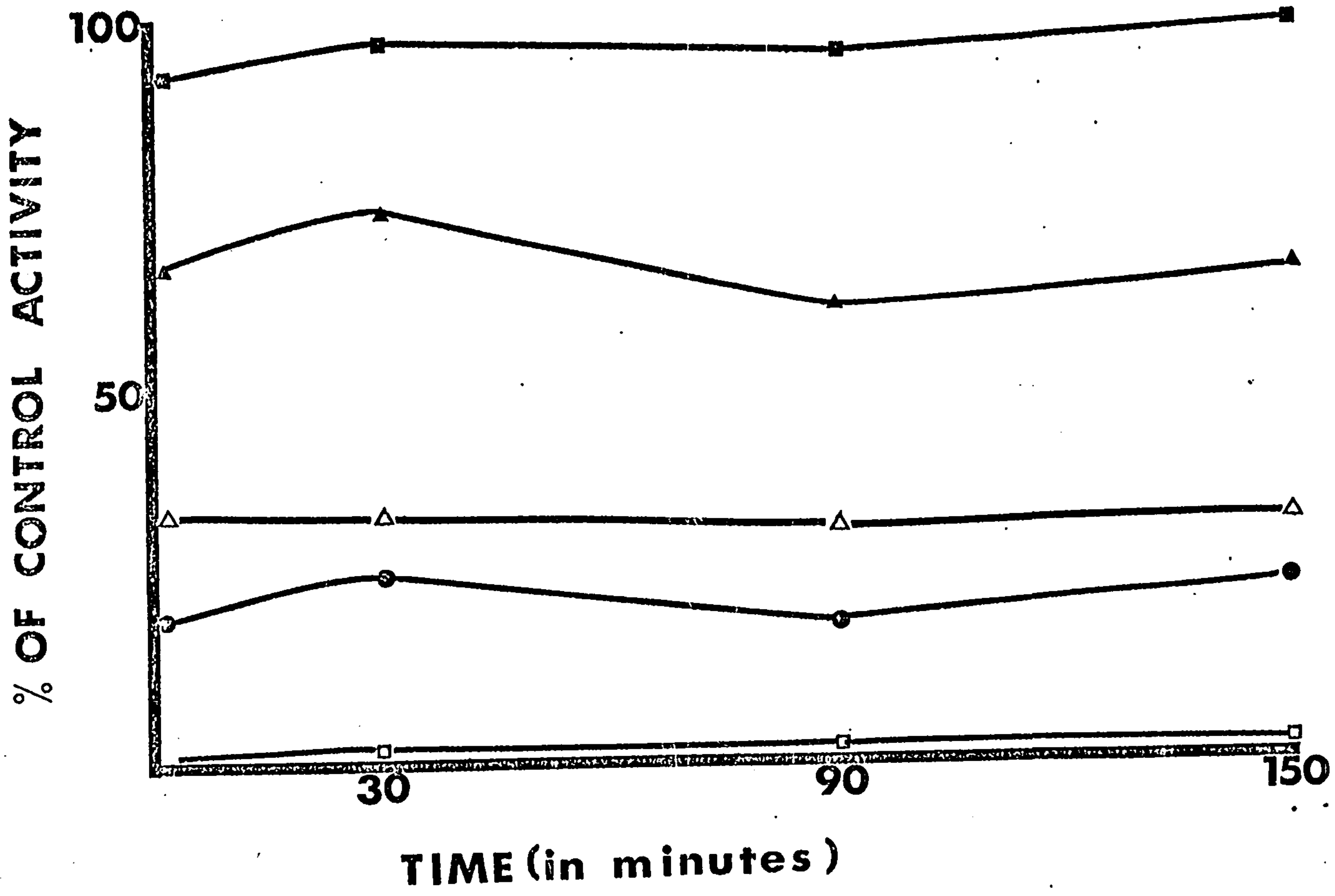

% OF CONTROL ACTIVITY
100
50
30
90
150
TIME (in minutes)

FIGURE 17

RELATIVE EXONUCLEASE ACTIVITY UNDER SEVERAL CONDITIONS DURING ACTIVATION INCUBATION OF PSEUDOMONAS AERUGINOSA CELL FREE EXTRACT

The experiment was that of Fig. 15. At the indicated times, assays were carried out under the following conditions and the activity relative to the standard exonuclease* assay determined.

■———■	10% TCA, 0.25% uranyl acetate used as precipitant
▲———▲	pH 9.0
△———△	denatured DNA as substrate
●———●	Ca^{++} 0.0025 M, no Mg^{++}
□———□	pH 5.2, 0.005 M EDTA, no Mg^{++}

*10% TCA was used as precipitant unless noted otherwise.

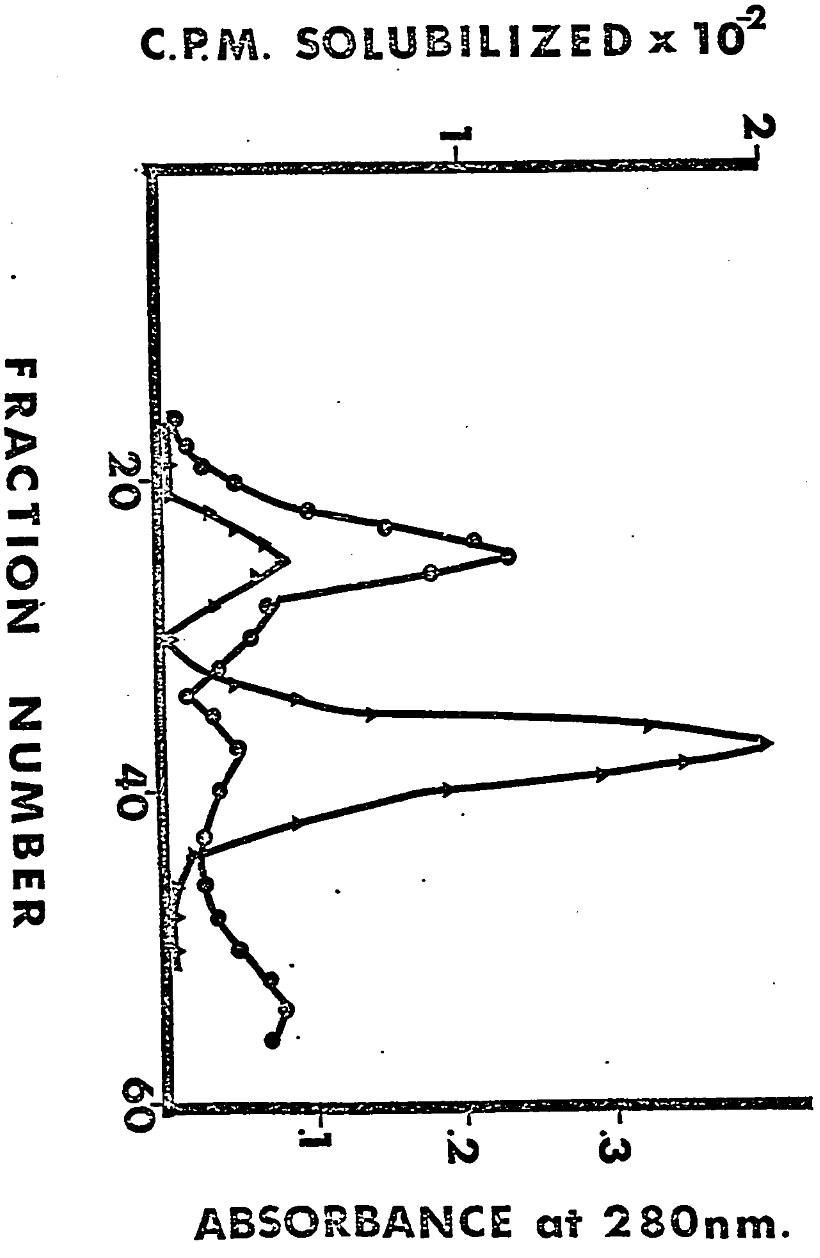

C.P.M. SOLUBILIZED x 10^{-2}
1
2
FRACTION NUMBER
20
40
60
.1
.2
.3
ABSORBANCE at 280nm.

FIGURE 18

THE FORMATION OF AN EXONUCLEASE FORM
EXCLUDED FROM G-75 SEPHADEX

0.4 ml of enzyme obtained from the G-75 Sephadex step in a normal purification sequence containing 0.7 mg/ml was incubated at 4° for 3 hours with E. coli DNA at a final concentration of 0.2 mg/ml. The DNA showed no exonuclease activity in the standard assay. The column specification and operation were identical to that of Fig. 9.

●——● Absorbance at 280 nm

▲——▲ Exonuclease activity

is taken to prevent proteolysis.

(ii) It is possible to reproduce a form of the enzyme which is excluded from G-75 Sephadex gel filtration by incubating E. coli DNA with the enzyme obtained from the last step of purification. Examination of Fig. 18 demonstrates the usual peak of activity eluting at the standard elution volume of 96 ml. In addition, a small activity peak eluting in the void volume of the column is also seen. In 8 columns run without prior DNA incubation, no activity has ever been detected in the void volumes. It therefore appears that the void volume peak is a complex of DNA and enzyme.

2. Proteolysis

The possibility that the exonuclease might be undergoing degradation was first suggested by the detection of activity associated with molecules considerably less than the estimated molecular weight of the exonuclease. The following investigation was undertaken to determine the nature of this modification of the enzyme.

(a) The detection of proteolysis

Examination for proteolytic activity was done in three ways. Each of these has definite problems associated with it, resulting primarily from the necessity of using crude cell free preparations.

(i) Casein hydrolysis

The casein assay depends upon the release of acid soluble products of the substrate which absorb at 280 nm. If cell free extract is incubated in buffer under assay conditions in the absence of casein, an endogenous release of acid soluble material absorbing at 280 nm occurs. This problem is overcome a) by estimating the endogenous release with appropriate blanks or b) by pretreating cell extract at pH 3.0 for 5 minutes at 25°.

(ii) Hydrolysis of benzoyl l-arginine ethyl ester

The hydrolysis of this substrate is specific for trypsin-like proteases in the absence of thrombin, plasmin and kallikrein (Dixon and Webb, 1958). Unfortunately, an endogenous hyperchromicity at 254 nm (which is the wavelength used in this assay) occurs under assay conditions but in the absence of the substrate, and is probably due to polynucleotide hydrolysis. That change is linear and is readily compensated for.

(iii) Proteolysis of endogenous protein

The ninhydrin assay was used to detect new α amino groups produced by proteolysis of endogenous protein when cell free extract was incubated at 37°. The major specific problem in using this assay was a small change in new amino groups with a high background level. However, the change was consistent.

Tables XII, XIII and XIV demonstrate evidence of protein hydrolysis using the casein assay, of ester hydrolysis involving

TABLE XII

THE PRESENCE AND INHIBITION OF PROTEOLYTIC ACTIVITY IN *PSEUDOMONAS AERUGINOSA* CELL FREE EXTRACT DETECTED BY THE HYDROLYSIS OF CASEIN

Preparation	Temperature of Incubation during Treatment with Inhibitor	Activity: ΔOD_{280}/20 min at specific times of incubation		Inhibition %
		0 MIN	90 MIN	
a) Cell free extract*	4°			
152 μg protein per assay		0.141	----	----
190 μg protein per assay		0.190	----	----
b) Cell free extract with 0.002 M PMSF	4°			
152 μg protein per assay		0.140	0.114	20
190 μg protein per assay		0.193	0.154	20
c) Cell free extract 380 μg protein per assay	37°			
(i) no additions		0.45	0.45	0
(ii) 0.002 M PMSF		0.45	0.18	60
(iii) in assay mix with no substrate			0.21	
(iv) (i) and (ii)			0.24	
corrected for (iii)			0.00	100

* No acid pretreatment

TABLE XIII

THE PRESENCE AND INHIBITION OF PROTEOLYTIC ACTIVITY IN *PSEUDOMONAS AERUGINOSA* CELL FREE EXTRACT DETECTED BY THE NINHYDRIN ASSAY

Preparation	ΔnM leucine equivalents after 90 minutes of incubation at 37°	Inhibition in 90 minutes at 37°
Cell free extract	875	----
Cell free extract with 0.002 M PMSF	200 - 400	77% - 54%

TABLE XIV

THE PRESENCE AND INHIBITION OF PROTEOLYTIC ACTIVITY IN *PSEUDOMONAS AERUGINOSA* CELL FREE EXTRACTS DETECTED BY THE HYDROLYSIS OF BENZOYL L-ARGININE ETHYL ESTER

Preparation	Temperature of Incubation during Treatment with Inhibitor	Activity (μM/mg/min) at specific incubation times				% Inhibition at 90 minutes
		0 min	30 min	60 min	90 min	
a) Cell free extract No additions	4°	$1.05x10^{-2}$	$1.05x10^{-2}$	$1.05x10^{-2}$	$1.05x10^{-2}$	0
	37°	$1.05x10^{-2}$	$1.00x10^{-2}$	$1.1\ x10^{-2}$	$1.05x10^{-2}$	0
b) Cell free extract plus PMSF 0.005 M	4°	$1.05x10^{-2}$			$1.05x10^{-2}$	0
	37°	$1.05x10^{-2}$	$3.2x10^{-3}$	$1.0\ x10^{-3}$	0.00*	100
c) Cell free extract plus 0.1 mg/ml trypsin	4°	18.2	17.8	17.6	18.1	0
	37°	18.2	18.0	17.8	17.6	3
plus 0.1 mg/ml trypsin and 0.005 M PMSF	4°	17.6	16.2	15.6	14.4	18
	37°	16.0	6.05	5.05	4.5	75

* No activity detected

the carboxyl group of arginine and the production of free α amino groups due to some degree of proteinase, peptidase or amidase activity in cell free extracts of P. aeruginosa.

(b) The inhibition of proteolysis

Phenylmethylsulfonylfluoride (PMSF), an inhibitor of many serine esterases (Meyers et al, 1954) was used to produce proteolytic inhibition. The limits of solubility are stated to be about 0.001 M in aqueous solution (Gold, 1967). However, if the inhibitor was initially dissolved in 95% ethanol at 0.12 M it could be added to aqueous preparations to yield a final concentration of 0.005 M with no evidence of crystallization. In addition, concentrations of 0.005 M were required to produce effective inhibition within 90 minutes at 37° in some crude preparations. Tables XII, XIII and XIV demonstrate that inhibition of 54 to 100% depending on the assay used was obtained at 37°. Inhibition at 4° is almost impossible to produce in crude preparations. The proteolytic enzyme activity assessed in this system resembles trypsin in that both enzymes have activity on benzoyl 1-arginine ethyl ester which as previously noted is reasonably specific for trypsin. In view of the resistance to proteolytic inhibition exhibited by the P. aeruginosa system, a comparison of the type of inhibition of exogenous trypsin added to the cell free extract was made.

Table XIV indicates that trypsin is only slowly inhibited at 37° and almost not at all at 0°. Thus, resistance to inhibition may be a function of the system in addition to an inherent resistance of trypsin (Fahrney and Gold, 1963). Reservation must be expressed in assuming an analogy for the endogenous protease as the characterization is inadequate to state that it is a close model of trypsin.

(c) Retention of exonuclease activity in the presence of trypsin

The endogenous protease demonstrated a possible resemblance to trypsin (part (a) - (ii) of this section) in its mode of action. If proteolysis was a cause of enzyme modification, it is necessary to assume that at least some exonuclease activity must be retained in spite of that modification. One way to ask this question was by the addition of trypsin at a concentration sufficient to produce activity on benzoyl l-arginine ethyl ester several times that of the endogenous protease. In doing this, the reservation that the endogenous enzyme is quite distinct from trypsin in specificity and that the results of such experiments may not be analogous must be firmly kept in mind.

Table XV demonstrates that it is possible for the exonuclease to be exposed to 0.1 mg/ml trypsin at 37° for up to 90 minutes and retain over one half of this activity. No loss of trypsin activity occurred over the incubation period.

(d) Evidence for proteolytic modification of the exonuclease

Included in the postulated effects of proteolytic modification of the exonuclease are degradation to molecules smaller than the parent enzyme and the production of molecules containing a different net charge.

Smaller forms of the enzyme as judged by gel filtration were detected in early purification trials and a spectrum of charged molecules has been produced very frequently much to my chagrin.

(i) detection of low molecular weight forms of the enzyme

1. Gel filtration

(a) Sephadex G-100: See section G - 1 part (b) and Fig. 14. Peaks 3 and 4 represent active enzyme which is smaller than that which is considered to be undegraded enzyme, represented by peak 2. The eluting solvent was 0.1 M Tris HCl buffer (containing 30% glycerol and 0.01 M mercaptoethanol) chosen to minimize electrostatic interaction of the gel and the protein.

(b) Biogel P-30 and P-6: Figures 19 and 20 demonstrate the elution profile from P-30 and P-6 columns of activity obtained after early purification as outlined in Table XVI. The P-30 column shows two peaks of activity and the P-6 column one peak not excluded from the column. The theoretical exclusion values are 30,000 molecular weight for P-30 and 4,600 for P-6.

Active enzyme of a molecular weight below that estimated by gel filtration (see section II, subsection J) for the intact exonuclease has thus been detected in two separate columns.

TABLE XV

RETENTION OF PSEUDOMONAS AERUGINOSA EXONUCLEASE ACTIVITY IN THE PRESENCE OF TRYPSIN[*]

Preparation	Time at 37° (min)	% Activity Retained
Cell free extract[**]	0	100
	30	75
	60	65
	90	62
G-75 (0.7 mg/ml)	0	100
	30	80
	60	75
	90	56

[*] Present in a concentration of 0.1 mg/ml.

[**] Prepared in the standard manner.

TABLE XVI

FLOW SHEET FOR THE PURIFICATION

USED IN P-30 AND P-6 COLUMNS

P. aeruginosa cells.
Grown and harvested as in Materials and Methods.

Resuspend as 1 g wet weight per 5 ml of Tris HCl 0.05 M pH 7.5,
mercaptoethanol 0.01 M and EDTA 0.0005 M.

Cell disruption in 50 ml volumes with the
Biosonik BP-I for 5 minutes at 4°.

100,000 x g Supernatant (see Materials and Methods)
80 ml volume containing 1580 mg protein.

Streptomycin sulfate treatment
(see Materials and Methods). Protein 1440 mg in 84 ml volume.

Applied supernatant to DEAE cellulose column 2.5 x 70 cm equilibrated with 0.05 M Tris HCl pH 7.5, 0.01 M mercaptoethanol and 30% glycerol followed by a 125 ml wash of the same buffer. A gradient consisting of 550 ml of buffer and 550 ml of 0.5 M NaCl in that buffer was run. Fractions of 20.0 ml were collected at a flow rate of 40.0 ml/hour. Temperature 4°.

Activity eluted as a single peak at 0.07 M NaCl.

Concentrated by ultrafiltration (Amicon UM 10) at 4°.
Protein 162 mg.

Dialyzed vs. 100 volumes of 0.005 M Na acetate pH 5.2,
0.01 M mercaptoethanol and 30% glycerol for 12 hours at 4°.

Centrifuged 10 minutes at 27,000 x g. Supernatant adjusted to 0.005 M
Tris HCl pH 7.5. Protein 50 mg.

DEAE cellulose column 1.5 x 12 cm. Equilibrated identically to
the first DEAE cellulose column. 50 mg of protein was applied,

TABLE XVI
(CONTINUED)

followed by a 15 ml wash, and a gradient of 50 ml equilibration buffer and 50 ml of 0.5 M NaCl in that buffer. Fractions were 2.0 ml and the flow 4 ml/hour. At the completion of the gradient 150 ml of 0.5 M NaCl in buffer was run into the column. Temperature 4°.

Activity eluted as 7 peaks. The first peak at 0.07 M NaCl was less than 5% of the total activity. Other peaks eluted at 0.2 M, 0.3 M, 0.35 M, 0.39 M, 0.42 M and 0.5 M.

The last 6 peaks eluting were combined and concentrated by ultrafiltration with a UM 2 filter. Protein 2.7 mg, volume 6.2 ml. The ionic strength was adjusted to 0.1 M Tris HCl by repeated concentration and dilution using a UM 2 filter.

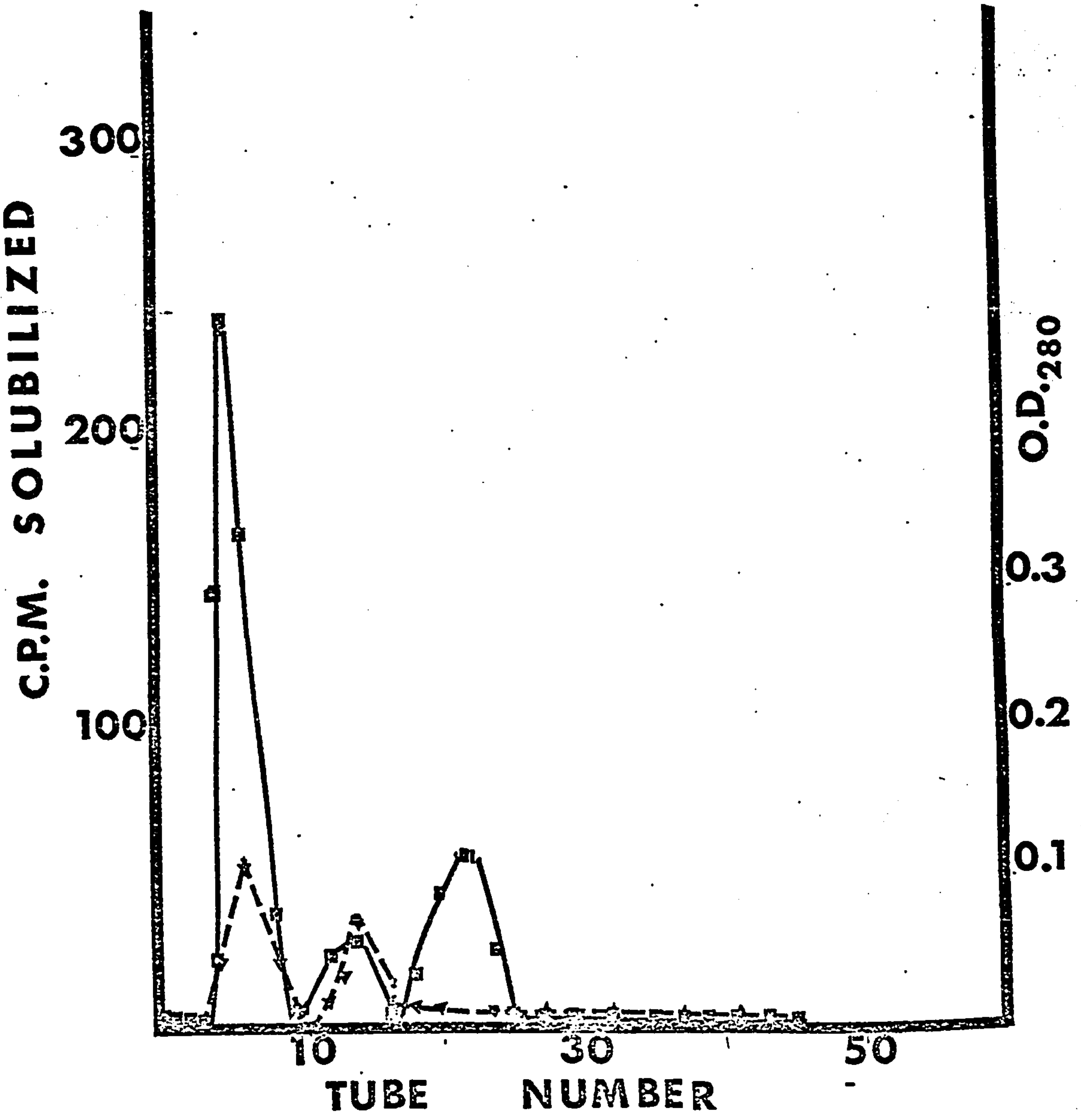

300
200
100
C.P.M. SOLUBILIZED
O.D.280
0.3
0.2
0.1
10
30
50
TUBE NUMBER

FIGURE 19

BIOGEL P-30 GEL FILTRATION OF THE PARTIALLY PURIFIED PSEUDOMONAS AERUGINOSA EXONUCLEASE

1.0 ml of a concentrate obtained from the second DEAE cellulose column specified in Table XVI containing 0.435 mg protein was applied to a 1.5 x 25 cm P-30 column. The buffer used for equilibration and elution contained 0.1 M Tris HCl pH 7.5, 0.01 M mercaptoethanol and 30% glycerol. Fractions of 1.7 ml were collected at a flow rate of 5.0 ml/hour. Temperature of operation was 4°. The void volume peak is tube 8.

■————■ exonuclease activity

▼- - - - -▼ absorbance (OD) at 280 nm

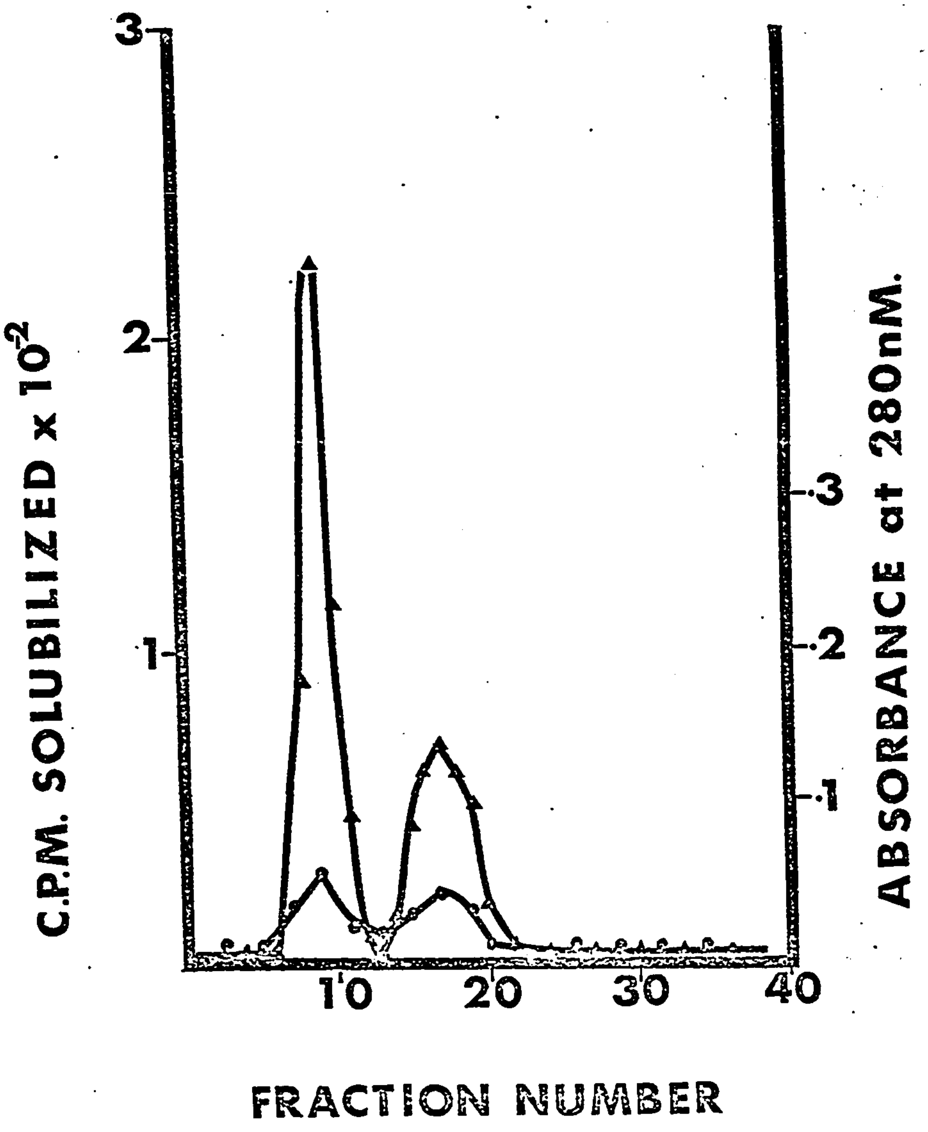

3
2
1
C.P.M. SOLUBILIZED x 10^{-2}
.3
.2
.1
ABSORBANCE at 280nM.
10
20
30
40
FRACTION NUMBER

FIGURE 20

BIOGEL P-6 GEL FILTRATION

OF PARTIALLY PURIFIED *PSEUDOMONAS AERUGINOSA* EXONUCLEASE

1.0 ml of the same preparation used in Fig. 19 was applied to a 1.5 x 25 cm P-6 column equilibrated and eluted with 0.1 M Tris HCl pH 7.5, 0.01 M mercaptoethanol and 30% glycerol. Fractions of 1.5 ml were collected at a flow rate of 10.0 ml/hour at 4°. The peak of the void volume is tube 9.

▲————▲ exonuclease activity

●————● absorbance at 280 nm

2. Ultrafiltration

Ultrafiltration with Amicon UM 10 filters provided an independent assessment of the presence of low molecular weight forms of the enzyme. UM 10 filters have a theoretical cutoff value for globular proteins of about 10,000 molecular weight. As a control, the filtration of ribonuclease (M.W. 13,680) (Hirs, Moore and Stein, 1956) was examined on the UM 10 and found to be greater than 90% retained in several trials.

The 6.2 ml volume effluent of the second DEAE cellulose column noted in Table XVI was refiltered using a UM 10 size filter in a 12.0 ml Amicon ultrafiltration unit.

Table XVII demonstrates that almost 25% of the total activity filtered. Such an observation, except for repeat trials of this experiment, was and has been the only occasion in over 20 trials in which more than 2% of activity filtered.

The observations of gel filtration and ultrafiltration have established the presence of enzyme activity less than the molecular weight of the intact enzyme. Such activity apparently exists in enzyme fragments.

TABLE XVII

AMICON UM 10 MEMBRANE FILTRATION OF

PSEUDOMONAS AERUGINOSA EXONUCLEASE ACTIVITY

Preparation	Activity per ml	Total Activity	Volume (ml)
1. DEAE cellulose #2 applied to UM 10 filter	58.8 units	364.6 units	6.1
2. Effluent of (1)	22.5 units	90 units	4.1
3. Concentrate	135 units	270 units	2.0

(ii) Multiple activity peaks on ion exchange chromatography

Multiple peaks of exonuclease activity on DEAE cellulose columns were observed on several occasions and in fact occurred without exception in purification sequences until the proteolytic inhibition of section I subsection G - 2 - (b) was used.

One example has been provided in Table XVI on the second DEAE cellulose column of a purification sequence.

A second example demonstrating 8 peaks is seen in Fig. 21. Three major points distinguish this example from that of Table XVI. The polymorphism resulted on the first DEAE cellulose column in Fig. 21 and the enzyme preparations used in that column had undergone an incubation at 37° for 90 minutes and had been stored at 4° for 7 days prior to activation. The preparation of Table XVI in contrast was used without activation and after storage at -20° for 3 days. The significance of these distinctions will become obvious in the results which follow.

These and other examples thus produced a population of species most of which (6 of 7 in Fig. 21) eluted at higher ionic strength than the species of Fig. 6 and therefore very likely contained a greater net negativity. The possibility that these additional peaks were distinct enzymes was eliminated

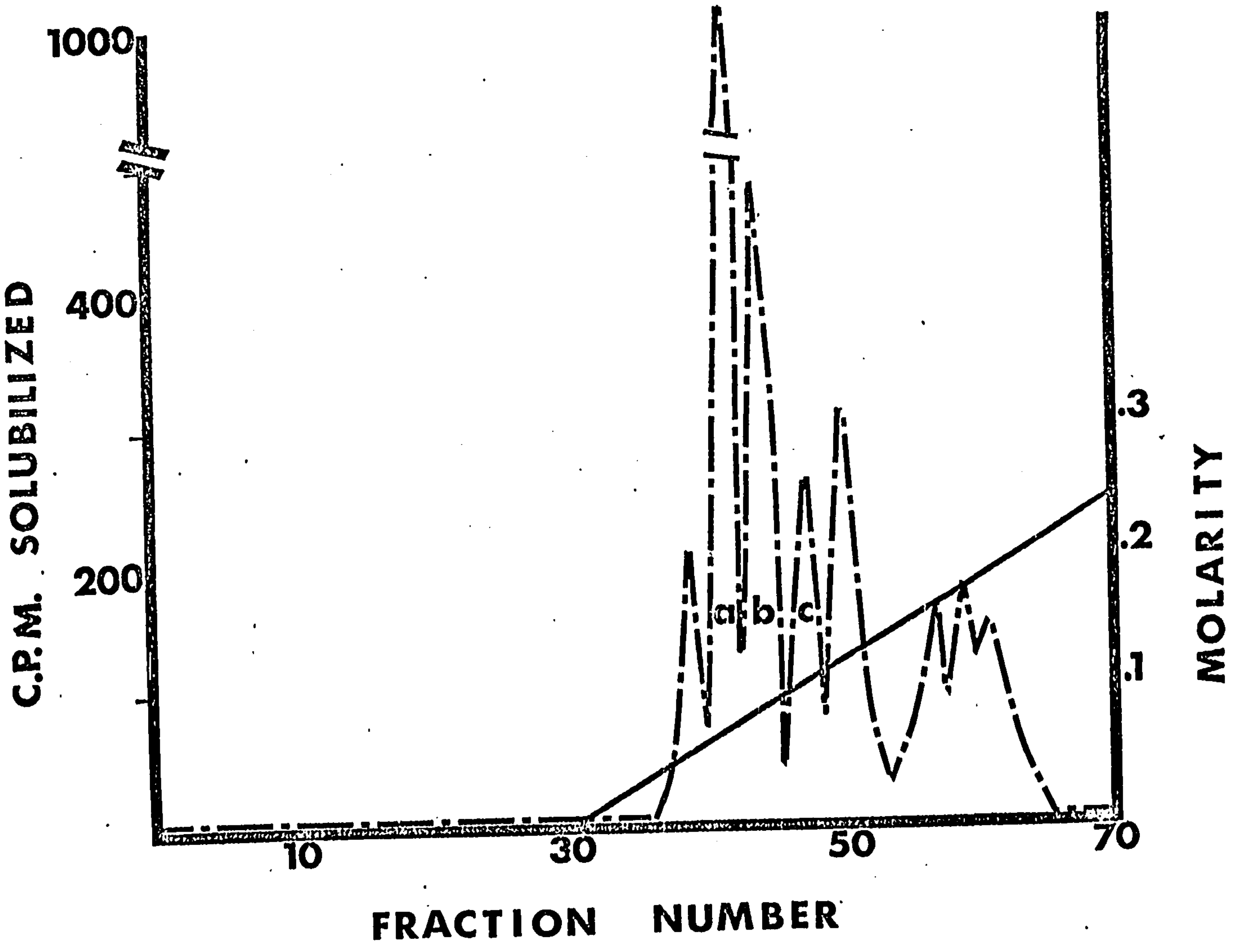

C.P.M. SOLUBILIZED
1000
400
200
MOLARITY
.3
.2
.1
a
b
c
10
30
50
70
FRACTION NUMBER

FIGURE 21

MULTIPLE PEAKS OF EXONUCLEASE ACTIVITY OBTAINED FROM DEAE CELLULOSE COLUMN CHROMATOGRAPHY

100,000 x g supernatant was prepared in the standard manner except that no PMSF was included in the activation step. The preparation was stored at 4° for 7 days and then added to a DEAE cellulose column #1 exactly as given in Materials and Methods. All tubes were assayed for exonuclease activity and examined for molarity.

— - —	exonuclease activity
———	molarity (NaCl)

to a substantial degree by the results of Figures 22, 23 and Table XVIII. The remarkable degree of similarity of activity demonstrated by tubes 42, 50 and 59 of Fig. 21 in pH profiles, cation requirements, the effect of EDTA, substrate specificity and exonucleolytic attack is a sound arguement that each of these fractions contains the same enzyme.

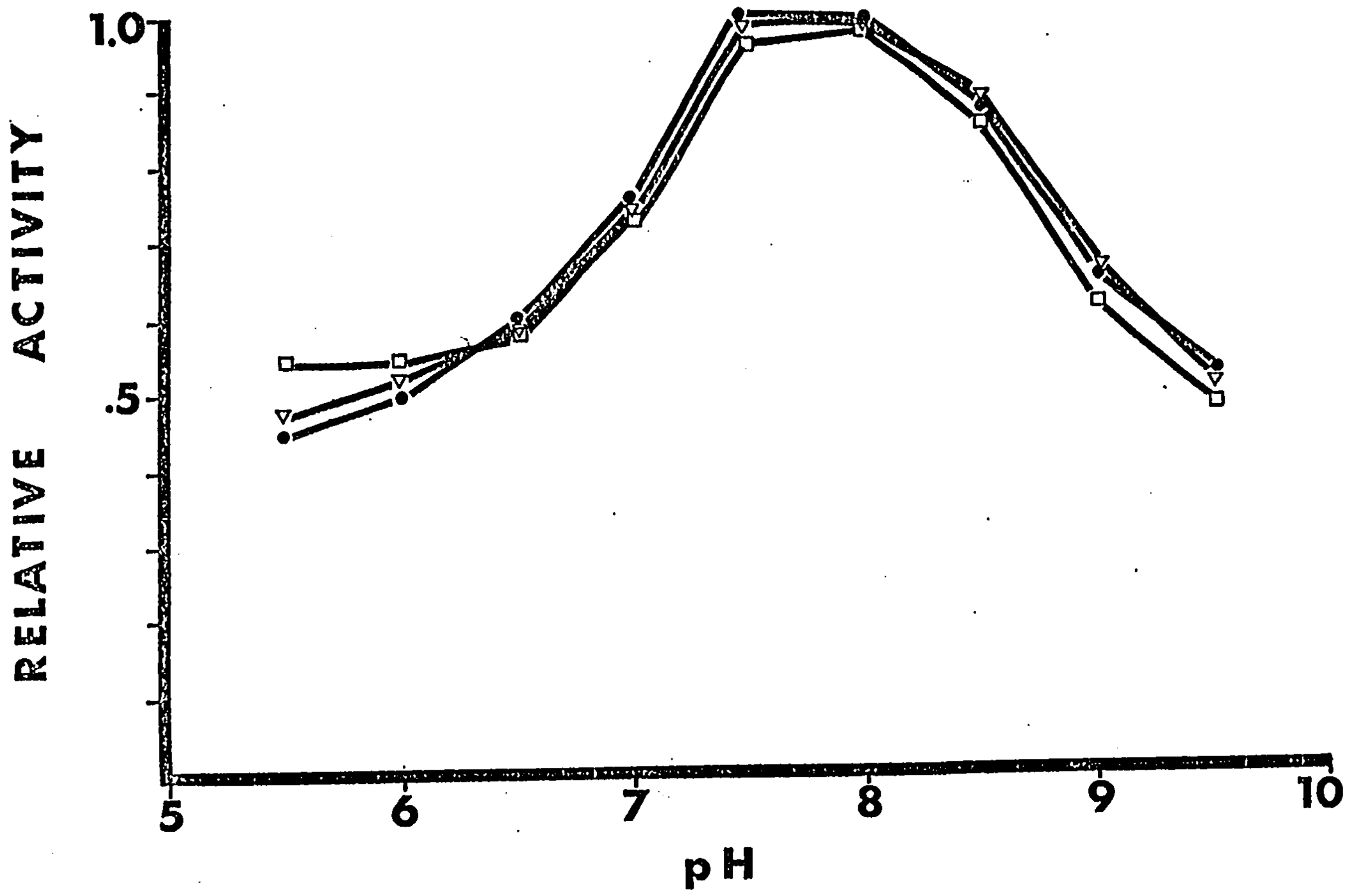
(iii) The elimination of multiple peaks of activity on DEAE cellulose by the use of phenylmethylsulfonyl fluoride (PMSF)

The effect of the inclusion of PMSF in the activation procedure at 37° and in all buffers of DEAE cellulose columns (as given in Materials and Methods) is clearly shown in Figures 24 and 25. Fig. 24 represents a portion of the DEAE cellulose elution profile of Fig. 6 on an expanded scale and provided for comparison with Fig. 25.

The DEAE elution profile of Fig. 25 is of 100,000 x g supernatant treated in an identical manner to that of Fig. 24 except that PMSF was not included in any stage of the procedure.

Several new small peaks eluting at an increased ionic strength have appeared in Fig. 25 in which no proteolytic inhibition was provided. In addition, less activity was recovered from the column.

These figures illustrate another observation which is that aging of cell extracts promotes an



RELATIVE ACTIVITY
1.0
.5
pH
5
6
7
8
9
10

FIGURE 22

pH PROFILES OF FRACTIONS 42, 50 AND 59 OF THE DEAE CELLULOSE COLUMN OF FIG. 21

pH profiles were carried out using a final concentration of 0.05 M Tris maleate in the exonuclease assays. 10 μl samples of each of the fractions were assayed so that the final concentration of the eluting Tris HCl buffer was 0.003 M.

●————●	tube 42
▽————▽	tube 50
□————□	tube 59

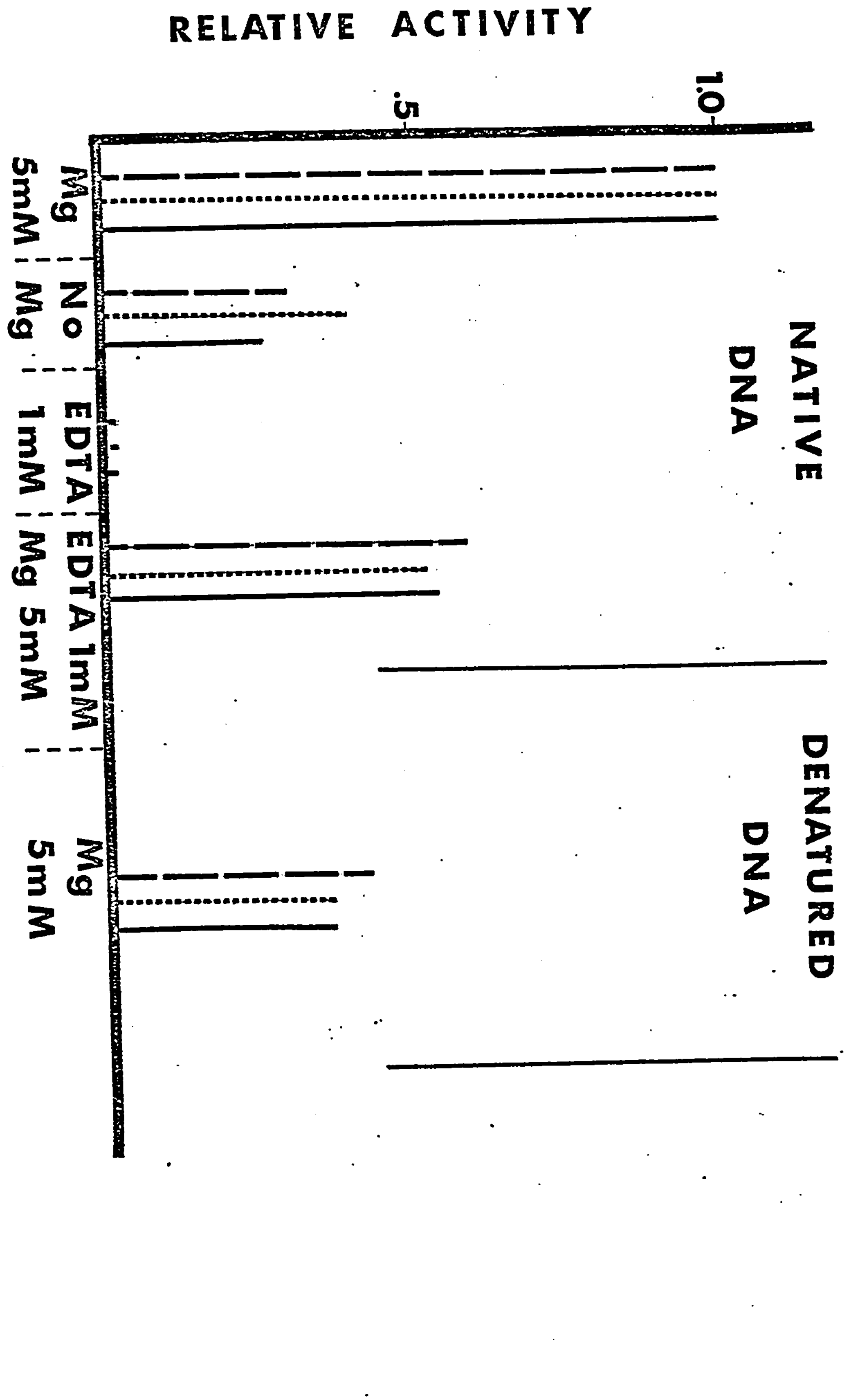

RELATIVE ACTIVITY
.5
1.0
NATIVE DNA
DENATURED DNA
Mg 5mM
No Mg
EDTA 1mM
EDTA 1mM Mg 5mM
Mg 5mM

FIGURE 23

THE RELATIVE ACTIVITY OF FRACTIONS 42, 50 AND 59
OF THE DEAE CELLULOSE COLUMN OF FIG. 21
UNDER A VARIETY OF ASSAY CONDITIONS

10 μl samples of the appropriate tubes were assayed in the standard exonuclease assay with the modifications indicated on the figure. Activities are relative to the standard assay containing native DNA and 0.005 M Mg^{++}.

— — — —	tube 42
-----------	tube 50
————	tube 59

TABLE XVIII

THE RELATIVE ACTIVITY OF TUBES 42, 50, AND 59 FROM THE DEAE CELLULOSE COLUMN OF FIG. 14 USING TCA* OR UTCA* AS A PRECIPITANT

Preparation	Relative Activity	
	TCA	UTCA
42	1.0	0.90
50	1.0	0.92
59	1.0	0.94

*The significance of the use of these reagents is shown in section II subsection D of the results.

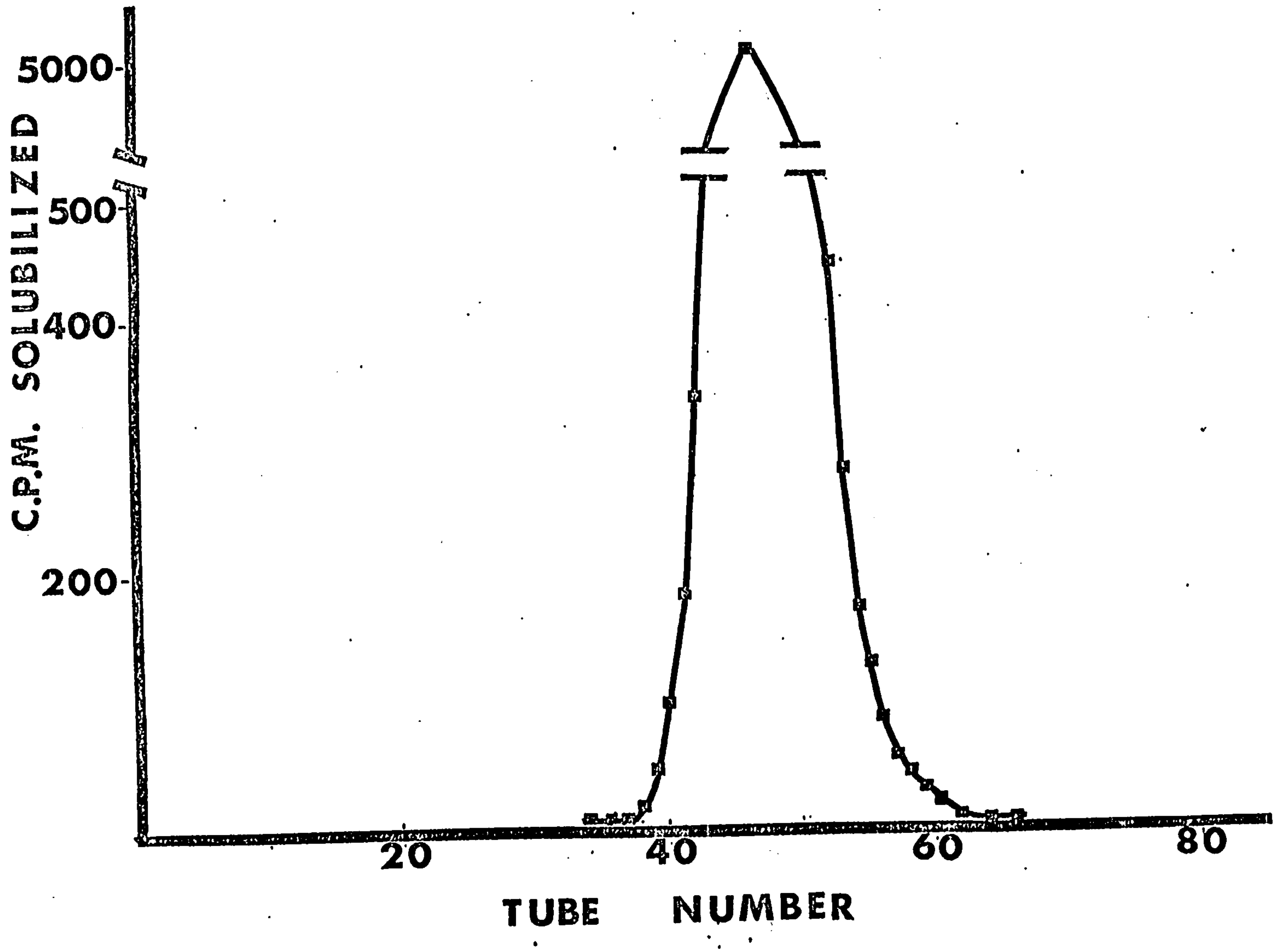

C.P.M. SOLUBILIZED
5000
500
400
200
20
40
60
80
TUBE NUMBER

FIGURE 24

ELUTION PROFILE OF THE DEAE CELLULOSE

COLUMN OF FIG. 6 ON AN EXPANDED SCALE

Conditions are identical to those of Fig. 6.

■——■ Exonuclease activity

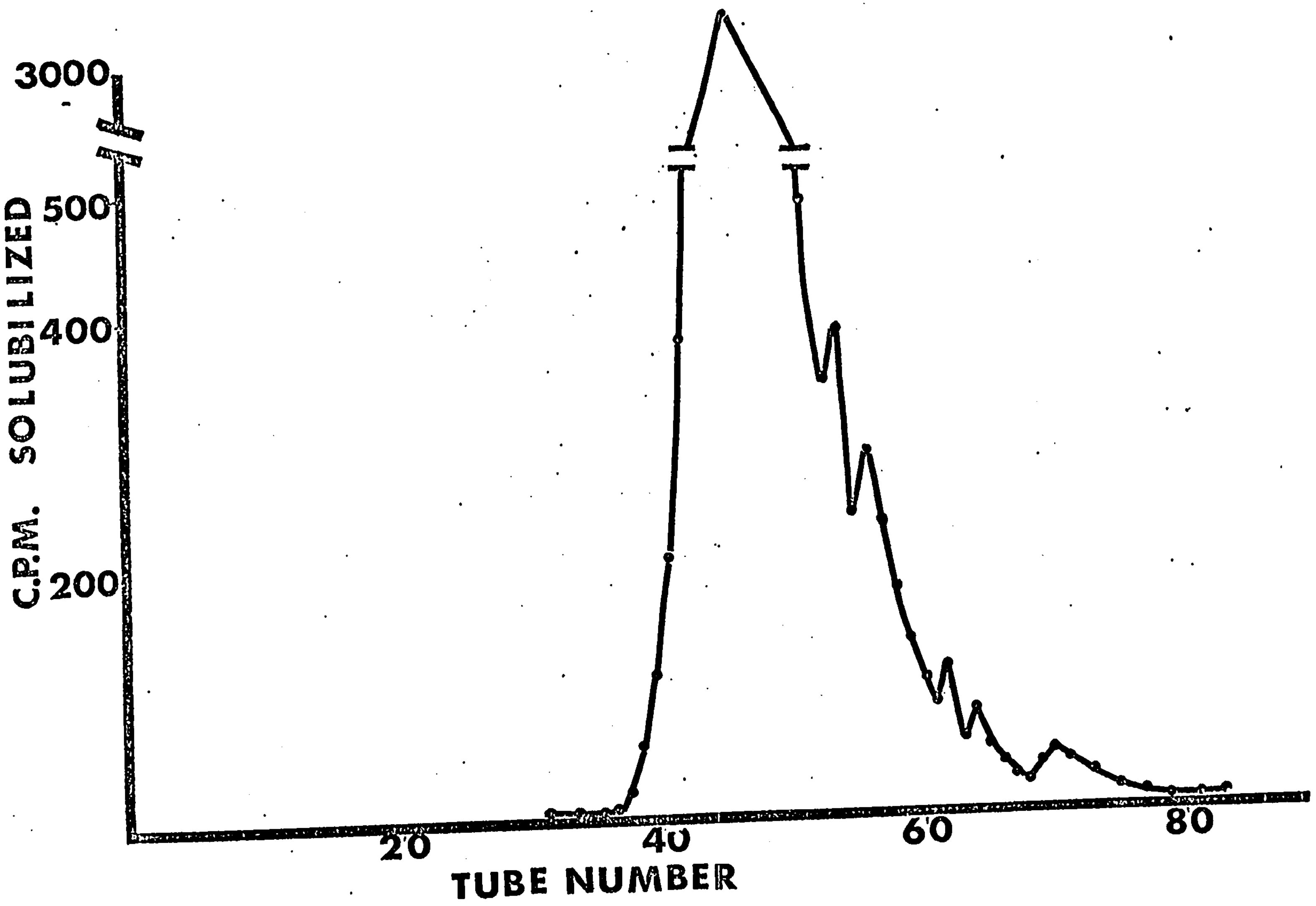

3000
500
400
200
C.P.M. SOLUBILIZED
20
40
60
80
TUBE NUMBER

FIGURE 25

ELUTION PROFILE FROM DEAE CELLULOSE CHROMATOGRAPHY OF "UNAGED" 100,000 x g SUPERNATANT CONTAINING NO PMSF

100,000 x g supernatant was prepared, activated and added to a DEAE cellulose column as given in Materials and Methods except that no PMSF was present at any time. Column operation was identical to that of Fig. 6.

●—● exonuclease activity

increased number of peaks. Comparison of Fig. 21 and Fig. 25 demonstrates this conclusion. The cell extract used in Fig. 21 was "aged" for 7 days at 4° as 100,000 x g supernatant whereas that of Fig. 25 was stored at -20° for 12 hours in the same state. In both cases, cells were harvested, broken and the 100,000 x g supernatant prepared within 12 hours of the termination of cell growth.

If PMSF is added to a preparation aged in an identical manner to that of Fig. 21, three main peaks elute from DEAE cellulose. These are indicated by the letters a, b and c in that figure.

These results demonstrate that inhibition of proteolysis can totally prevent polymorphism on DEAE cellulose in "unaged" preparations and substantially diminish it in "aged" preparations. The effect of aging is presumably to modify the exonuclease by proteolysis and not to activate the protease. That conclusion is based on the observation that proteolytic activity does not increase in new cell extracts over a 7 day period. In addition, prevention of proteolysis causes some preservation of exonuclease activity while on DEAE columns (Fig. 24 vs. Fig. 25).

(iv) The persistence of a single peak of exonuclease activity throughout the complete purification sequence using PMSF

The persistence of a single activity peak on gel filtration and two ion exchange steps (see Figures 6, 8 and 9) in the purification has only been possible since the use of PMSF was instituted up to and including the ammonium sulfate step. The inhibitor is normally no longer used after that step.

Exceptions to a single peak have been detected occasionally when a second small peak of activity elutes from the first DEAE cellulose column in the purification sequence. It elutes at 0.11 M NaCl in buffer compared to 0.07 M for the main peak. The second peak was examined by Sephadex G-200 and found to elute at a position identical to the main peak.

The occasional presence of this additional peak does point out one problem. Effective proteolytic inhibition requires incubation at 37° so that it is very likely that some proteolytic modification of the exonuclease can and does occur before total proteolytic inhibition is achieved. The additional peak is, therefore, probably an example of such an effect.

II. CHARACTERIZATION OF AN EXONUCLEASE OF PSEUDOMONAS AERUGINOSA

All characterizations were obtained using exonuclease-phosphatase from the final purification step.

A. Assay of activity

1. Kinetics and the effect of mercaptoethanol

The assay used is dependent on the production by the enzyme of acid soluble fragments from the substrate: tritiated E. coli DNA. The carrier initially utilized in these assays was calf thymus DNA at a final concentration of 1 mg/ml. It was replaced by bovine serum albumin at a final concentration of 4 mg/ml for several reasons. These were:

(i) difficulty in complete solubilization of the DNA

(ii) pipetting error due to high viscosity of the stock solution of 2.5 mg/ml DNA

(iii) occasional examples of high and inconsistent zero time radioactivity were seen when viscous enzyme preparations were assayed.

Bovine serum albumin eliminated each of those objections. Zero time values were consistently less than ½ of 1% of the total counts in the assay.

Fig. 26 demonstrates an example of a standard exonuclease assay. In such assays, replicate tubes were set up for removal at the designated time intervals rather than removing aliquots. Linearity with respect to time exists for about 15 minutes in the presence of mercaptoethanol and was always present for at least 12 minutes in several identical experiments. Activity levels off rapidly after 2 hours (see Fig. 35). In the absence of mercaptoethanol, deviation from linearity begins before 12 minutes and levels off rapidly thereafter. As a results, assays of 12 minutes duration were selected for the standard assay.

Crude preparations demonstrate deviation from linearity in the presence of mercaptoethanol usually later than 15 minutes. For example, assays of cell free extract remained linear for 25 to 30 minutes. In addition, mercaptoethanol effects were not evident until at least after 30 or more minutes.

The effect of varying concentration of sulfhydryl reagents is seen in Table XIX-A. The assays in that experiment were purposely allowed to extend to 30 minutes so that the effect of the reagent in correcting the deviation from linearity could be assessed. Under those conditions the improvement in activity is present to a concentration of at least 0.05 M mercaptoethanol. However, as noted in Table XIX-B, 420 units/ml or 94% of maximal activity is obtained at a concentration of 0.005 M mercaptoethanol so that for the standard assay duration of 12 minutes 0.01 M was considered an adequate concentration.

Dithiothreitol is also effective as noted in Table XIX-A.

On several occasions during purification, despite the presence of 0.01 M mercaptoethanol in all fluids, it was noted that fresh addition of that compound would produce slight improvements in activity. The experiment in Table XX illustrates this phenomenon. In view of this finding, fresh mercaptoethanol was added at intervals so as to yield a final concentration of "fresh" mercaptoethanol of 0.01 M. For example, active fractions obtained from a column were treated with fresh mercaptoethanol before concentration, as were preparations thawed and frozen at -20° five times.

The proportionality between activity detected and the quantity

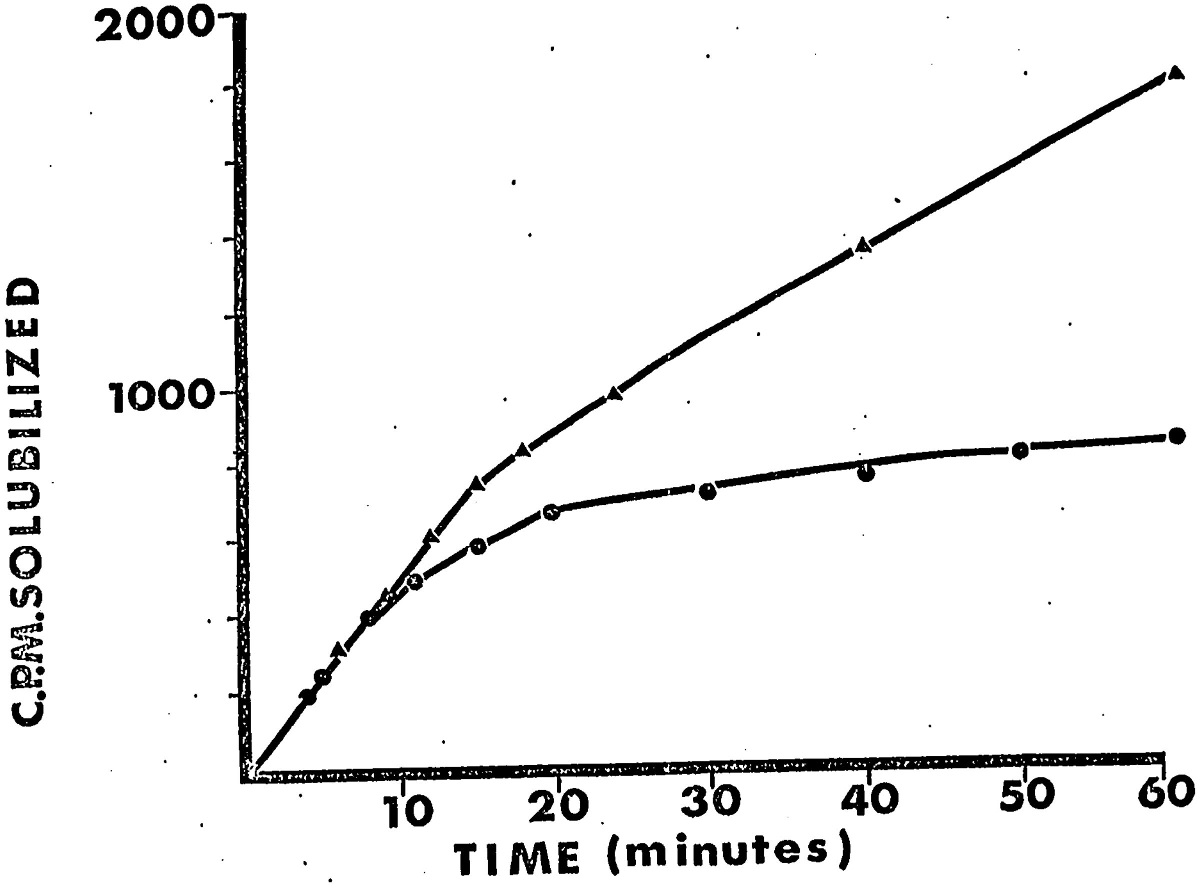

2000
1000
C.P.M. SOLUBILIZED
10
20
30
40
50
60
TIME (minutes)

FIGURE 26

THE RELEASE OF ACID SOLUBLE TRITIATED PRODUCTS WITH TIME BY *PSEUDOMONAS AERUGINOSA* EXONUCLEASE IN THE PRESENCE AND ABSENCE OF MERCAPTOETHANOL

Assay mixes of 100 μl containing 1.8 units of exonuclease were prepared, incubated for the indicated time, stopped and counted as outlined in Materials and Methods for the standard exonuclease assay. Mercaptoethanol was deleted from one set of assays.

▲———▲ standard assay mix

●———● standard assay mix minus mercaptoethanol

TABLE XIX

THE EFFECT OF SULFHYDRYL REAGENTS ON ACTIVITY OF THE PSEUDOMONAS AERUGINOSA EXONUCLEASE

Preparation	Final concentration in assay	Activity* (units/ml)
A. 30 MINUTE ASSAY		
Mercaptoethanol	0.001 M	295
	0.005 M	343
	0.01 M	355
	0.05 M	434
	0.1 M	453
	0.2 M	450
Dithiothreitol	0.005 M	410
	0.01 M	454
	0.05 M	458
B. 12 MINUTE ASSAY		
Mercaptoethanol	0.001 M	275
	0.005 M	420
	0.01 M	440
	0.05 M	448
	0.1 M	454
	0.2 M	452

*Activity units for 30 minute assays were determined using the cpm solubilized in 30 minutes and making a time correction.

TABLE XX

THE DECREASE IN SPECIFIC ACTIVITY SEEN WITH STORAGE OF A G-75 SEPHADEX PSEUDOMONAS AERUGINOSA EXONUCLEASE PREPARATION AND THE RECOVERY WITH MERCAPTOETHANOL

Time at 4°	Specific Activity	
	No additional mercaptoethanol* added	Additional mercaptoethanol added to a final concentration of 0.01 M
1 hr	1650	1650
24 hr	1425	1620
36 hr	1360	1640
48 hr	1180	1580
72 hr	1074	1565

* Mercaptoethanol was kept in all buffers during the purification at a concentration of 0.01 M.

of enzyme used is illustrated in Fig. 27. A constant specific activity is obtainable using 0.5 to 2.5 μg of protein per assay. This represents a solubilization range of 6.3% to 32% of the total DNA present.

The demonstration of proportionality in crude preparations was a more difficult problem. It was present over a very narrow range of activity. In general, solubilization of 3 to 12% of the total DNA present was the extreme limit using crude cell free extract. The range gradually increased through purification up to that given above.

2. The effect of pH and ionic strength

pH profiles of activity have been obtained in Tris HCl over the range pH 7.0 to pH 9.5 and Tris maleate from pH 5.5 to pH 9.0 as seen in Fig. 28. The pH optimum is about 7.8 to 8.0 with Tris HCl. The profiles are similar in both buffers. The buffer concentration is significant so that all final buffer concentrations were 0.05 M. If 0.1 M final concentration of Tris maleate was used, the activity at pH 8.0 and 7.5, for example, was reduced to 46% and 43.5% respectively with a general reduction at all pH values. If phosphate buffer pH 7.5 was used, a final concentration of 0.05 M produced only 65% of the activity of the same assay with 0.05 M Tris HCl pH 7.5. However, if the buffer strength was lowered to 0.025 M the result was 97%. Phosphate could not be used below pH 7.0 as Mg^{++} was precipitated and activity was greatly reduced.

The examples of the effect of increased concentration of Tris maleate and of phosphate buffer are evidence of an untoward effect of increased ionic strength. The significance of such an effect is seen in Fig. 29. The addition of NaCl to the final concentrations as noted in a standard assay produced a remarkable decline in activity at even low concentration of NaCl. For example, less than 50% of activity was

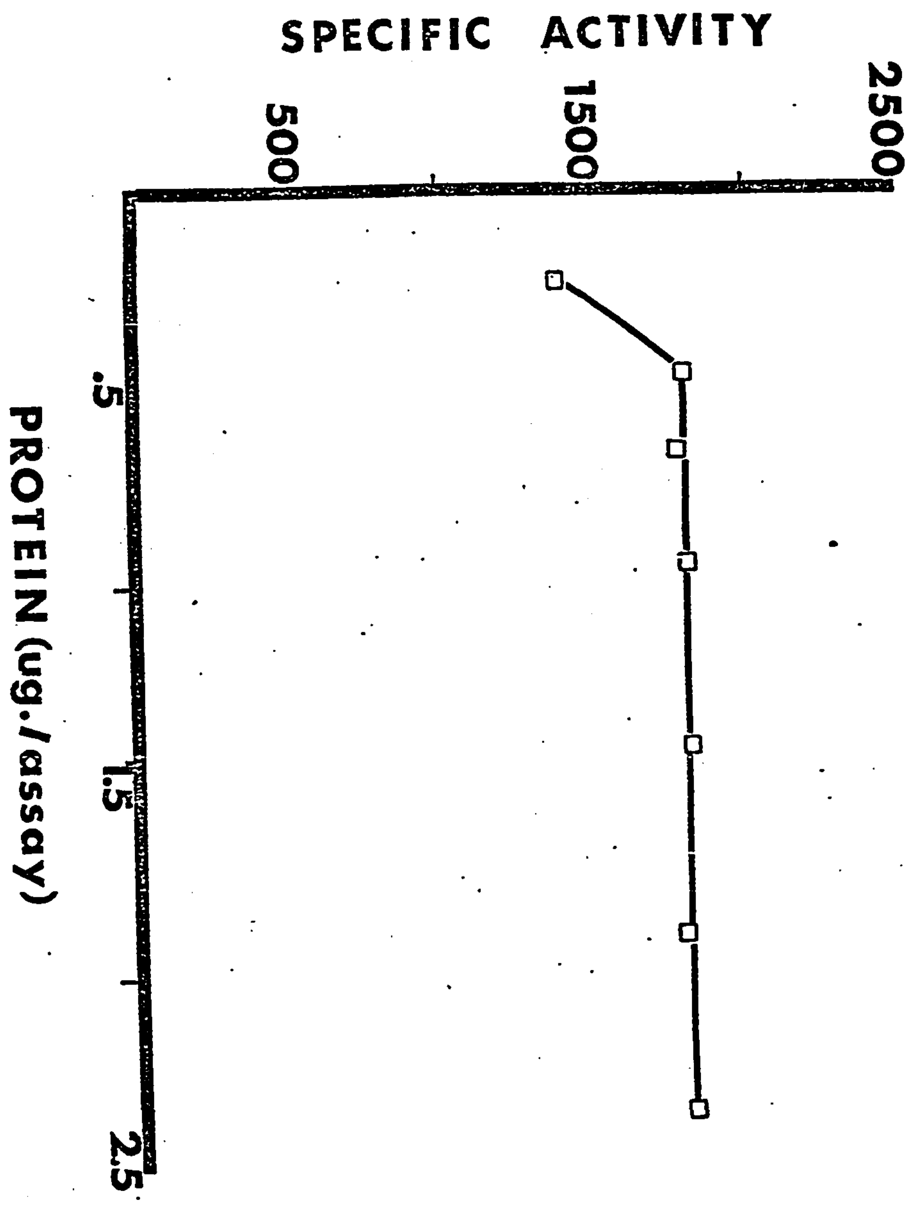

SPECIFIC ACTIVITY
2500
1500
500
PROTEIN (ug./assay)
.5
1.5
2.5

FIGURE 27

PROPORTIONALITY BETWEEN ACTIVITY AND PROTEIN CONTENT OF THE PSEUDOMONAS AERUGINOSA EXONUCLEASE

□——□ Specific activity (units/mg protein)

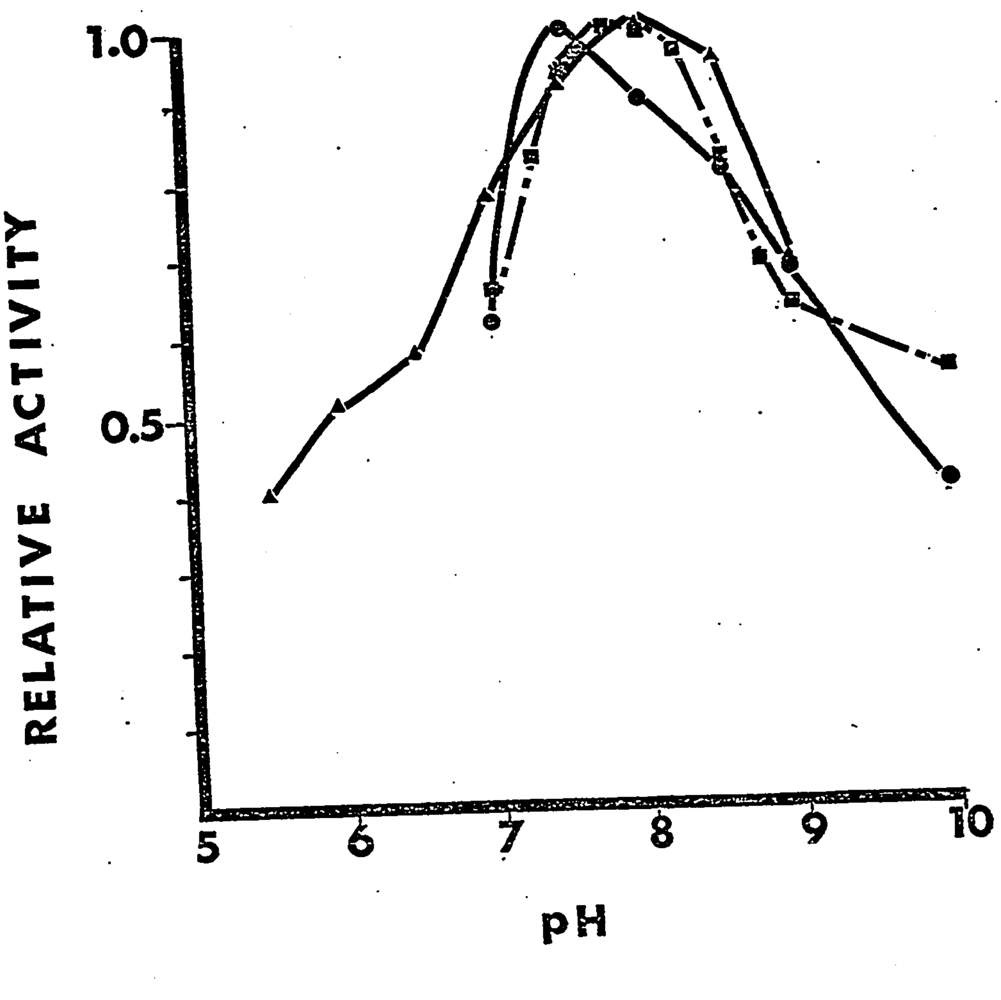

1.0
0.5
RELATIVE ACTIVITY
5
6
7
8
9
10
pH

FIGURE 28

pH PROFILES OF EXONUCLEASE AND
PHOSPHATASE FUNCTIONS OF
PSEUDOMONAS AERUGINOSA EXONUCLEASE

●————● Phosphatase using 0.05 M Tris HCl

▲————▲ Exonuclease using 0.05 M Tris maleate

■— — — —■ Exonuclease using 0.05 M Tris HCl

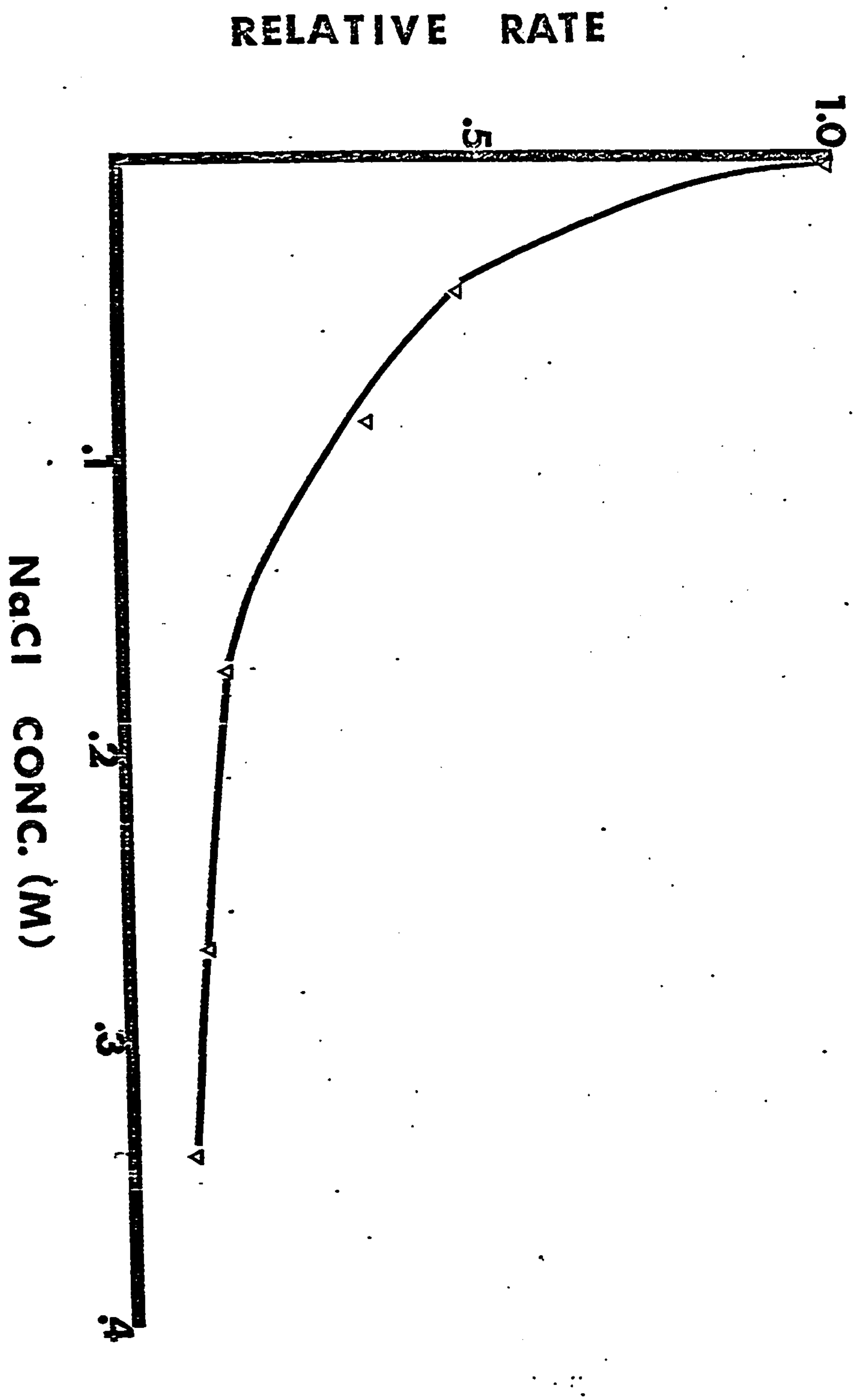

RELATIVE RATE
.5
1.0
NaCl CONC. (M)
.1
.2
.3
.4

FIGURE 29

THE EFFECT OF NaCl ON

PSEUDOMONAS AERUGINOSA EXONUCLEASE ACTIVITY

NaCl concentrations are final concentrations in the assay mix.

present at approximately 0.04 M NaCl. At 0.175 M, 10% of initial activity remained. The reduction of Tris HCl below 0.05 M did not affect activity.

As noted in Table V, this effect was seen even in the cell free extract. In an effort to minimize such an effect during chromatography, the smallest assay volumes feasible were utilized thus producing the lowest possible ionic strength increase. All quantitative assays were done in 0.05 M Tris buffer with final phosphate concentration (due to the enzyme additions) kept below 0.0025 M.

Fig. 38 represents a K_m and V_{max} determination for the purified enzyme with 0.07 M NaCl present in the assay. As can be seen in Table XXVIII the K_m is markedly increased while the V_{max} is unchanged. Thus the effect of salt appears to inhibit binding affinity.

3. Cation requirements

Table XXI demonstrates the relative exonuclease activity with several cations compared to the standard assay containing Mg^{++}. In the absence of added cations using preparations of enzyme and DNA substrate extensively dialyzed, values of 10 to 20% of the activity of the control preparation using Mg^{++} were obtained.

Mn^{++} is the only cation, of the group tested, partly effective in replacing Mg^{++}. All of the other cations are totally ineffectual with the exception of Co^{++} which produces slight stimulation.

The effect of combinations of cations is seen in Table XXII. As expected, all cations mixed with Mg^{++} produced some decrease in activity. Zn^{++} appears to produce total inhibition when used at equimolar concentrations. However, under these conditions the DNA wholly or partly precipitates. At a 1:5 molar ratio to Mg^{++} and a final concentration of 0.0005 M Zn^{++} produces a 28% reduction so that it can be

TABLE XXI

THE ACTIVITY OF PSEUDOMONAS AERUGINOSA EXONUCLEASE* WITH VARIOUS CATIONS

Cation**	Relative Exonuclease Activity	Relative phosphatase Activity
Mg	1.0	1.0
Mn	0.66	0.65
Co	0.21	0.23
Ca	0.10 to 0.20	0.106
Zn	0.01	0.03
Fe	0.05	---
Ni	0.05	---
Cu	0.00	---
None	0.10 to 0.20	0.07

*The exonuclease and DNA substrate were dialyzed against 100 volumes of 0.0025 M potassium phosphate, 0.01 M mercaptoethanol and 0.005 M EDTA followed by dialysis with the EDTA deleted.

**Final concentration in the assay is 0.0025 M in the case of the exonuclease and 0.001 M for the phosphatase.

TABLE XXII

ACTIVITY OF *PSEUDOMONAS AERUGINOSA* EXONUCLEASE WITH CATION MIXTURES

Cations	Final concentration (M) in assay	% of Control*
(1) Mn^{++} Mg^{++}	0.0025 0.0025	79
(2) Mg^{++} Zn^{++}	0.0025 0.0025	0
(3) Mg^{++} Zn^{++}	0.0025 0.0005	72
(4) Mg^{++} Ca^{++}	0.0025 0.0025	62
(5) Mg^{++} Ca^{++}	0.0025 0.0005	85

*The control is the standard exonuclease assay.

statistically considered inhibitory.* Calcium, on the other hand, produces 38% and 15% reductions under the same conditions so that it obviously fails to activate the reaction but apparently does not inhibit it.

The effect on activity of varying the Mg^{++} concentration is seen in Fig. 30. Optimal concentration is from 0.001 to 0.003 M. At 0.005 M activity is approximately 95% of the optimum.

4. Temperature optimum

The optimal temperature, shown in Fig. 31, of those examined is 37°. A Q_{10} of 2.2 exists between 27° and 37°. The fall-off of activity at 45° is at least partly due to inactivation. If the examined enzyme preparation is heated to 45° for 12 minutes in the presence of 30% glycerol and 0.01 M mercaptoethanol, there is a loss of about 40% of the activity present.

B. Enzyme inhibition

The inhibition of the exonuclease by several means was attempted and four of these were effective.

(i) Zinc has already been referred to in the section (3) on cation requirements.

(ii) EDTA is an effective inhibitor as shown in Table XXIII-A which demonstrates that EDTA produces from 90 to 100% inhibition at equimolar concentrations of Mg^{++}. It is probable that the EDTA in the assay functions as an inhibitor by chelating

---

*If zinc failed to activate the mixture, a decline of activity in (3) in Table XXII of 5/30 or about 17% would be expected if the enzyme is assumed to have no activity in the absence of Mg^{++} (or Mn^{++}).

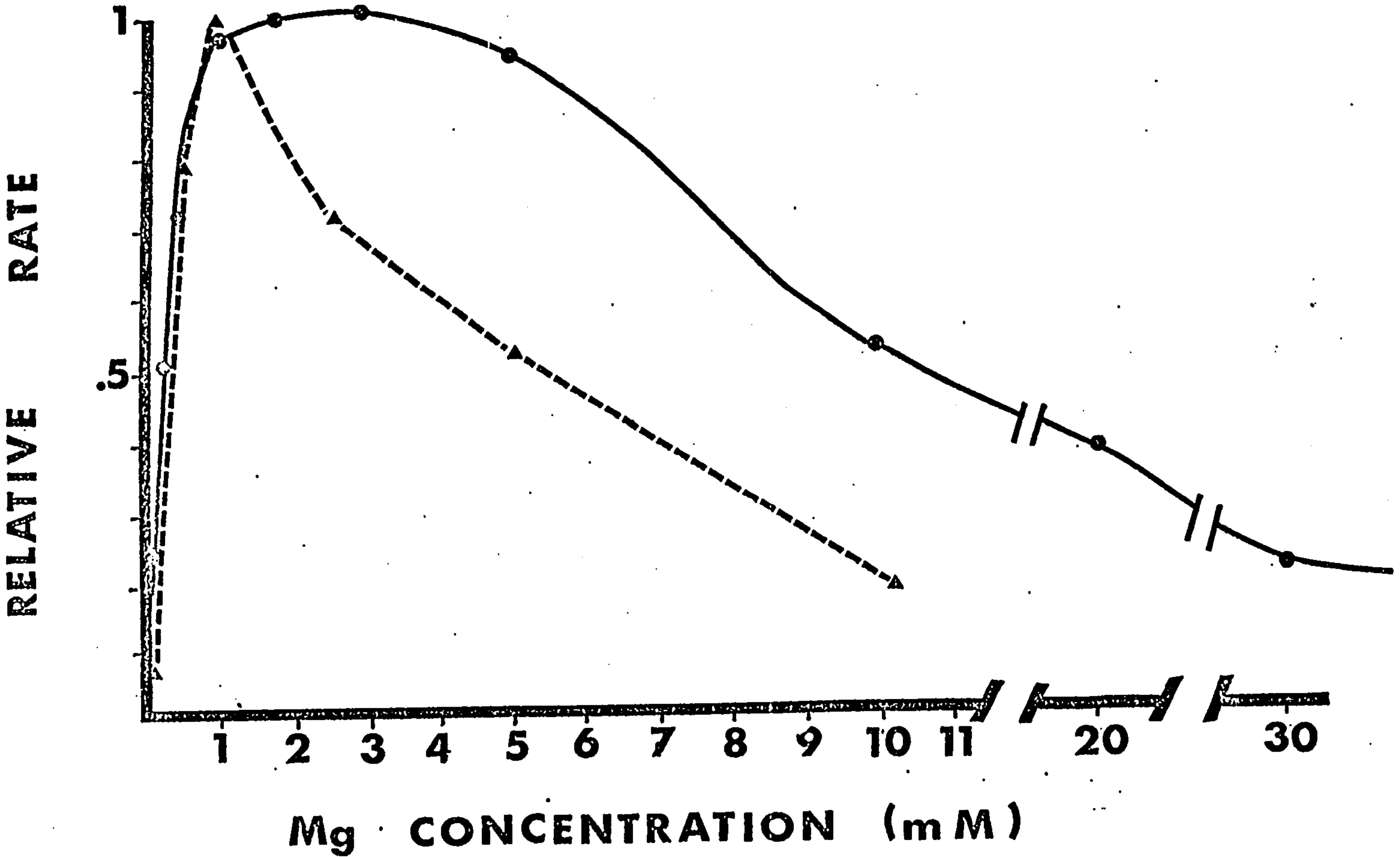

1
.5
RELATIVE RATE
1
2
3
4
5
6
7
8
9
10
11
20
30
Mg · CONCENTRATION (mM)

FIGURE 30

THE EFFECT OF VARYING Mg^{++} CONCENTRATION ON *PSEUDOMONAS AERUGINOSA* EXONUCLEASE AND PHOSPHATASE ACTIVITY

Mg^{++} concentrations are final concentrations in the assay.

▲----------▲ Phosphatase

●————● Exonuclease

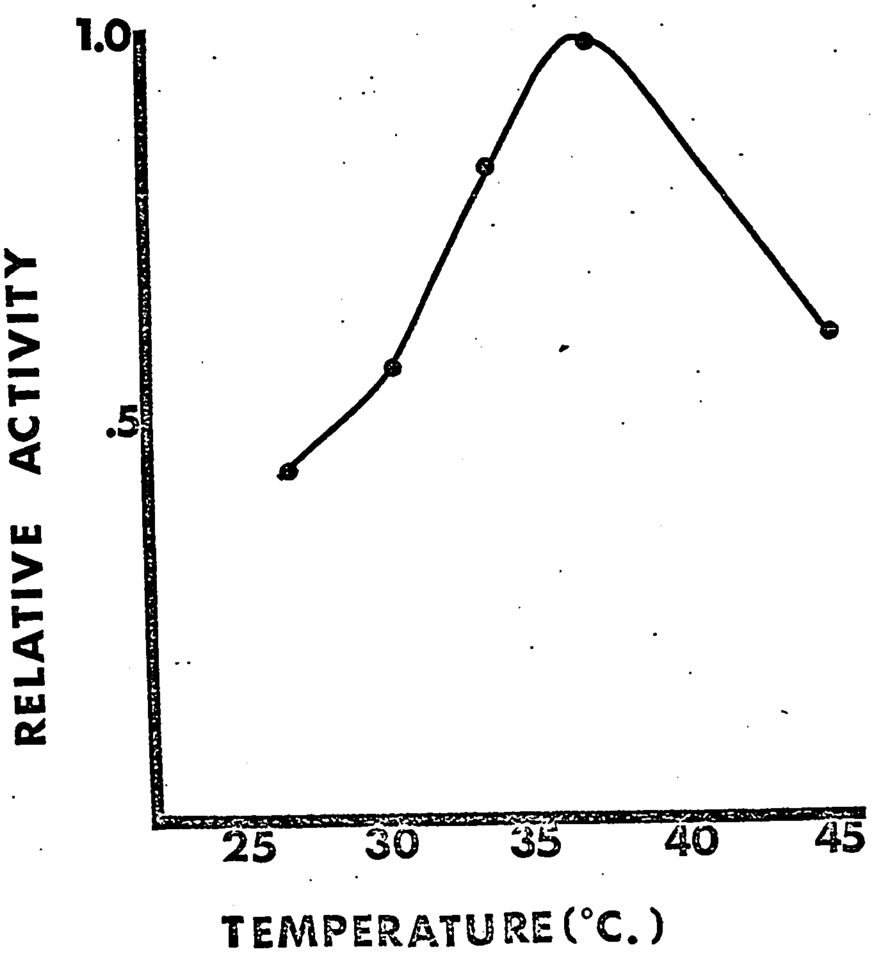

1.0
.5
RELATIVE ACTIVITY
25
30
35
40
45
TEMPERATURE (°C.)

FIGURE 31

THE TEMPERATURE OPTIMUM OF THE PSEUDOMONAS AERUGINOSA EXONUCLEASE

All assays were prewarmed to the indicated temperatures for 5 minutes prior to the addition of the enzyme.

TABLE XXIII

INHIBITION OF PSEUDOMONAS AERUGINOSA EXONUCLEASE AND PHOSPHATASE ACTIVITY

Agent	Final Concentration (M) in assay	% Inhibition
A. EXONUCLEASE		
EDTA*	0.01	100
	0.005	97.7 to 100
	0.0025	93 to 100
PCMB	1×10^{-7}	0
	1×10^{-6}	45
	1×10^{-5}	84
	5×10^{-5}	87
	1×10^{-4}	94
	5×10^{-4}	100
B. PHOSPHATASE		
EDTA**	0.0025	100
	0.001	100
PCMB	1×10^{-7}	7
	1×10^{-6}	49
	1×10^{-5}	78

*Mg^{++} present at 0.0025 M

**Mg^{++} present at 0.001 M

all Mg^{++} available for the enzyme reaction. This further suggests that the enzyme has no activity in the absence of Mg^{++}.

(iii) Parachloromercurobenzoate (PMCB) is an effective inhibitor producing greater than 90% inhibition at 1×10^{-4} M and almost 50% at 1×10^{-6} M. The results are seen in Table XXIII-B.

(iv) RNA also effects an inhibition of activity. The production of this situation was somewhat unique. The addition of RNA at concentrations up to four times that of the DNA substrate to the assay mixture at 37° did not produce inhibition. However, if RNA was pre-incubated with the enzyme at 4° for 15 minutes prior to the addition of the substrate, inhibition could be produced. The results of that are shown in Table XXIV. In the sample of the enzyme utilized, RNase activity at 20 units/mg was present. The presence of that activity does impair the examination of the problem of the inhibitor at 37°. In addition, the question of relative enzyme-nucleic acid binding affinities must also be considered.

All four deoxyribonucleotides and ribonucleotides up to a final concentration in the assay of 0.5 μM/ml did not produce inhibition or for that matter stimulation. Also dATP, dCTP, ATP, CTP, CDP and dCDP up to the same concentrations produced no apparent effect on the enzyme activity.

C. Stability of the purified exonuclease

The exonuclease is a labile protein. Two significant stabilizing reagents must be utilized to maintain activity. One is mercaptoethanol, and has been explored in section I, subsection A - 1. The second is glycerol which has been successfully used to stabilize several enzymes. The effectiveness of glycerol has been alluded to in the section on stabilization of crude preparations. The results presented here pertain to purified enzyme.

Table XXV examines stability at low temperatures in the presence and absence of glycerol. The enzyme is fully stable for at least 4 weeks in 30% glycerol at -20° and loses activity only slowly with multiple thawing or storage at 4°.

In contrast, removal of the glycerol results in a marked loss of activity with a single freezing and thawing or with storage at 4°.

Heat stability was tested at several temperatures, but only at 37° in the absence of glycerol. Fig. 32 is a plot of the logarithm of the percent activity retained vs. time. The first 10 minutes of the plot produces a linear loss of activity with 50% loss occuring in 7.5 minutes. After 10 minutes, the rate of loss declines slowly, however, by 30 minutes 100% of activity was lost. In contrast, in 30% glycerol, less than 5% activity is lost at 7.5 minutes or for that matter in 30 minutes.

Heating to 45° and 50° in 30% glycerol produces a much larger activity loss which is not linear with time, however. The profile of activity loss at 50° is seen in Fig. 33. The 45° profile is similar except, for example, at 5 minutes the loss is 33%. The non-linear

TABLE XXIV

INHIBITION OF PSEUDOMONAS AERUGINOSA EXONUCLEASE BY PRE-INCUBATION AT 4°* WITH YEAST RNA

Final RNA Concentration in assay (μg/ml)	Final DNA Concentration in assay (μg/ml)	% Inhibition
50	50	22
50	37.5	32
50	25	37
50	12.5	48

*For 15 minutes

TABLE XXV

STABILIZATION OF PURIFIED *PSEUDOMONAS AERUGINOSA* EXONUCLEASE BY GLYCEROL

Preparation	Temperature of Storage	Duration	Retention of Activity
1.) 30% glycerol 0.01 M mercaptoethanol 0.025 M potassium phosphate pH 7.5 Protein 0.5 mg/ml [CONTROL]	a) -20°	4 weeks	95-100%
	b) 4°	24 hours	95%
		48 hours	93%
		72 hours	87%
	c) -20° rethawed 7 times	7 days	90%
2.) CONTROL dialyzed 8 hours against 0.01 M mercaptoethanol 0.025 M potassium phosphate [NO GLYCEROL]	a) 4° dialysis		100%
	b) 4°	48 hours	43%
	c) -20°	2 hours	40.5%
	d) -20° 4°	2 hours 48 hours	27%

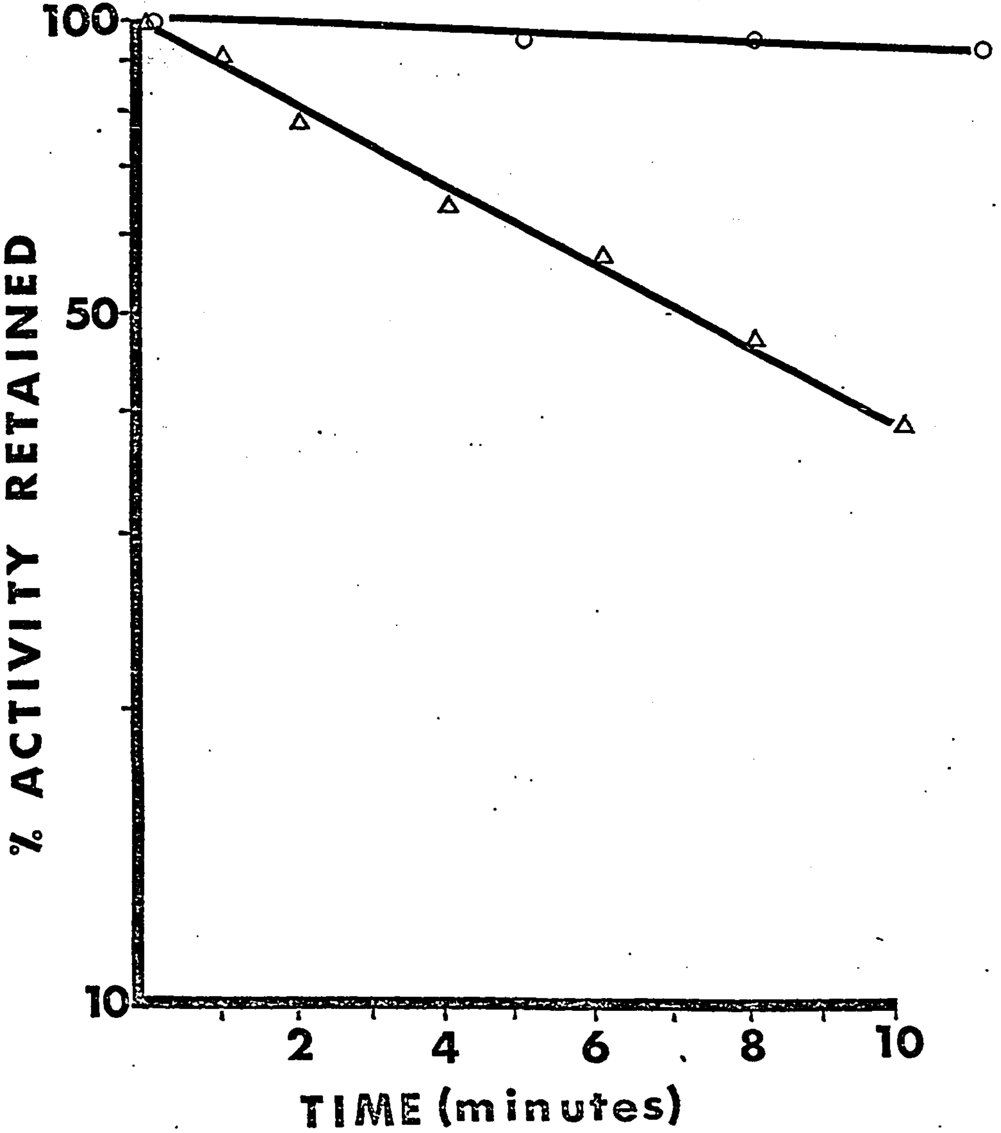

100
50
10
% ACTIVITY RETAINED
2
4
6
8
10
TIME (minutes)

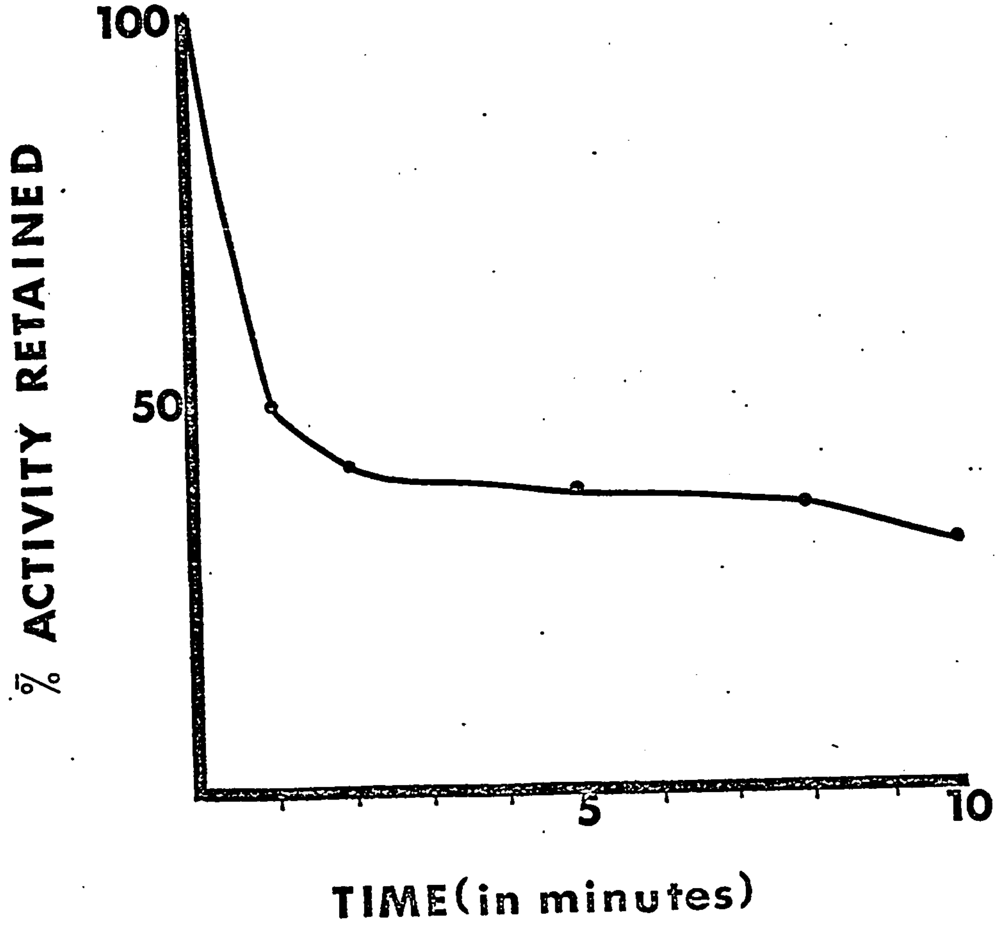
FIGURE 32

LOSS OF PSEUDOMONAS AERUGINOSA EXONUCLEASE ACTIVITY
DUE TO INCUBATION AT 37° IN THE
PRESENCE AND ABSENCE OF GLYCEROL

Enzyme purified in 30% glycerol containing 0.5 mg/ml protein was incubated at 37° for the indicated times and samples removed to an ice bath and assayed. The same preparation dialyzed against 0.025 M potassium phosphate pH 7.5 and 0.01 M mercaptoethanol was incubated at 37° and assayed at the indicated times.

○————○ Preparation containing 30% glycerol

△————△ Preparation containing no glycerol



100
50
% ACTIVITY RETAINED
5
10
TIME(in minutes)

FIGURE 33

THE EFFECT OF HEATING *PSEUDOMONAS AERUGINOSA* EXONUCLEASE AT 50°

A preparation of the exonuclease containing 30% glycerol was incubated at 50° for the indicated times, transferred to an ice bath and assayed.

TABLE XXVI

PROTECTION AGAINST INACTIVATION OF *PSEUDOMONAS AERUGINOSA* EXONUCLEASE BY 50° FOR 5 MINUTES

Preparation	% Activity Retained
Control (30% glycerol, 0.025 M phosphate)	44
Control + 0.01 M mercaptoethanol	70.4
Control + 0.0025 M Mn^{++} or 0.0025 M Mg^{++}	55
Control + 0.005 M Mn^{++} or 0.005 M Mg^{++}	60
Control + 0.0025 M Mn^{++} and 0.0025 M Mg^{++}	59
Control + 0.0025 M Ca^{++}	53
Control with phosphate reduced to 0.015 M	42
Control with phosphate increased to 0.2 M	55

effect prevents a quantitative comparison of the loss of activity in the presence of a variety of agents but some qualitative estimate can be made and these are seen in Table XXVI.

The reason these heat inactivation studies were not expanded was that they require the preparation and destruction of an inordinate amount of high purity enzyme and the information thus obtained would not warrant this expense (i.e., the loss of that much material which could be put to more productive use).

From Table XXVI, the effect of mercaptoethanol is obvious. Also all the cations tested have a slight protective effect as does 0.2 M potassium phosphate buffer.

D. Evidence for exonucleolytic hydrolysis of DNA by the P. aeruginosa exonuclease

If but one nuclease is present in a system, the comparison of the relative effectiveness of precipitation by 10% TCA or 10% TCA containing 0.25% uranyl acetate of the enzymatic hydrolysis products is a simple and reasonably reliable method to determine whether an enzyme is an exo- or endonuclease. This method described by Helleiner (Helleiner, 1955) works on the principle that some oligonucleotide products of an endonuclease are soluble in 10% TCA but not in UTCA. However, the products of an exonuclease, being only mononucleotides, are equally soluble or nearly so in both. The behavior of the Pseudomonas enzyme (Fig. 35) is compared to that of a well known endonuclease (bovine pancreatic DNase I) (Laskowski, 1961) and a well known exonuclease (smake venom phosphodiesterase) (Singer et al, 1958; Razzell and Khorana, 1958) in Fig. 34. The Pseudomonas enzyme is clearly exonucleolytic by these criteria.

E. Substrate specificity and the limit of substrate hydrolysis

The exonuclease has exhibited at all stages of purification

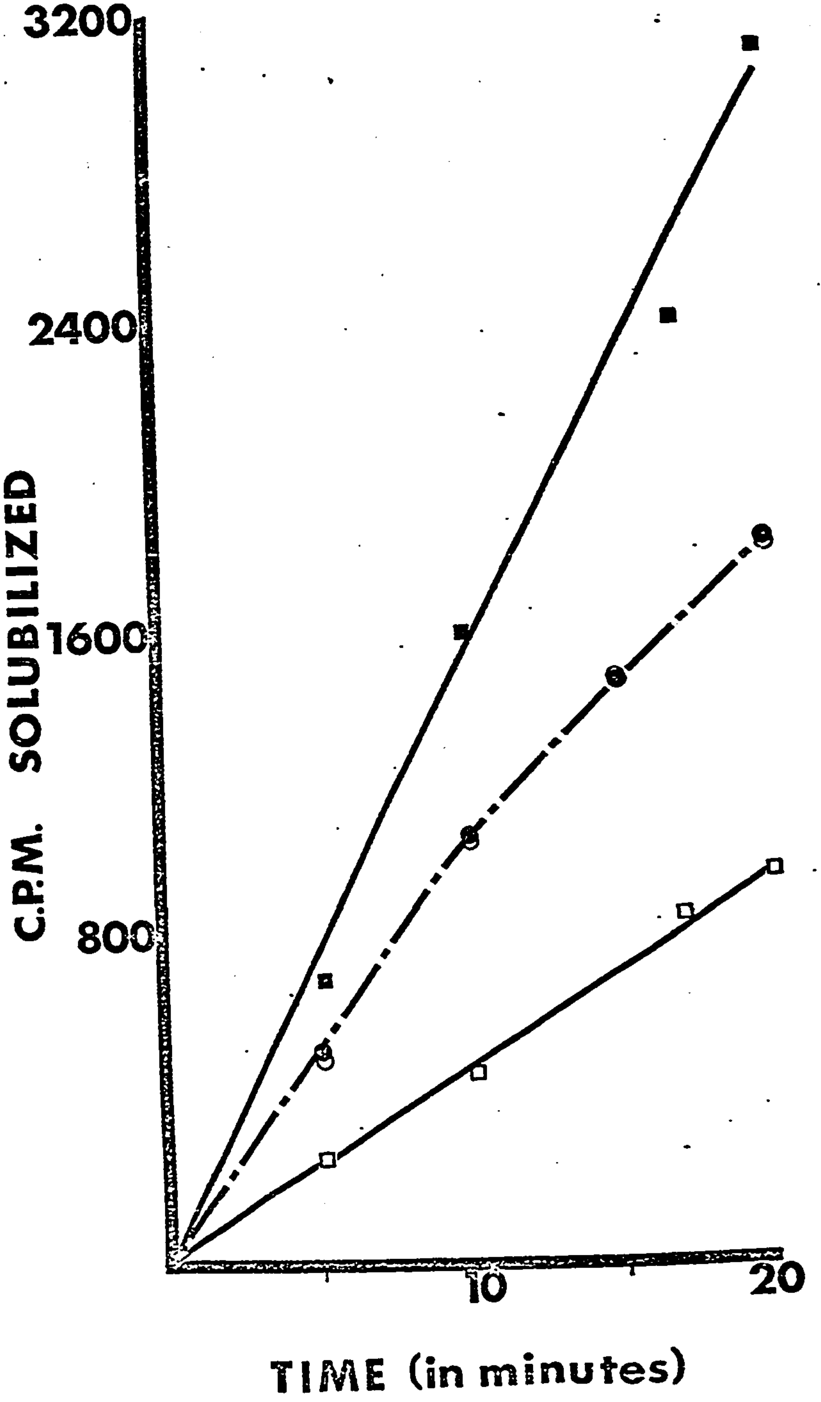

3200
2400
1600
800
C.P.M. SOLUBILIZED
10
20
TIME (in minutes)

FIGURE 34

COMPARISON OF THE MODE OF ACTION OF TWO DNASES ON DENATURED DNA

Pancreatic DNase was assayed using the standard exonuclease assay with heat denatured DNA, as was snake venom phosphodiesterase except in that case, 0.05 M Tris HCl pH 8.9 and Mg^{++} 0.001 M were used. Identical assays were stopped with 10% TCA or 0.25% uranyl acetate in 10% TCA (UTCA).

■———■ Pancreatic DNase 10% TCA

□———□ Pancreatic DNase UTCA

●— – —● Snake venom phosphodiesterase 10% TCA

○— – —○ Snake venom phosphodiesterase UTCA

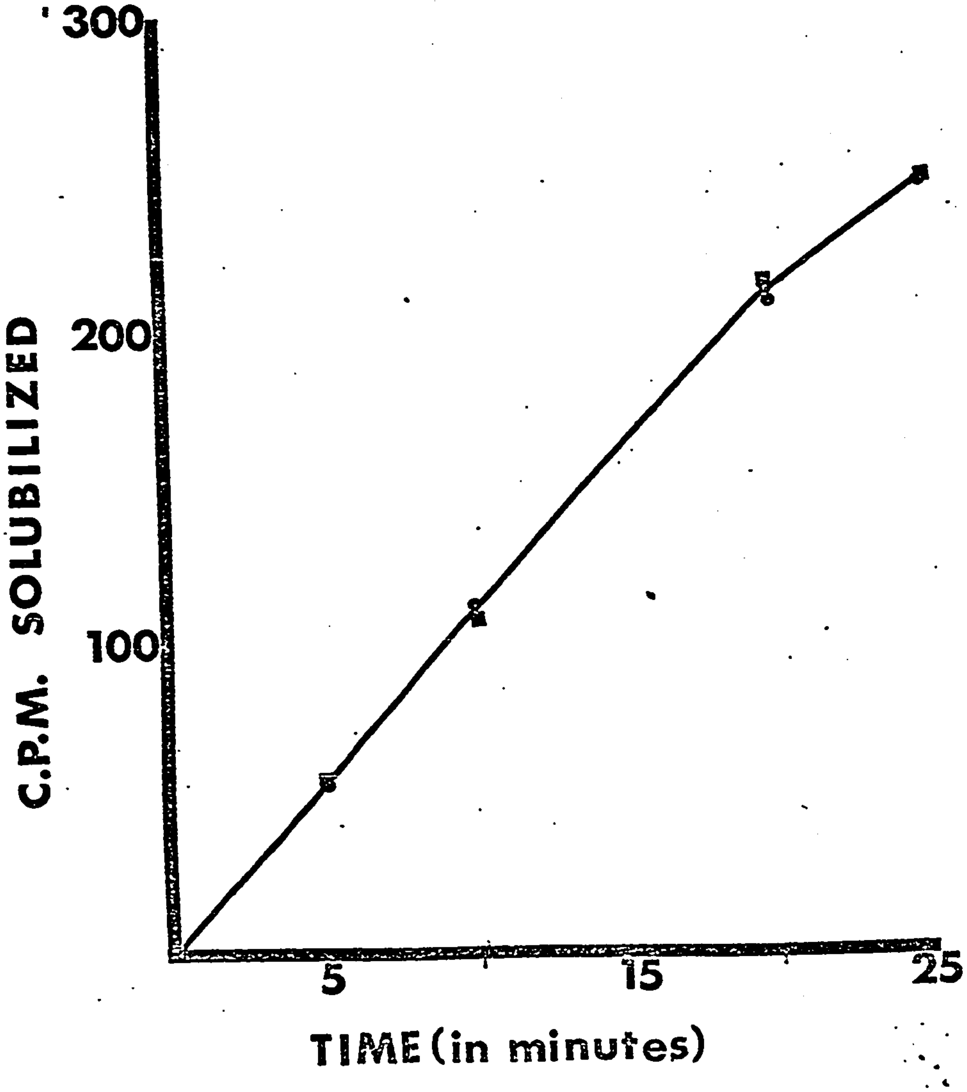

300
200
100
C.P.M. SOLUBILIZED
5
15
25
TIME (in minutes)

FIGURE 35

THE MODE OF ACTION OF

PSEUDOMONAS AERUGINOSA EXONUCLEASE

ON DENATURED DNA

The standard exonuclease assay was used and was stopped with 10% TCA or 0.25% uranyl acetate in 10% TCA (UTCA).

■——■ 10% TCA

●——● UTCA

a well defined preference for native DNA, in that the initial rate is 3 to 4 times faster on native than denatured DNA. This can be seen by referring to Table XXVII and comparing the values at 12 minutes. The remainder of Table XXVII demonstrates that 100% of the native DNA can be eventually solubilized. However, several additions of fresh enzyme and mercaptoethanol are required to achieve complete hydrolysis. The effect of such additions is seen in Fig. 36. The rate of solubilization in the absence of additional enzyme is seen to fall precipitously after 2 hours. Upon additions of new enzyme, the rate increases but remains significantly lower than the initial rate.

The effect of the addition of new substrate to an assay after 2 hours incubation is also seen in Fig. 36. The rate increment in 12 minutes approaches the initial rate but declines more rapidly.

These findings permit at least three conclusions.

(i) The exonuclease appears to undergo progressive inactivation during prolonged incubation. This is supported by the failure of a single enzyme addition to cause total substrate hydrolysis and by the rate increment produced by fresh enzyme (Fig. 36).

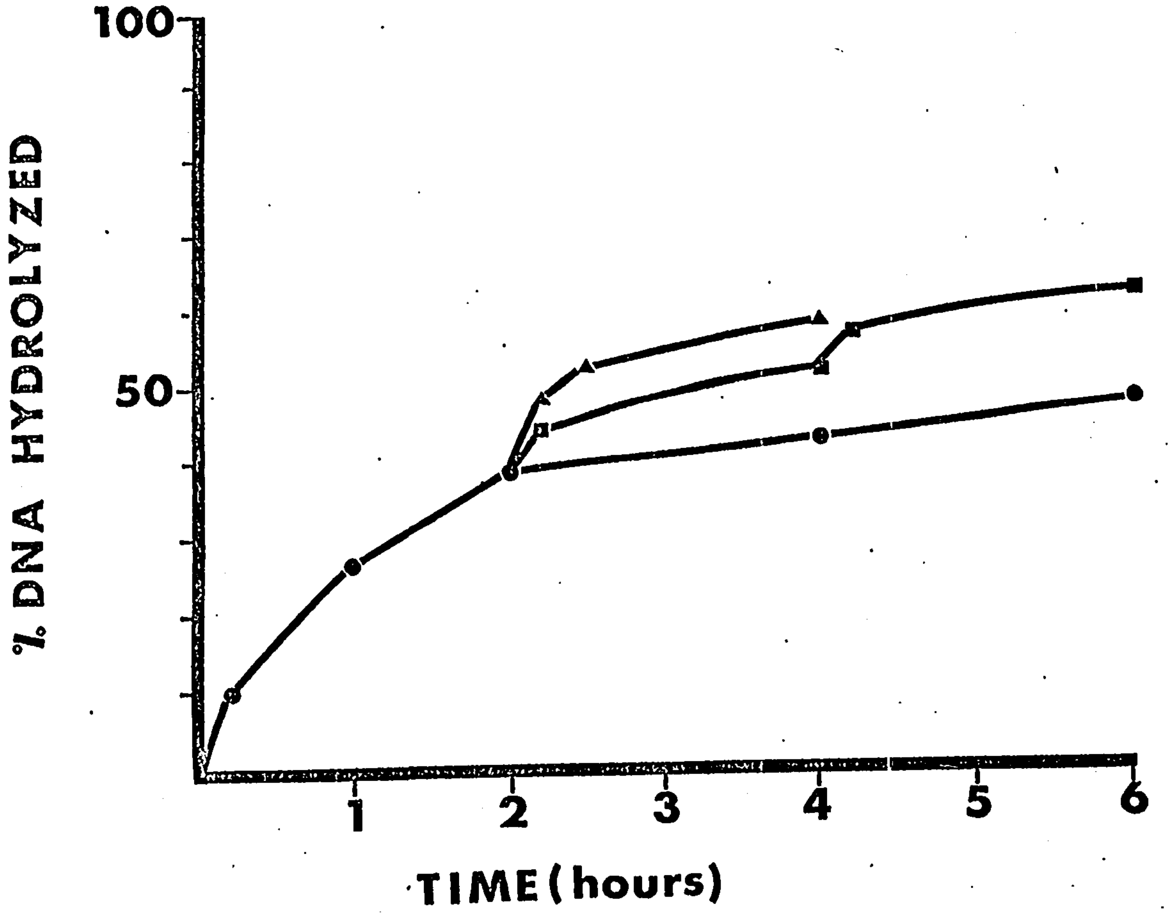
(ii) The substrate is modified to or initially contains portions which serve as less effective substrate. This is supported by the lowering of rate with time and the rate increment resulting from the addition of new substrate. The fact that the rate on denatured DNA is about 1/4 to 1/3 that of native DNA suggests that the less effective

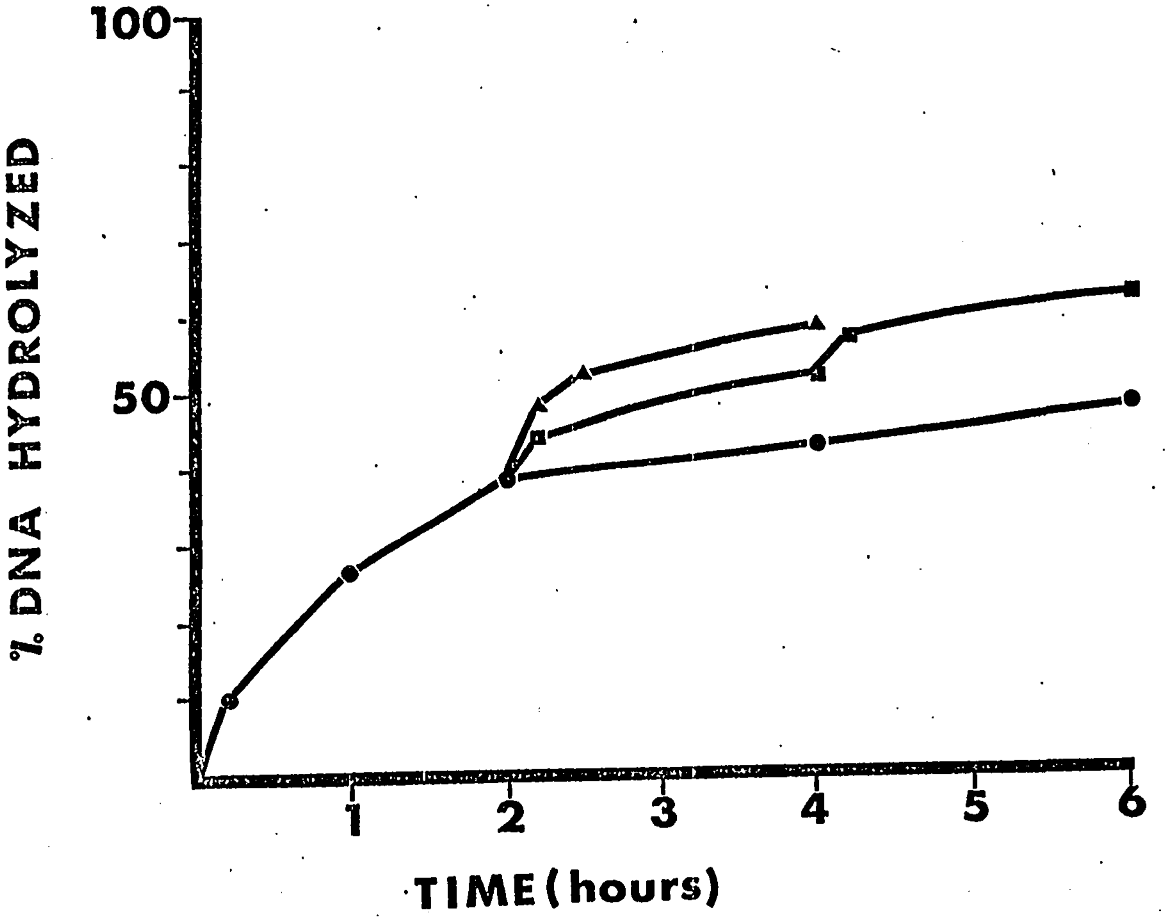
TABLE XXVII

THE EXTENT OF DNA SOLUBILIZATION POSSIBLE WITH THE *PSEUDOMONAS AERUGINOSA* EXONUCLEASE

Type of DNA	Time	% Solubilization
1. Native	12 minutes	8.8
	12 hours	33.5
	16 hours	45.2
	20 hours	52
	45 hours	100
2. Denatured	12 minutes	2.9
	12 hours	22
	20 hours	31
	45 hours	65
3. Native	45 hours (enzyme addition only at 0)	40 to 48

Fresh enzyme and mercaptoethanol added at 0, 12, 16 and 20 hours in 1 and 2, but only at zero time in 3.


100
50
% DNA HYDROLYZED
1
2
3
4
5
6
TIME (hours)



100
50
% DNA HYDROLYZED
1
2
3
4
5
6
TIME (hours)

FIGURE 36

THE EFFECT OF ADDITIONAL ENZYME AND SUBSTRATE ON THE PSEUDOMONAS AERUGINOSA EXONUCLEASE ACTIVITY RATE

Standard assays containing 1.8 units of exonuclease were used to follow the rate of exonuclease activity at the indicated times for 6 hours. At 2 hours and at 2 and 4 hours incubation, an additional 1.8 units of exonuclease was added to some assays which were allowed to proceed for the specified times. At 2 hours, an additional 5 μg of ^{3}H-DNA was added to other assays and they were stopped at the times noted.

●———●	Standard assay - no additions
■———■	Standard assay - addition of additional enzyme at 2 and at 2 and 4 hours.
▲———▲	Standard assay - addition of 5 μg of ^{3}H-DNA at 2 hours.

substrate may be single stranded.

(iii) Products of the hydrolysis are not inhibitory. This is supported by the solubilization of about 10% of substrate in the initial 12 minutes and again after the addition of new substrate DNA. In addition, mononucleotides are not inhibitory (see section II, subsection B).

Variations in individual batches of DNA have been found responsible for small changes in activity of identical enzyme samples. Differences of ±20% have been detected. These can be minimized by dividing each DNA batch into 2.0 ml fractions and freezing at -20°. Each tube is thawed, kept at 4° and used within 7 days.

Modification of DNA can significantly affect activity. In view of evidence presented in section II, subsection I that the exonuclease attacks 3' to 5' and first releases a phosphate if present, the role of modification of the 3' terminus was examined.

The standard DNA used in all assays unless otherwise indicated, can be modified by micrococcal nuclease which produces endonucleolytic scissions leaving 3' phosphoryl termini. The micrococcal nuclease does not have to be removed from DNA preparations as it is totally inactive in the exonuclease assay.

The effect of the introduction of new 3' phosphoryl termini is seen in the Lineweaver-Burke double reciprocal plots in Fig. 37 and the K_m and V_{max} values listed in Table XXVIII. There is no effect on K_m so that binding affinity is likely unchanged. On the other hand, the V_{max} is more than doubled (Table XXVIII). The question of whether the increase in V_{max} was due to many new termini or specifically to new

TABLE XXVIII

K_m AND V_{max} VALUES OBTAINED WITH STANDARD AND MODIFIED DNA

Type of DNA	K_m (μg/ml)	V_{max} (units/ml)
Standard native	38.8	400
3' phosphoryl	38.8	955
3' hydroxyl	38.8	955
Standard denatured	35.0	65.0
Standard native with 0.07 M NaCl in assay	140	400

TABLE XXIX

REMOVAL OF PHOSPHATE GROUPS FROM DNA MODIFIED BY MICROCOCCAL NUCLEASE

Enzyme Preparation	Temperature of Incubation	Time of Incubation (minutes)	Total Δcpm
Alkaline phosphatase	37°	40	(i) 1053
			(ii) 1075
Alkaline phosphatase	65°*	40	(i) 874
			(ii) 796
P. aeruginosa exonuclease	37°	40	820
		60	1030
		240	1038

*Fresh enzyme every 10 minutes

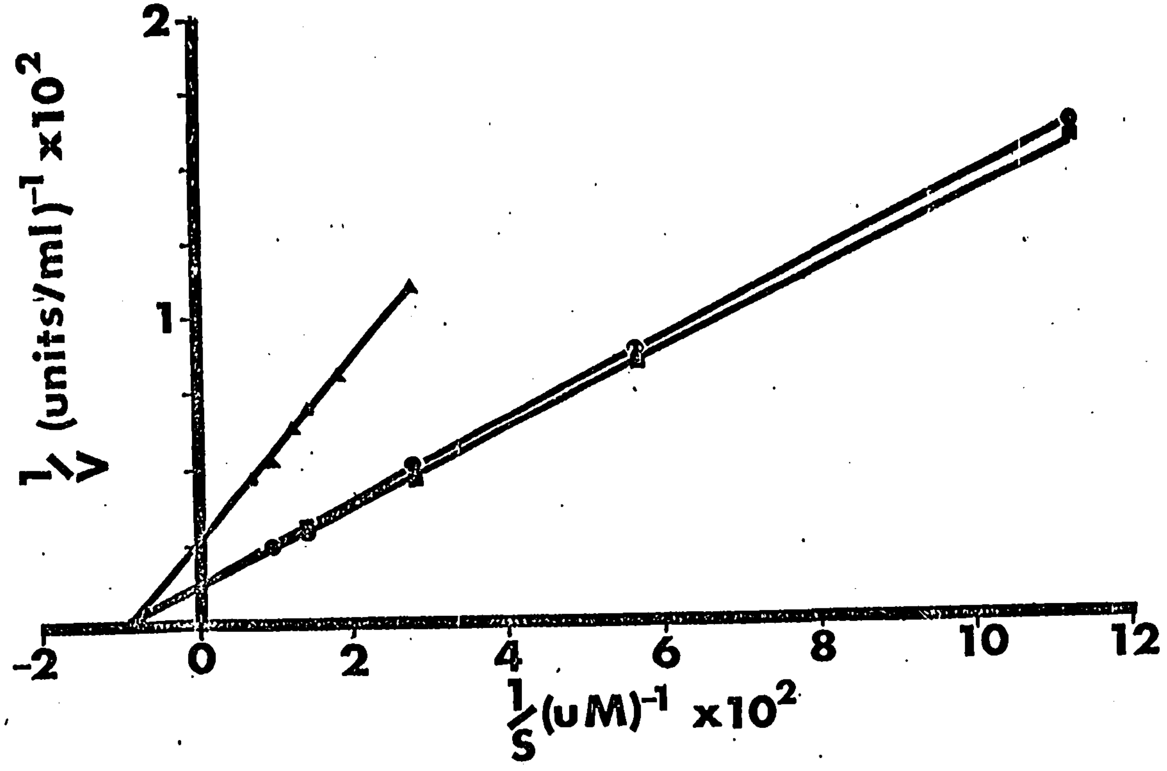

2
1
1/V (units/ml)^-1 x10^2
-2
0
2
4
6
8
10
12
1/S (uM)^-1 x10^2

FIGURE 37

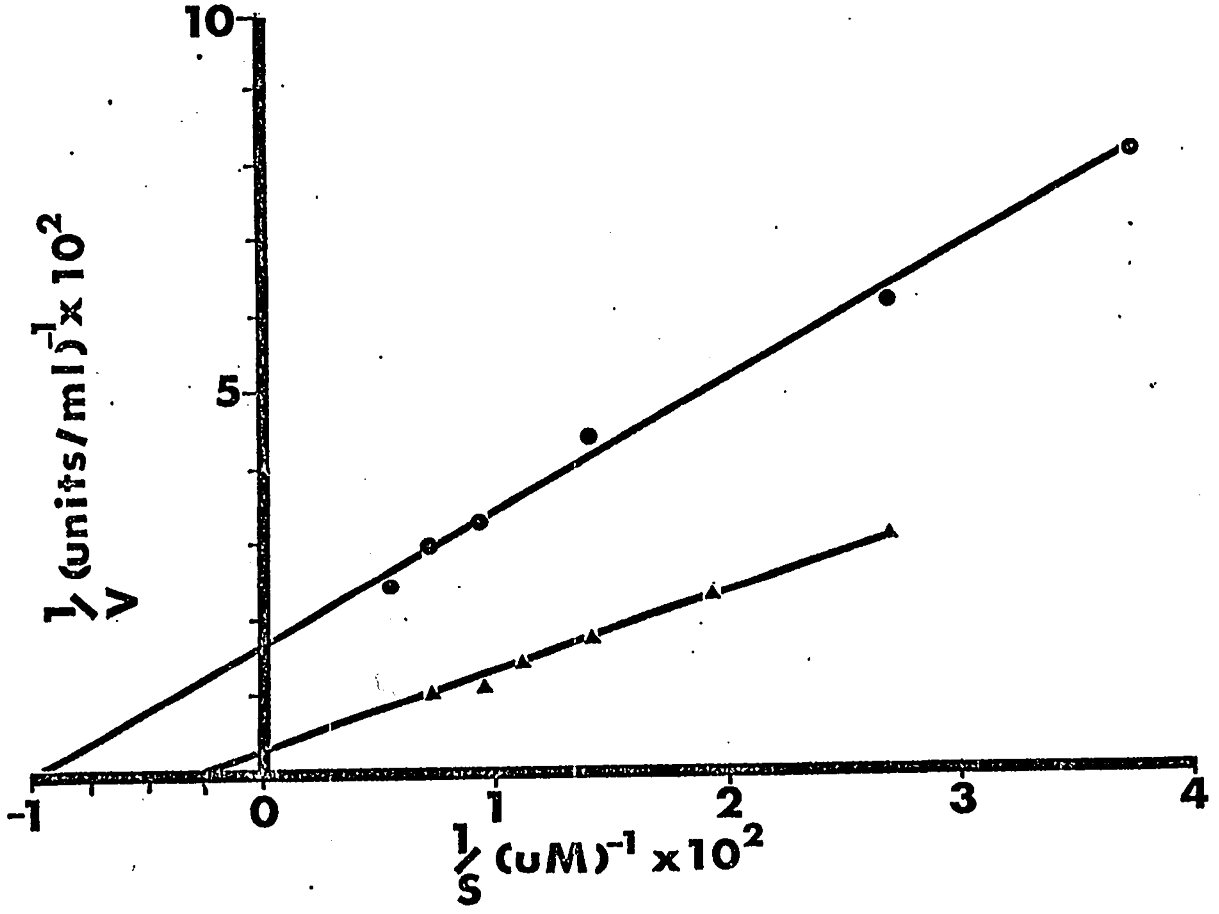
LINEWEAVER-BURK PLOTS OF VELOCITY VS. CONCENTRATION OF NATIVE AND MODIFIED DNA

Assays were carried out in the standard manner. DNA concentration is expressed in nucleotide equivalents. DNA was modified as given in the Materials and Methods.

▲———▲ Standard native DNA

●———● 3' phosphoryl DNA (native)

■———■ 3' hydroxyl DNA (native)



10
5
$\frac{1}{V}$ (units/ml)$^{-1}$ x 10^2
-1
0
1
2
3
4
$\frac{1}{S}$ (uM)$^{-1}$ x 10^2

FIGURE 38

LINEWEAVER-BURK PLOTS OF VELOCITY VS. CONCENTRATION OF DENATURED DNA IN STANDARD ASSAYS AND NATIVE DNA IN ASSAYS WITH INCREASED IONIC STRENGTH

Assays were carried out in the standard manner. DNA concentration is expressed in nucleotide equivalents. Denatured DNA was prepared as given in Materials and Methods.

●———●	Standard heat denatured DNA
▲———▲	Standard native DNA in assays containing a final concentration of 0.07 M NaCl

3' phosphoryl groups was asked. The approach to that problem was to use alkaline phosphatase to remove the phosphoryl groups. The removal of phosphate groups was monitored on ^{32}P-labelled identically prepared DNA specimens. However, contrary to the findings of Weiss et al (1968) the removal of phosphate groups was more effective at 37° than 65°. That group postulated that alkaline phosphatase removed external phosphomonoesters only at 37° and 65° was required to attack internal phosphomonoesters. Attempts to confirm these findings under identical conditions and with higher concentrations of alkaline phosphatase were unsuccessful (Table XXIX).

The effect of removal of phosphoryl groups at 37° to produce 3' OH groups is seen in Fig. 37 and Table XXVIII. The K_m and V_{max} do not change to any significant degree. Thus, the effect of micrococcal nuclease to increase the V_{max} appears to be strictly due to new ends being available.

Finally, Table XXVIII and Fig. 38 demonstrate the K_m for denatured standard DNA is very similar but that the V_{max} is very much lower. Thus, the Pseudomonas enzyme possesses similar affinity for denatured standard DNA but the enzymatic mechanism of hydrolysis is slower than with native DNA.

F. The products of the exonuclease activity

Examination of the products was carried out employing several procedures. The method used to produce the results shown in Figures 39, 40 and 41 was fractionation of reaction products on DEAE cellulose columns in 7 M urea and 0.02 M Tris HCl pH 7.5. The reason for choosing that particular system was the fact that oligonucleotides separate according to size (Tomlinson and Tener, 1962; 1963). Thus, one could

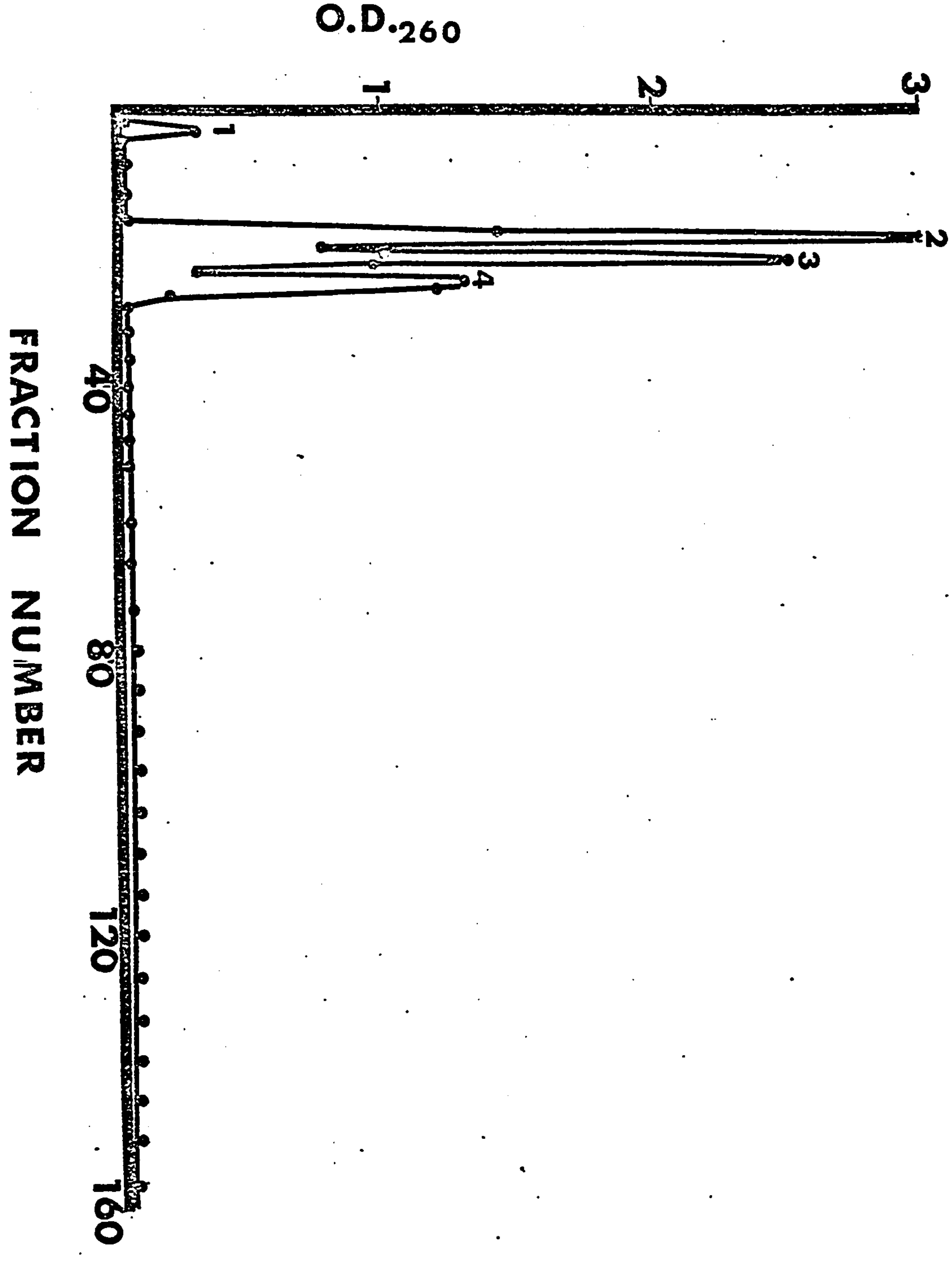

O.D.260
1
2
3
1
2
3
4
40
80
120
160
FRACTION NUMBER

FIGURE 39

DEAE CELLULOSE - 7 M UREA CHROMATOGRAPHY
OF DEOXYRIBOMONONUCLEOTIDE STANDARDS

A 1.2 ml sample containing 1.25 μmoles of each of the four deoxyribomononucleotides was mixed with 1.2 ml of 7 M urea in 0.02 M Tris HCl pH 7.5 and added to a DEAE cellulose column. The preparation, size and operation of the column is given in Materials and Methods. Samples were examined for absorbancy at 260 nm.

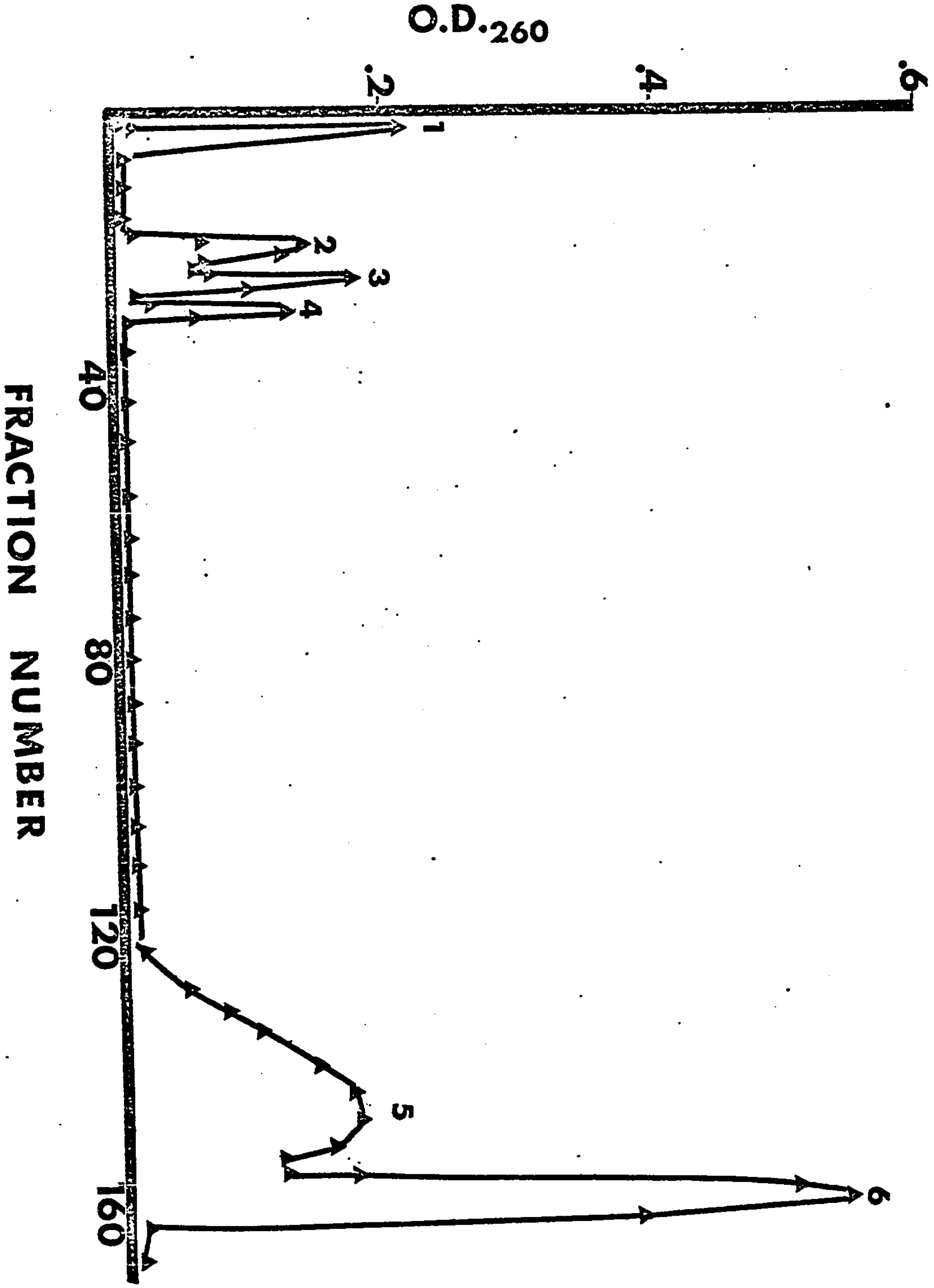
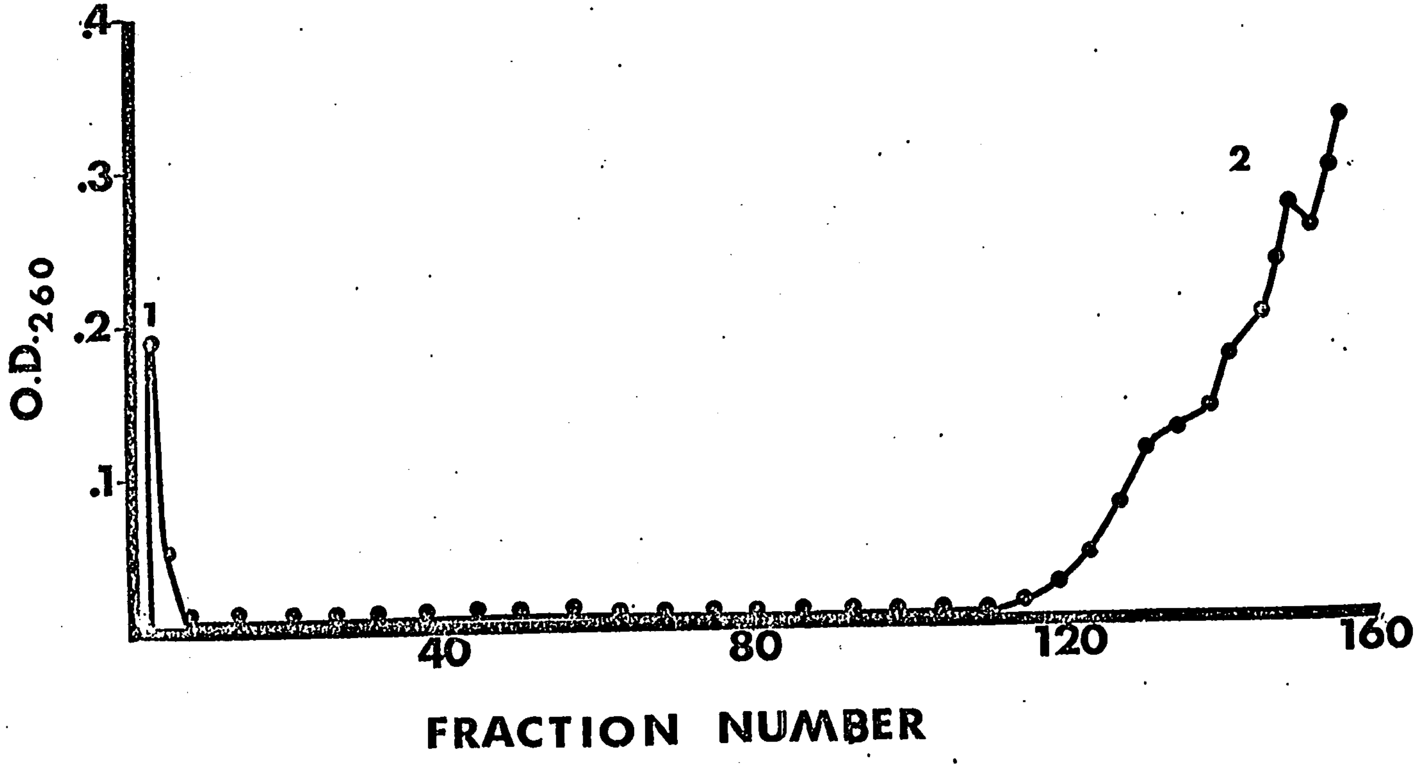

O.D.260
.2
.4
.6
1
2
3
4
5
6
40
80
120
160
FRACTION NUMBER

FIGURE 40

DEAE CELLULOSE - 7 M UREA CHROMATOGRAPHY OF A LARGE SCALE PSEUDOMONAS AERUGINOSA EXONUCLEASE ASSAY MIX

The mixture and incubation was identical to that specified in section H - 3 of Materials and Methods. At the termination of incubation, 1.8 ml of that mixture diluted with 1.8 ml of 7 M urea in 0.02 M Tris HCl pH 7.8 was added to the column and washed in with 10 ml of the same buffer. Column operation is specified in section H - 2 of Materials and Methods. The indicated tubes were examined for absorbancy at 260 nm, and 200 µl of each were counted in 5 ml of Bray's solution in the liquid scintillation counter. Acid insolubility was performed on every fourth tube after tube 110 and selected tubes by concentrating by pervaporation the volume to 0.5 ml, removing 0.2 ml and adding an equal volume of cold 6% PCA. The mixture was placed in ice and 0.1 ml of bovine serum albumin 10 mg/ml was added. After 5 minutes, it was centrifuged for 1 minute in a Beckman Microfuge and the absorbancy at 260 nm was determined on 250 µl of the supernatant.



O.D.260
.4
.3
.2
.1
1
2
40
80
120
160
FRACTION NUMBER

FIGURE 41

DEAE CELLULOSE - 7 M UREA CHROMATOGRAPHY OF A LARGE SCALE ASSAY MIX CONTAINING NO *PSEUDOMONAS AERUGINOSA* EXONUCLEASE

The procedure was identical to that of Fig. 40 except that the 37° incubation (see section H - 3 Materials and Methods) contained no exonuclease.

TABLE XXX

RECOVERY OF ^{3}H LABEL FROM DEAE CELLULOSE - 7 M UREA COLUMNS

Preparation	Total cpm	% of total cpm	% of acid soluble cpm
A. DIGEST			
(i) Total acid soluble cpm added	1.26×10^4	41	100
(ii) Peak I	60	--	0.47
(iii) Peak II	1.22×10^4	40	96.5
B. CONTROL			
(i) Total acid soluble cpm added	80	--	100
(ii) Peak I	56	--	70
C. DIGEST CORRECTED FOR CONTROL			
(i) Peak I	4	--	0.031
(ii) Peak II	1.22×10^4	40	96.5

detect the presence of products of varying size. In Fig. 40, the result of a fractionation of a DNA digestion by the exonuclease, which produced 40% solubilization (see Table XXX), added to a DEAE cellulose - urea column is seen. As stated in Materials and Methods, some of the DNA contained ^{3}H-labelled thymine. Fig. 40 shows an initial peak not held up on the cellulose. A second set of 3 peaks and a final large double peak absorbing at 260 nm is also seen. ^{3}H appeared in peak 1, and peaks 5 and 6, and in peak 2.

A set of mononucleotide standards was fractionated under identical conditions and the results shown in Fig. 39. The 4 peaks represent:

(i) a peak with contaminating nucleosides
(ii) dCMP and TMP
(iii) dAMP
(iv) dGMP

Fig. 41 illustrates the fractionation of 7 M urea DEAE cellulose of an incubation identical to that shown in Fig. 40, except that no exonuclease was present. In contrast, however, in this case only two peaks are observed. Peak one, which does not hold up and peak two, which is DNA. Peak 1 represents acid soluble material which was shown to be mainly nucleosides. (Tube 3 was concentrated by pervaporation to 1.0 ml, adjusted to pH 2.0 and added to Norit. The Norit was washed with water twice and eluted three times with 50% ethanol containing 1% concentrated NH_4OH. The eluant was concentrated to 100 μl and chromatographed using PEI cellulose. The UV absorbing spots ran with nucleoside standards near the solvent front.)

A comparison of the sample digest (Fig. 40) to the control (Fig. 41) and the mononucleotide standards clearly demonstrates the only products of a digest containing the Pseudomonas enzyme are mononucleotides. Tubes of each of the peaks of the mononucleotides from the digest column (Fig. 40) were pooled separately, diluted 1:10 with water and added to separate 1.4 x 10 cm DEAE cellulose columns equilibrated with 0.001 M ammonium carbonate pH 8.0. These were washed with 50 ml of 0.001 M ammonium carbonate and eluted with 0.1 M ammonium carbonate. These were repeatedly evaporated to dryness and redissolved with water until no further decline in salt occurred. They were analyzed by paper chromatography as indicated in the Materials and Methods. Peak 2 contained dCMP and TMP, peak 3 dAMP and peak 4 dGMP. The presence of TMP in peak 2 was further confirmed by the presence of ^3H label. The acid absorption spectrum from 220 to 300 nm of each of the appropriate tubes was compatible with that of dAMP and dGMP in peaks 3 and 4 respectively.

The first identical peaks containing nucleosides seen in the test digest (Fig. 40) and the control incubation (Fig. 41) indicate that nucleosides are not a product of the exonuclease digest. This was further confirmed on a quantitative basis. Table XXX demonstrates that essentially all of the material in the peak 1 of the digest column can be accounted for in the control. Nucleosides thus are not a product of the exonuclease.

In addition, acid soluble material of normal assays which produced from 10 to 40% solubilization and which had the perchlorates removed by neutralizing with 5 N KOH and subsequent centrifugation was examined on PEI cellulose thin layer chromatography. Controls containing no enzyme were also run. The nucleoside fraction with an Rf of 0.95 under the conditions used never contained counts in excess of the controls.

The final peaks of the control (Fig. 41) and digest columns (Fig. 40) contain acid-insoluble material containing label and presumably is the DNA beginning to elute. The division of that peak into two sections in Fig. 40 may indicate the presence of two size ranges -- one which is partly digested and other molecules perhaps not attacked. However, this remains as speculation.

A further confirmation of the products using ^{32}P-DNA is seen in Fig. 42. This is a photograph of Actigraph tracings of ^{32}P-labelled products chromatographed by paper. In Fig. 42, the assays were stopped with the addition of EDTA and the whole assay mixture chromatographed. A control containing no enzyme was provided.

In Fig. 42, there are 4 peaks present in the digest and not present in the control and a peak which is significantly increased over that in the control. The latter material is ^{32}P-labelled phosphate. The release of this material is discussed in more detail in the sections on the phosphatase action of the exonuclease. The other 4 new peaks are mononucleotides which were identified by comparison to standards run under identical conditions but at higher concentration so that they were detectable by UV light.

The next problem in the product identification was to determine whether the phosphate group was present on the 3' or 5' carbon of the deoxyribose of the mononucleotide. That question was pursued by treating perchloric acid soluble products after neutralization and removal of perchlorates with the nuclease of Micrococcus sodonensis (Berry and Campbell, 1967a, b) which has a specific 5' nucleotidase activity.

To show the products were 5' mononucleotides, the M. sodonensis nuclease was used under the conditions given in Materials and Methods.

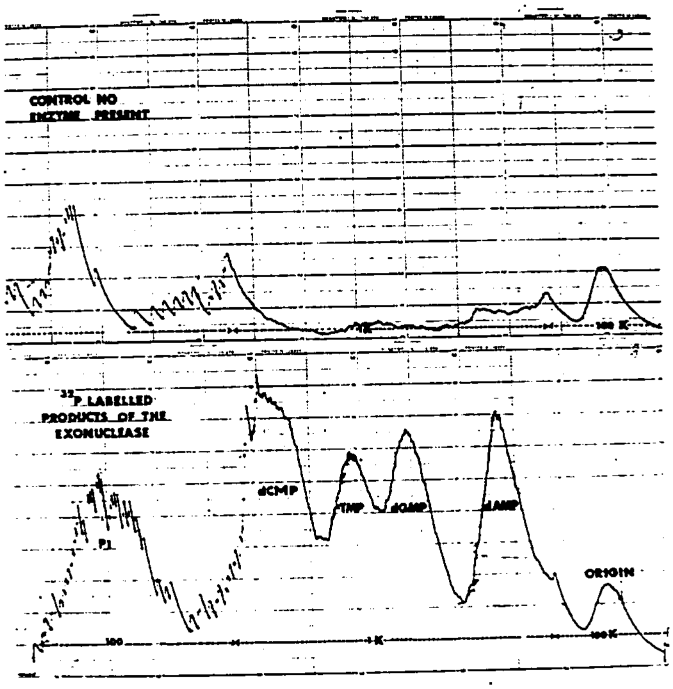

CONTROL NO
ENZYME PRESENT
100 K
32P LABELLED
PRODUCTS OF THE
EXONUCLEASE
Pi
dCMP
TMP
dGMP
dAMP
ORIGIN
100
K
100 K

FIGURE 42

RESULTS OF PAPER CHROMATOGRAPHY OF COMPLETE ASSAY MIXES OF *PSEUDOMONAS AERUGINOSA* EXONUCLEASE

100 µl assays were prepared in a manner identical to that of the standard exonuclease assay except that ^{32}P-DNA was used as substrate. After 12 minutes incubation to produce 10% solubilization (in identical controls) EDTA was added to a final concentration of 0.01 M. The entire sample was chromatographed by paper as indicated in Materials and Methods. The 1.5 inch wide paper strips were scanned on a Nuclear Chicago Actigraph III. Scale settings are indicated on Fig. 42, and are the same for each of the three corresponding sections of the two tracings. The control containing no exonuclease was treated in an identical manner.

The estimation of the 4 mononucleotides present was as noted there based on an average extinction coefficient of 11. Obviously, this estimation is open to considerable error. However, using that information, the release of phosphate as detected by the Ames-Dubin method was essentially mole for mole of nucleotide as indicated in Table XXXI, thus strongly suggesting the products are 5' mononucleotides.

In view of the criticism which might be levelled at the estimation of total nucleotide present another method of demonstrating the 5' linkage of the mononucleotides was carried out. Fig. 43 is a demonstration of Actigraph tracings of the acid soluble ^{32}P products treated as noted in the figure legend. The control preparation demonstrates 4 nucleotides and a very low phosphate level. It should be noted the sensitivity used for the phosphate here is 1/3 that of the assay employed in the experiments of Fig. 42. After digestion with the 5' nucleotidase activity , the ^{32}P chromatographs completely as inorganic phosphate at an Rf of 0.9. The ^{32}P previously seen in the nucleotides has fully disappeared.

G. The presence of other enzymatic activities

1. RNase

Most G-75 purification preparations contain RNase activity as detected by the solubilization of yeast RNA. The RNase is, however, a distinct enzyme from the exonuclease. That conclusion is warranted because of several findings.

(a) Fig. 44 shows a comparison of activity using TCA and UTCA. It can be seen that the RNase activity acts like bovine pancreatic deoxyribonuclease in that the products of hydrolysis are oligonucleotides -- i.e., about 75% of the products soluble

TABLE XXXI

RELEASE OF INORGANIC PHOSPHATE BY MICROCOCCUS SODONENSIS 5' NUCLEOTIDASE FROM MONONUCLEOTIDE PRODUCTS OF PSEUDOMONAS AERUGINOSA EXONUCLEASE

Estimated nM Mononucleotide Present	nM Inorganic Phosphate Released	Ratio Phosphate Released to Mononucleotide present
Experiment 1		
21.0	23.5	1.110
21.0	20.75	0.985
Experiment 2		
45.0	41.0	0.915
45.0	43.0	0.960

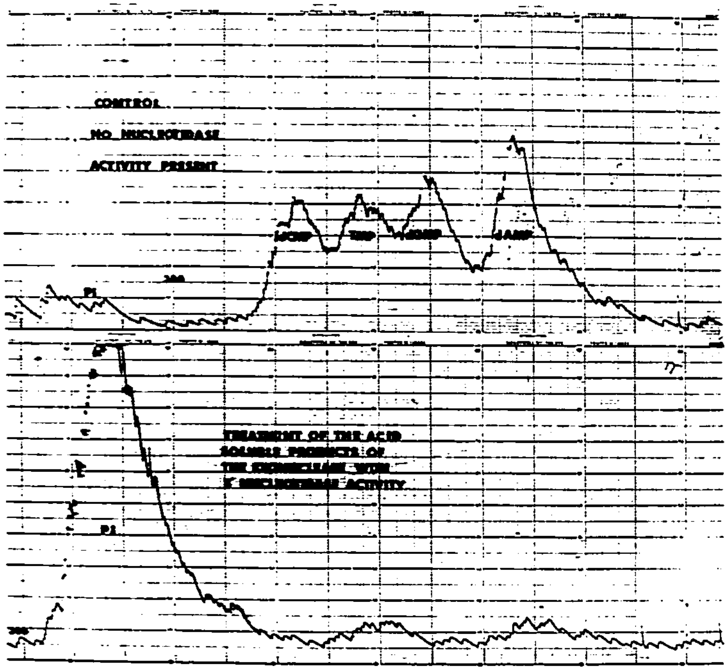

CONTROL
NO NUCLEOTIDASE
ACTIVITY PRESENT
dCMP
TMP
dGMP
dAMP
Pi
300
TREATMENT OF THE ACID
SOLUBLE PRODUCTS OF
THE ENDONUCLEASE WITH
5' NUCLEOTIDASE ACTIVITY
Pi
300

FIGURE 43

PAPER CHROMATOGRAPHY OF THE ACID SOLUBLE PRODUCTS OF THE *PSEUDOMONAS AERUGINOSA* EXONUCLEASE AFTER TREATMENT WITH A 5' NUCLEOTIDASE

100 μl assays containing ^{32}P-DNA were prepared, incubated and stopped exactly as for the standard exonuclease assay. The acid soluble supernatants were neutralized with 5 N KOH and the perchlorates removed by three centrifugations for 1 minute in the Beckman Microfuge. Identical 300 μl mixtures containing 200 μl of the acid soluble supernatant and concentrations of the required reagents as noted in section H - 3 of the Materials and Methods were incubated for 120 minutes (i) in the presence of 0.10 units of *M. sodonensis* nuclease and (ii) with no *M. sodonensis* nuclease present. The samples were spotted and run on paper chromatography as given in Materials and Methods and the chromatographs examined on the radioactive chromatogram scanner (Actigraph III) and tracings made.

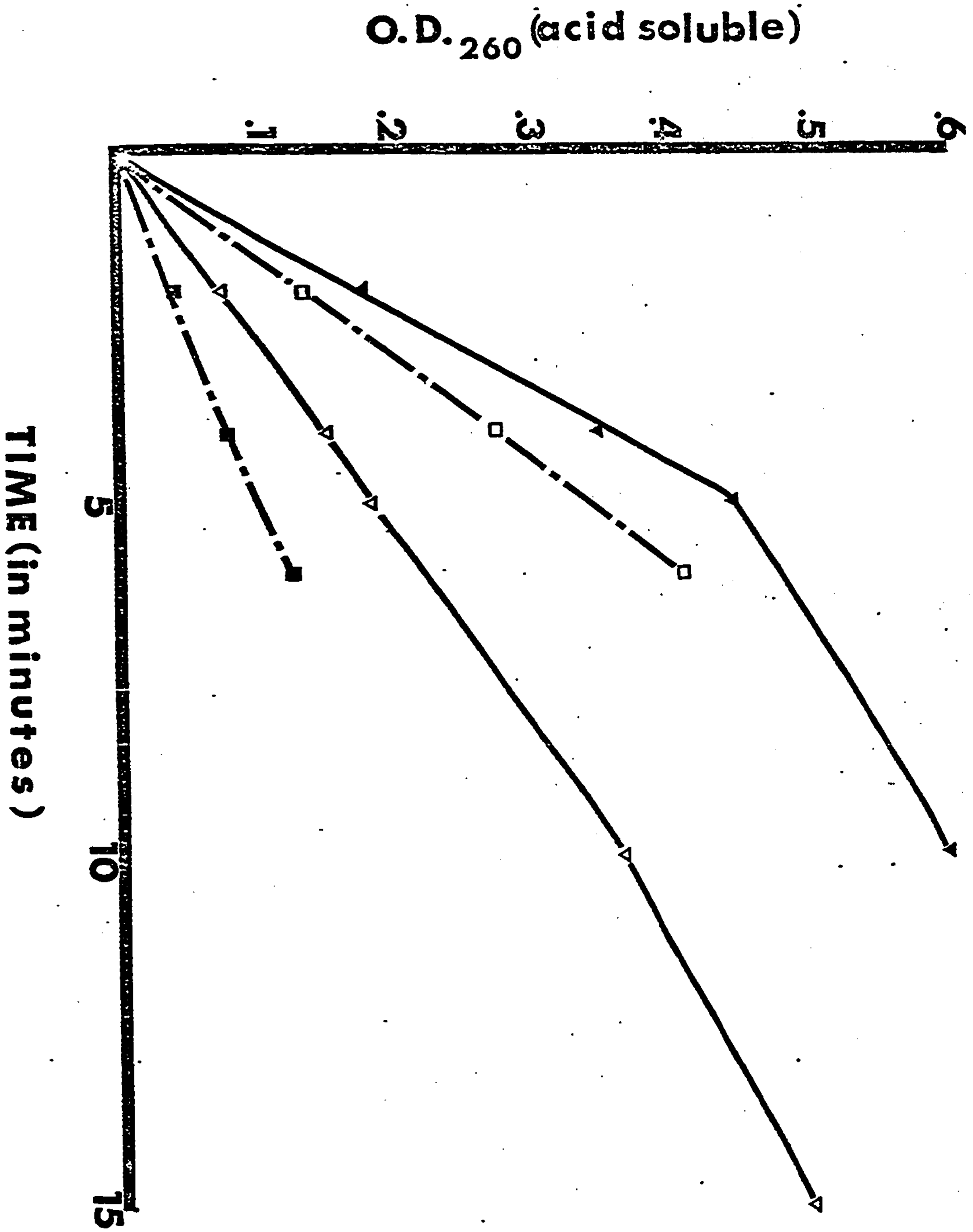

O.D.$_{260}$ (acid soluble)
.1
.2
.3
.4
.5
.6
TIME (in minutes)
5
10
15

FIGURE 44

MODE OF ACTION OF CONTAMINATING RNASE ACTIVITY IN *PSEUDOMONAS AERUGINOSA* EXONUCLEASE PREPARATIONS

Samples of exonuclease containing 0.5 mg/ml protein were assayed for RNase activity as indicated in Materials and Methods under the conditions given below.

▼————▼ Standard assay (with EDTA)

▽————▽ Assay contains no EDTA, Mg^{++} 0.0025 M

□— – —□ Standard assay, TCA used as precipitant

■— – —■ Standard assay, UTCA used as precipitant in TCA are insoluble in UTCA. Thus the enzyme is an endonuclease.

(b) Fig. 44 also demonstrates RNase activity is stimulated by EDTA whereas it has been shown the exonuclease is inhibited completely by that reagent.

(c) Pretreatment of the exonuclease at a concentration of 0.5 mg/ml with 1 N HCl to produce a final pH of 3.0 and kept at 25° for 5 minutes failed to inactivate the RNase. The preparation was first readjusted to pH 7.5 with 2.0 M Tris and then assayed as in Materials and Methods. The activity loss was nil, whereas in contrast, the exonuclease is completely inactive under these conditions.

These points plus the elution at a distinct section of the G-75 column confirm that the RNase is a contaminant.

2. Phosphomonoesterase

Table XXXII demonstrates activity on para-nitrophenol phosphate by the exonuclease. The absence of activity on 5' nucleotides of either the ribo- or deoxyribo- series has been confirmed on several occasions at several pH's, with enzyme concentration up to 25 μg per assay and with incubations up to 6 hours. 3' nucleotidase activity has not been examined for, as no preparations of the substrate were available.

The persistence of low phosphomonoesterase activity is believed to be a manifestation of the phosphatase activity of the exonuclease. See part H of this section for a discussion of phosphatase activity.

3. Phosphodiesterase

Phosphodiesterase activity on p-nitrophenyl thymidine 5' PO_4 and p-nitrophenyl thymidine 3' PO_4 at pH 7.5 and 9.3 and 5.9 and 7.5 respectively is not detectable after incubations for up to 6 hours, with up to

TABLE XXXII

ACTIVITY ON p-NITROPHENOL PHOSPHATE BY
PSEUDOMONAS AERUGINOSA EXONUCLEASE

Purification Step	Activity
Ammonium sulfate	1.85×10^{-3} units/mg
Hydroxylapatite	1.2×10^{-3} units/mg
Sephadex G-75	5.4×10^{-3} units/mg

25 μg of G-75 enzyme per assay.

4. DNA polymerase activity

Using the assay described in Materials and Methods, no evidence of polymerase activity was detected at pH 7.5 and 9.2 in 40 minutes incubations over a range of exonuclease protein per assay of 0.25 to 25 μg. A positive polymerase control was included in the form of purified E. coli DNA polymerase. The incorporation of ^{3}H-TTP was linear over 40 minutes giving 390 units/ml which was the stated activity of the enzyme.

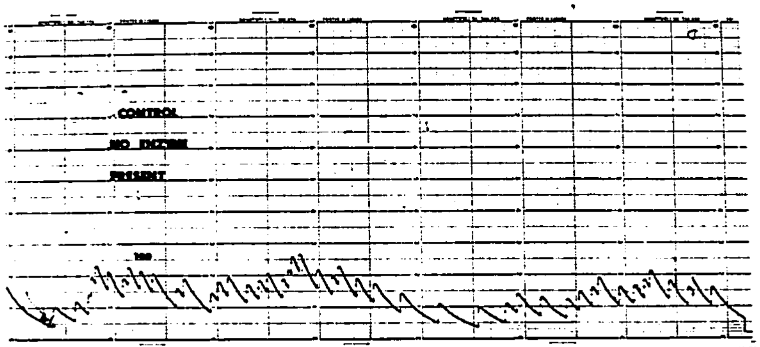
H. Phosphatase activity

The exonuclease exhibits a 3' polynucleotide phosphatase activity which is detected by the release of acid soluble ^{32}P-phosphate from ^{32}P-DNA by the assay given in Materials and Methods. That assay has the following characteristics:

(i) Proportionality between phosphate release and enzyme concentration exists over a range of 10 to 50% of the total 3' phosphate present.

(ii) It is linear with time at a DNA concentration of 37.5 μg/ml for at least 15 minutes under the conditions of (i) above.

(iii) The assay is effective in measuring only ^{32}P-phosphate release and no other ^{32}P products. Fig. 45 demonstrates an Actigraph tracing of a paper chromatogram of the product of the standard charcoal adsorption assay for the phosphatase with and without enzyme present. The only labelled product detected is ^{32}P-labelled inorganic phos-


CONTROL
NO ENZYME
PRESENT
100

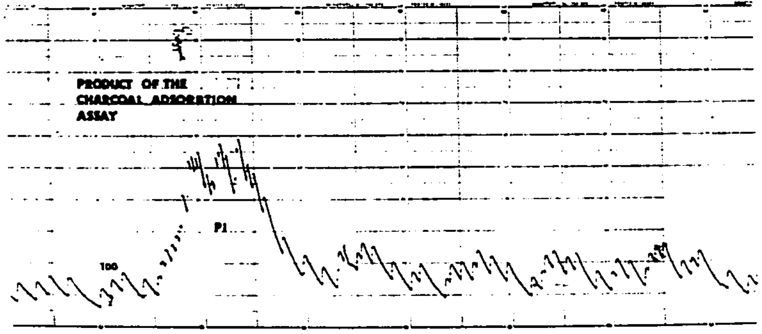

PRODUCT OF THE
CHARCOAL ADSORPTION
ASSAY
P1
100

FIGURE 45

PAPER CHROMATOGRAPHY OF THE PRODUCT OF THE CHARCOAL ADSORPTION ASSAY FOR PHOSPHATASE ACTIVITY IN *PSEUDOMONAS AERUGINOSA* EXONUCLEASE PREPARATIONS

100 μl assay mixes were prepared, incubated and stopped exactly as for the standard phosphatase assay given in Materials and Methods. After the two charcoal adsorption steps, the entire supernatant was chromatographed by paper. The paper strips were scanned on the Nuclear Chicago Actigraph. A control containing no exonuclease was also examined in an identical manner.

phate with an Rf of 0.9 on the chromatogram. Evidence is also presented in Fig. 42 demonstrating low levels of ^{32}P-labelled inorganic phosphate as a product of the exonuclease.

(iv) the pH optimum as shown in Fig. 28 is 7.5.

(v) the cation requirements as shown in Table XXI indicate an almost identical relative activity with the different cations to that shown by the exonuclease.

(vi) Fig. 30 exhibits the Mg^{++} requirements of the phosphatase function. The optimum is narrower than the exonuclease being 0.001 M, activity in the absence of added Mg^{++} is below 10% and the fall-off in activity at higher concentrations is more rapid.

The probability that the exonuclease and phosphatase are activities of a single enzyme is suggested by the following findings:

(i) The pH optima are similar at 7.8 to 8.0 and 7.5 respectively.

(ii) The relative activity with several cations is very similar (Table XXI).

(iii) In Table XXIII-B it is seen both activities are inhibited by EDTA at equimolar concentration of Mg^{++} present. Also both activities are inhibited to similar extents by 10^{-5}, 10^{-6} and 10^{-7} M PCMB.

(iv) A near constant ratio of exonuclease to phosphatase is maintained in the last three purification steps as is seen in Table XXXIII.

(v) Activity in very low levels has been recovered from polyacrylamide gel electrophoresis in a band of gel #4 seen in Fig. 10. It was recovered as indicated in Materials and Methods. Under these conditions, both phosphatase and exonuclease activity were detectable in the same gel slice again at similar ratios, as indicated in Table XXXIII.

(vi) The phosphatase activity shows a distinct preference for native DNA as seen in Table XXXIV which is of a magnitude similar to that shown by the exonuclease.

The phosphatase activity is almost surely limited to an activity on 3' phosphoryl termini of DNA. The reasons for that conclusion are listed:

1. The activity is negligible unless the substrate DNA has been modified to produce 3' phosphoryl ends. This is seen in Table XXXIV. An experiment documented in subsection I of this section demonstrates the release of specifically labelled 5' terminal phosphates is very slow.

2. The release of inorganic phosphate by the the enzyme never exceeds that produced by alkaline phosphatase even after 4 hour incubations as shown in Table XXVIII. Alkaline phosphatase used in

TABLE XXXIII

RELATIVE PHOSPHATASE - EXONUCLEASE ACTIVITY IN FINAL PURIFICATION PROCEDURES AND GEL ELECTROPHORESIS

Procedure	Phosphatase Activity (units/ml)	Exonuclease Activity (units/ml)	Ratio Phosphatase to Exonuclease
Hydroxylapatite	142	760	0.187
DEAE Cellulose #2	81	406	0.198
G-75 Sephadex	27.4	146.5	0.187
Polyacrylamide gel	3.0	15.7	0.19

TABLE XXXIV

RELATIVE PHOSPHATASE ACTIVITY ON NATIVE AND MODIFIED DNA BY *PSEUDOMONAS AERUGINOSA* EXONUCLEASE

Type of DNA	Units/ml	Relative Activity
Native 3' phosphoryl	21.2	1.0
Denatured 3' phosphoryl	6.3	0.30
Standard (not modified with micrococcal nuclease)	2.3 - 2.7	0.1 - 0.13
Native 5' phosphoryl	2.0	0.095

these experiments had no nuclease activity and the great majority of phosphates available to be released were those at the 3' termini. Thus by analogy, the exonuclease - phosphatase activity hydrolyzes 3' phosphates.

3. Release of phosphate from 5' phosphoryl DNA is negligible.

4. The products of the exonuclease are 5' mononucleotides. The exonuclease has had at no time a 5' nucleotidase activity demonstrated though extremely low rates cannot be excluded. Thus, no products are available to account for the release of inorganic phosphate.

I. The Direction of enzymatic attack

The modification of DNA with micrococcal nuclease is known to produce 3' phosphoryl groups. There is almost no phosphatase activity unless such modification is carried out. The probability of initiation of enzymatic attack at the 3' phosphoryl end therefore seems likely. Strong evidence in favour of that hypothesis is seen in Fig. 46. In that experiment, the 5' termini of DNA have been phosphorylated using γ-labelled ATP and polynucleotide kinase as indicated in Materials and Methods. The phosphorylation resulted in 320 cpm of acid insoluble ^{32}P in each 5 μg of DNA. If the initial phosphate removal occurred at the 5' termini, early release of a considerable fraction of the ^{32}P counts would be expected. On the other hand, by using the DNA modified by micrococcal nuclease to produce 3' phosphates, the reverse would be expected. Fig. 46 demonstrates that attack does not occur from the 5'

FRACTION OF TOTAL 3′ OR 5′ PHOSPHORYL GROUPS RELEASED PER nM OF NUCLEOTIDE HYDROLYZED

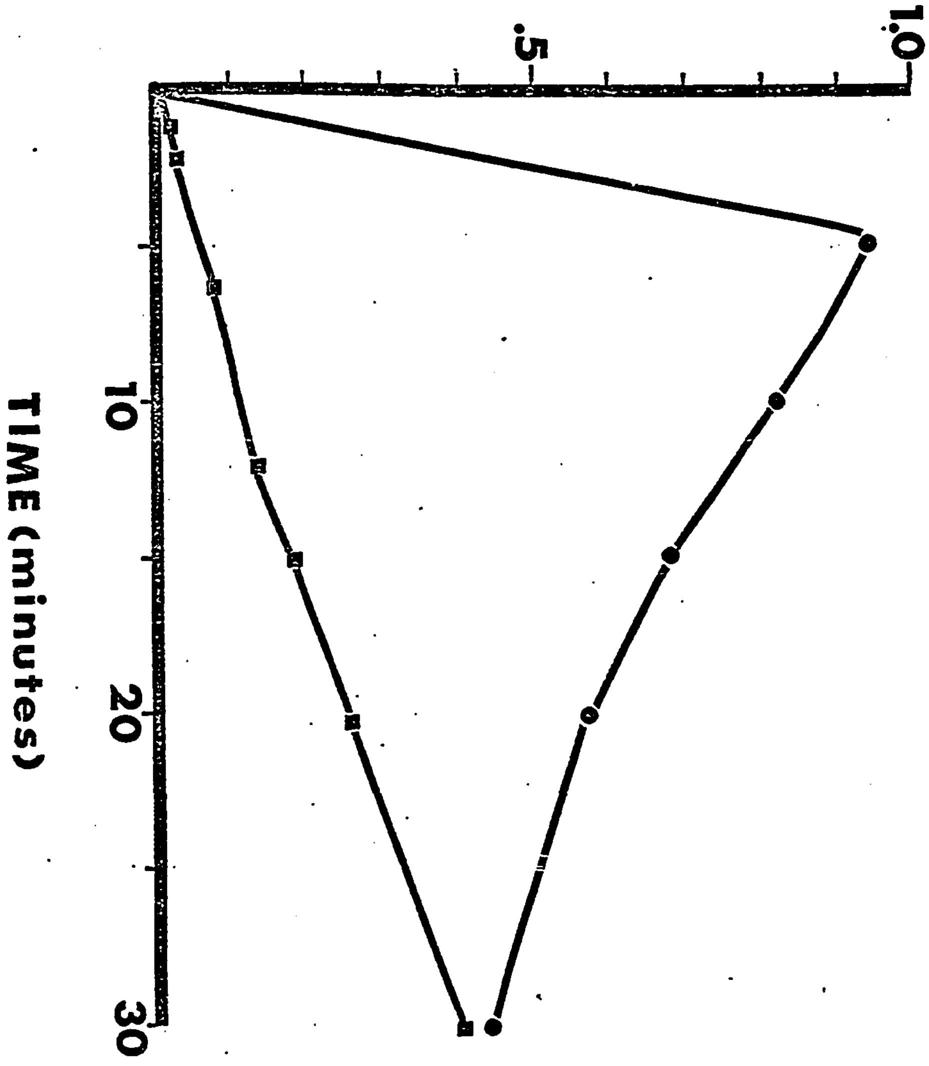


FIGURE 46

THE RELEASE OF TERMINAL PHOSPHORYL GROUPS FROM SPECIFICALLY LABELLED DNA BY THE *PSEUDOMONAS AERUGINOSA* EXONUCLEASE

^{3}H-DNA was labelled at 5' termini with ^{32}P as given in Materials and Methods. Standard exonuclease assays were prepared and stopped at the indicated times as usual. Counting was carried out in the Nuclear Chicago Mark I Liquid Scintillation counter so that no ^{3}H counts were recorded on the ^{32}P channel and 5% of the ^{32}P counts were recorded on the ^{3}H channel.

^{32}P inorganic phosphate release from ^{32}P-labelled 3' phosphoryl DNA was detected by the standard phosphatase assay and exonuclease activity was corrected for the release of ^{32}P inorganic phosphate.

The total ^{32}P present as 3' phosphoryl termini was estimated by the total release of phosphate using alkaline phosphatase.

■———■ ^{32}P labelled 5' phosphoryl termini of ^{3}H-DNA

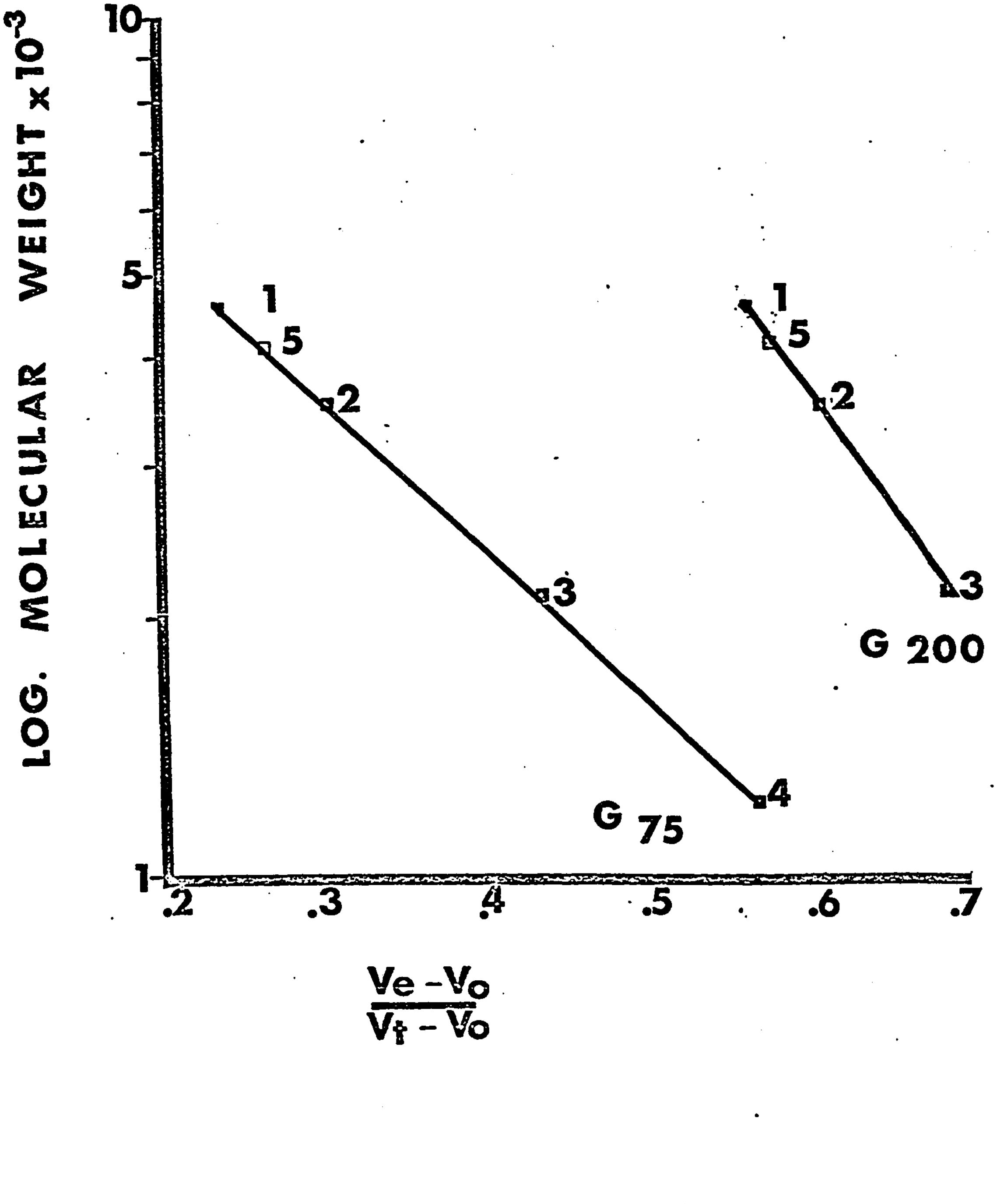
●———● ^{32}P release from 3' phosphoryl DNA terminus. A much higher percentage of the total ^{32}P as 3' phosphate per nM of nucleotide is released at all times than of ^{32}P as 5' phosphate. The total ^{32}P as 3' phosphate was estimated by the amount of phosphate hydrolyzed by alkaline phosphatase and detected by the phosphatase assay. As previously noted, the alkaline phosphatase used contained no nucleolytic activity and removed only terminal phosphates. As shown in Fig. 45, the phosphatase assay is specific for phosphate. The specificity of micrococcal nuclease to produce 3' phosphoryl groups and not 5' phosphates is well established (Cunningham et al, 1956; Ohsaka et al, 1964).

J. Molecular weight determination be gel filtration

Gel filtration studies using G-75 and G-200 Sephadex columns calibrated with standards were used to estimate the molecular weight of the exonuclease. The results of those procedures are seen in Fig. 47. The K_{av} from G-75 Sephadex was 0.26 and from G-200 was 0.57 for the exonuclease. The molecular weights corresponding to those values are 41,500 and 43,500 respectively so that close agreement was obtained.

K. Ultracentrifugal studies

The results obtained from the sedimentation equilibrium procedures were inconclusive. Several serious problems resulted in that outcome. An initial attempt to determine the molecular weight while in 30% glycerol was unsuccessful. It was found that the glycerol underwent re-distribution and produced fringe displacement in the absence of the protein. Lowering the glycerol concentration failed to overcome that problem. Thus, it was necessary to do the runs in 0.05 M phosphate and 0.01 M mercaptoethanol which is a distinct system from that used for gel filtration. That procedure resulted in the inactivation of 75 µg



LOG. MOLECULAR WEIGHT x 10^{-3}
10
5
1
.2
.3
.4
.5
.6
.7
$\frac{Ve - Vo}{Vt - Vo}$
1
5
2
3
4
G 75
1
5
2
3
G 200

FIGURE 47

DETERMINATION OF THE MOLECULAR WEIGHT
OF *PSEUDOMONAS AERUGINOSA* EXONUCLEASE
BY GEL FILTRATION

Columns were prepared and operated as given in Materials and Methods. The samples are:

1. ovalbumin
2. pepsin
3. soybean trypsin inhibitor
4. cytochrome c
5. *P. aeruginosa* exonuclease

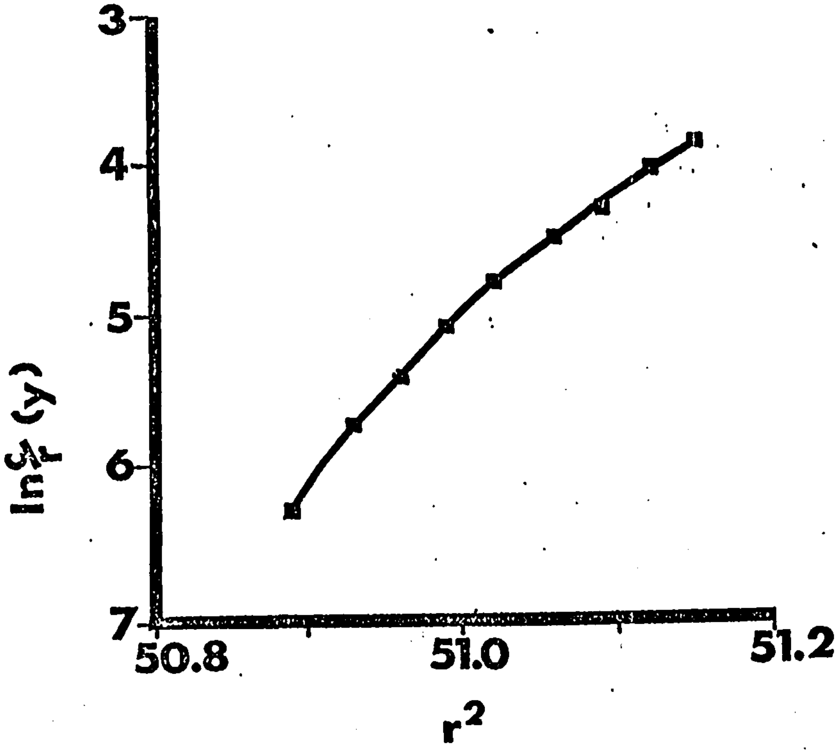

3
4
5
6
7
$\ln\frac{c}{r}$ (y)
50.8
51.0
51.2
r^2

FIGURE 48

PLOT OF ln c/r VS. r^2

Sedimentation equilibrium was carried out for 51 hours at 22,000 rpm as outlined in Materials and Methods. A 12 mm cell was used and the temperature was 2°.

c/r = concentration at point r

r = distance from the center of the axis of rotation of RNase-free exonuclease, which is the yield from 20 litres of culture. Under these conditions, 0.25 ml of 0.3 mg/ml protein solution was obtained using the extinction ratio of 280 nm: 260 nm.

The molecular weight estimations underwent a decrease with increasing protein concentration in the cell. A plot of ln y vs. r^2 (y = protein concentration in fringes; r^2 = distance from the center of the axis of rotation) undergoes a decreasing slope with increasing concentration (Fig. 48) which indicates a problem of non-ideality (Williams et al, 1958; Chervanka, 1969). However, a technical problem is present because of the extremely low concentrations present near the meniscus. Fringe deviations of 100 microns or less should not be used because of the error of neasurement (Chervanka, 1969) which leaves only 4 - 6 points available for plotting in each of the 22, 30 and 36,000 rpm trials, if those values are excluded.

An extreme non-ideality effect would be unexpected in view of the very low protein concentration used (Van Holde, 1967).

Another problem is an apparent loss of mass. The concentration of protein used was 0.3 mg/ml but the highest concentration obtained in any run was 0.25 mg/ml. That concentration occurred at the cell bottom and should be in excess of 0.3 mg/ml as it represents an area of concentration. The reason for mass loss is not clear, though an inaccurate estimation by optical density, aggregation or deposition on the tube walls or bottom might account for that loss.

The molecular weights obtained by extrapolation of 1/molecular weight* vs. protein concentration in the 22, 30 and 36,000 rpm runs do not agree nor do they agree with apparent molecular weights utilizing the slope of the ln y vs. r^2 plots. Finally, all the molecular weights obtained from sedimentation equilibrium data are in excess of that by gel filtration by a range of 1.5 to 9 fold.

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*weight average molecular weight at a given point in the cell

Therefore, a conclusion cannot be made without more experimentation. However, the protein available prevents that being done until 100 or more litre batches of cultures can be handled which is not physically possible at this time.

DISCUSSION

Enzyme purifications may be easy, difficult, or impossible. The purification undertaken in this study came close to qualifying as the last alternative. The extreme polymorphism due to polynucleotide binding and proteolytic modification coupled with a very low stability of the enzyme presented a problem of remarkable severity. The nature of the mechanisms causing the polymorphism were such that they produced effects which were in direct opposition to each other. Confusion freely arose from a process which resulted in molecules larger and smaller than the enzyme. It was even extremely difficult for many months to identify the enzyme itself in several purification methods.

The binding of polynucleotides to a nuclease is not surprising in view of the fact that they are substrates. The evidence presented in this study strongly suggests the major structure being bound was a refractive form of DNA. What is surprising is that the complex of DNA and enzyme should be sufficiently stable to affect behavior in several purification techniques. The stability of the complex may come from the fact that the enzyme probably carries out processive* (Nossal and Singer, 1968) degradation. Under these circumstances, the enzyme is bound to a strand of substrate and produces extensive or total hydrolysis before release and binding to a new fragment occurs.

The hypothesis of processive degradation is supported by two observations.

(i) Complete inhibition of the release of radioactive acid soluble products of the ^{3}H-DNA in a standard exonuclease assay can be produced as discussed in section I subsection C - vi, by unlabelled DNA. However, it is also obvious from hyperchromic assays that the unlabelled DNA is being hydrolyzed. It therefore appears an enzyme molecule is bound to DNA substrate and extensively hydrolyzes it before being freed to attack other DNA molecules.

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*Processive degradation is the term used by Nossal and Singer to indicate complete hydrolysis of one strand of RNA before a new strand is attacked.

(ii) The addition of micrococcal nuclease for a period and at a concentration sufficient to produce near-maximal solubilization of products absorbing at 260 nm as shown in Table IX does not accelerate the activation rate of the enzyme. It is thus strongly suggested that the DNA molecules responsible for the apparent inhibition relieved by the activation procedure are not hydrolyzed by micrococcal nuclease. A probable reason for this is that these molecules are already bound to the exonuclease. If this is true, and there is no reason to expect it not to be so, then the exonuclease must act sequentially. In this model it would remain bound to a DNA fragment until it was extensively hydrolyzed. If this model is not true and release occurred after each or a few hydrolytic events, the inhibitory DNA should be subject to attack by micrococcal nuclease, which attacks oligonucleotides to produce eventual complete hydrolysis (Sulkowski and Laskowski, 1968), and DNA fragments with no exonuclease bound initially would be partly hydrolyzed by that enzyme. The net effect would be an acceleration of activation which was not observed.

The DNA bound to the exonuclease in crude preparations is bound, apparently, non-productively to the catalytic site. The provision of conditions of Mg^{++} and temperature which allow the enzyme to hydrolyze actively allows the solubilization of the bound inhibitor. The fact that the destruction of the DNA fragments produces an increase in total activity is due only to relief from apparent DNA inhibition, (Fig. 16). The inhibition is only apparent because the DNA is unlabelled and its destruction is not detected in the standard assay. Once again, this observation argues in favour of a processive (or modified processive) degradation of DNA in that the unlabelled fragments are destroyed and then activity with radioactive substrate can be detected.

Polynucleotide binding has been recognized by many other investigators. However, in those cases, precautions were taken to overcome the problem in the following ways.

(i) Destruction of polynucleotides with RNases and DNases (usually endogenous).

(ii) Removal by precipitation with multivalent cations such as streptomycin or protamine.

(iii) Reaction with anion exchange materials for example DEAE cellulose.

In the case of the P. aeruginosa exonuclease, these methods were tried and discarded because (a) precipitation of nucleic acid as in (ii) was incomplete before the enzyme precipitated. Co-precipitation of the enzyme and nucleic acids was prevented by the low recovery of activity when the enzyme was eluted from the precipitate.

(b) (iii) was not used as the possibility that the multiple peaks on DEAE cellulose might be a result of various sized polynucleotide fragments bound to the enzyme was not eliminated for some time.

(c) (i) was used because nucleic acid precipitation was almost complete, and an increase in activity was produced. However, it also worsened the proteolytic degradation.

The effect of binding polynucleotides is clearly shown in this investigation to produce polymorphism on gel filtration, and sucrose density gradients.

Modification of the exonuclease due to limited proteolysis was the second cause of polymorphism. Manifestations of this process were seen in DEAE cellulose chromatography and gel filtration procedures. As demonstrated in this study, the inhibition of proteolysis eliminated such heterogeneity.

Proteolytic modification resulted in the sequential production of a population of species. The first member to appear is that form which occasionally cannot be prevented even by the method used for proteolytic inhibition, and which elutes at a slightly higher ionic strength on DEAE cellulose. Thereafter, new members were produced which required increasing ionic strength to be eluted and thus were more negatively charged. This can be concluded from the data of Fig. 25, from the work on aged preparations and the occasional appearance of a second DEAE peak in standard purification. The increased negativity may be due to one or more of several reasons.

(i) scission of peptide bonds releasing fully dissociated carboxyl groups and partly dissociated amino groups at pH 7.5.

(ii) the release of positively charged amino acids as lysine and arginine. Sasaki _et al_ (1966) found that with yeast phosphoglyceric acid mutase, 7 molecules of lysine were released in the transition of the parent enzyme through four additional forms as a result of limited proteolysis.

(iii) the release of amide nitrogens to produce carboxyl groups which are negatively charged (Sasaki, 1961).

(iv) minor local conformational changes so that new charged groups might be exposed.

In addition to a population of negatively charged species, several size variants were also detected. The estimation of size was done partly by gel filtration which is prone to many errors (see later in this discussion). However, on a relative basis it is quite obvious that active molecules smaller than the unassociated intact enzyme exist. In this regard, Sasaki (1961) found that 51 amino acids were released from yeast phosphoglyceric acid mutase with retention of activity and no major conformational change detected by O.R.D. studies. Klenow and Hennigsen (1970) have isolated DNA polymerase molecules from _E_. _coli_ estimated at 70,000 molecular weight by gel filtration with essentially no exonuclease function present in addition to the conventional enzyme of 150,000 molecular weight with both exonuclease and polymerase activity. Such molecules can also be produced by digestion with subtilisin. In each case, the separate DNA polymerase molecules have undergone an

109,000

increase in specific activity. This represents a size reduction of about one half with an actual increase in specific activity. Thus precedent for a significant size change and activity retention does exist. One other example is provided by Chesbro (1966) who described by gel filtration studies a population of different sized species of extracellular Staphylococcus aureus (Micrococcus aureus) nuclease. He did not invoke proteolysis as an explanation but did demonstrate a range of molecules from the major component at 16,000 molecular weight through 12,000, 7,500 and 2,500, and in one case a molecule of 850. Some reticence in accepting such low molecular weight samples is, of course, to be expected but the possibility exists that significantly smaller forms than the parent form were present and retained activity.

Thus the existence of molecular weight forms significantly smaller than the parent enzyme has been suggested and reasonably well demonstrated in cases other than P. aeruginosa exonuclease.

The significance of such a distribution of molecules with a difference of charge and size is profound in a purification system. The effects produced in this system have been outlined in section I, subsection G of the Results, and the effects in other systems have been noted in the Introduction.

It is clear that the significance of modification of enzymes has only in recent years been recognized in spite of the important effects. Those effects, in summary, include polymorphism, which impairs purification and can result in several isozymes being described, a loss of stability, an inability to define unity of an enzyme and false molecular weights.

Several other facets of the proteolysis are worth mention. The proteolytic activity is difficult to inhibit (see section I, subsection

G - 2 - b of the Results) perhaps due to the system and/or the nature of the protease. The selection of PMSF as an inhibitor was made because of the relative lack of activity on acetylcholinesterase so that its toxicity to the investigator was very low, in addition to a high melting point (91-92°) and being crystalline.

The possibility that the proteolytic enzyme may resemble trypsin exists and is of interest. Schulze and Colowick (1969) found that the enzyme responsible in the yeast system was trypsin-like also. Proteolysis of this nature has not been demonstrated in _P. aeruginosa_ previously. However, evidence of extracellular enzymes showing no activity on benzoyl l-arginine ethyl ester is well documented (Morihara, 1963). If the protease should prove a close model of trypsin it may be of evolutionary interest. In addition, the role in the host and its control are questions of substantial importance.

The low stability of the _P. aeruginosa_ exonuclease and the dramatic stabilizing effect of glycerol both in crude and purified preparations is demonstrated in this study. Increasing concentration of glycerol up to 40%, the highest concentration produced increased stability. It was also noted that dihydric alcohols (ethylene glycol) were of low effectiveness and that monohydric alcohols produced destabilization. Similar effects were seen by Gerlsma (1967) in a study of the denaturation of RNase (pancreatic) as influenced by polyhydric alcohols. He noted that stabilization against heat, pH and urea denaturation was produced by polyhydric alcohols but not against the destabilizing effect of propanol. He concluded the stabilizing effect of glycerol was principally due to a decreased bond rupturing capacity of the solvent, particularly H bonds.

The alcohols examined in this study and by Gerlsma contain a hydrophobic component represented by the methyl and methylene groups in addition to the hydroxyl or hydroxyls. Gerlsma postulated that the increased number of hydroxyls in polyhydric alcohols resulted in a greater interaction with water and thus diminished the hydrophobic interaction of the methyl or methylene groups and the protein. The failure of polyhydric alcohols to stabilize against propanol destabilization prompted his suggestion that the effect was mainly to reduce the H bond rupturing capacity of the medium. That form of denaturation may be due to increased hydrophobic interaction of the propanol solvent and the protein.

The effect of freezing on the enzyme in non-glycerol solvent was disastrous. Enzymes which have low stability in the cold are generally felt to depend relatively more for stability on hydrophobic bonds. However, such enzymes would be expected to have an enhanced stability at higher temperatures where the hydrophobic bond is more stable (Schachman, 1963). However, _P. aeruginosa_ exonuclease does not exhibit any heat stability, in fact the contrary is true so that on a theoretical basis hydrophobic bonding may be less significant in this enzyme than in many. Physical effects may also account for some of the low stability to freezing. These include concentration variations, ice crystal formation which has a shearing and compressing effect (Porter _et al_, 1953) and disturbance of the solvation shell. Thirty per cent glycerol prevents complete solidification during freezing at -20° and this may eliminate these effects.

The purification of the exonuclease was as noted interfered with by its modification and low stability. The 350 fold purification is, in my opinion, an underestimation. Two reasons suggest the purification is actually more. If one examines the polyacrylamide gels of the DEAE cellulose #2 column and the G-75 column (Fig. 10) it is apparent from the decrease of the RNase band relative to the increase of the exonuclease band in the two steps that a substantial purification has occurred. In addition, several additional bands disappear between the two steps. However, numerically that purification is less than 2 fold. It is, I feel, obvious that the actual purification is much greater. A recovery of activity of 38% occurs in these two steps so that it is quite possible that inactive exonuclease is being purified with active enzyme. That possibility is further suggested by the finding on one occasion of a specific activity of 2,500 from the G-75 step. It is, of course, quite possible that inactive enzyme accumulates throughout the purification, thus tending to reduce the final purification. Special precautions were taken to retain 90% or more of the active fractions from each procedure so that only a low percentage of activity is discarded. In this same vein, it is interesting to note that the total protein reduction is 6,200 fold which is high relative to the activity purification in view of the precautions taken not to discard activity.

A dual activity is associated with the exonuclease. It is both a DNA phosphatase and exonuclease. The evidence presented in favour of that premise is, I feel, very convincing. Several enzymes possess dual functions including the exonuclease - polymerase activity of *E. coli* DNA polymerase (Richardson, Schildkraut, Aposhian and Kornberg, 1964; Klett and Cerami, 1968). At least 4 other enzymes possessing combined DNA phosphatase - exonuclease functions have been detected. These are exonuclease III of *E. coli* (Richardson *et al*, 1964), a pneumococcal enzyme (Greenberg and Lacks, 1967), lambda exonuclease (Korn and Weisbach, 1963; Little et al, 1967; Little, 1967b) and one detected in crude materials only in Bacillus subtilis (Okazaki and Kornberg, 1964). Of these, the lambda exonuclease differs in attacking from the 5' end of the DNA.

The exonuclease described herein resembles exonuclease III to a considerable extent. The preference for native DNA, the attack from the 3' terminus with release of a phosphate (if present), the release of 5' mononucleotides, the requirement for sulfhydryl reagents to produce full activity, and general similarities of cation requirements, pH and inhibition by zinc are seen for the two enzymes. In addition, both enzymes are of low stability though the Pseudomonas enzyme is less stable.

In spite of the close resemblance, several well defined distinctions also exist. One of the most significant of those is the difference in the final extent of substrate solubilization. Exonuclease III solubilizes less than 50% of native DNA substrate even with prolonged incubation or new enzyme additions. The P. aeruginosa exonuclease, on the other hand, can produce total solubilization of native DNA. Similarly, the limits with denatured DNA are different being 18% for exonuclease III and 60% in 48 hours for the P. aeruginosa exonuclease. The model proposed by Richardson et al (1964a, b) for the action of exonuclease III is an attack from 3' termini leaving a single stranded structure resistant to attack. Those workers obtained a rate preference for native over denatured DNA of about four fold which is alsotrue of the enzyme of this study. The explanation for the rate on denatured DNA was said to be due to residual double stranded areas. Obviously with the P. aeruginosa exonuclease such a proposal is not possible for the extent of hydrolysis of native DNA and heat denatured DNA at 48 hours rules out the possibility that residual double stranded regions account for the attack rate. Denaturation was accompanied by a hyperchromicity of 37 to 39% indicating the heat denaturation was successful. It thus appears that the heat denatured DNA is attacked but at a slower rate. K_m values for denatured DNA are almost identical to those for native DNA indicating that binding is not dependent on the secondary structure of DNA. However, the V_{max} is considerably reduced, so that the catalytic function is impaired by the change in secondary structure.

The question of whether this enzyme could separate double stranded DNA from single stranded DNA in the same mixture by destroying the former was not examined in this study. However, it appears unlikely to be true, in that some single stranded DNA would be destroyed.

If denatured DNA is not resistant to attack, the question of why activity levels off after 30 to 45% solubilization occurs (Fig. 36). A very slow increase results after this extent of solubilization but it is considerably slower than the rate shown by denatured DNA. Thus the relative resistance of denatured (mainly single stranded) DNA cannot wholly account for this observation. The other major possibilities are that the enzyme is inactivated extensively or that products are inhibitory. Evidence presented in section II subsection E suggests that the rate decline is a product both of some enzyme inactivation, and the production of resistant substrate. The substrate remaining after extensive partial hydrolysis is not totally comparable to denatured DNA. Richardson (1964b) has shown that heat denatured DNA does contain some double stranded regions. In addition, it would be expected also to contain DNA of a length similar to that of native DNA. The partially hydrolyzed substrate, however, probably contains many shortened fragments and DNA areas which are totally single stranded due to partial enzyme hydrolysis. This substrate may well be a much poorer substrate than denatured DNA. A combination of these effects thus would seem to account for the levelling off of activity with extensive hydrolysis.

Another difference exhibited by P. aeruginosa exonuclease from exonuclease III is the failure to produce a change in maximal velocity using DNA modified with micrococcal nuclease to produce new 3' phosphoryl termini. As pointed out in the Results (Table XXVIII) the V_{max} of P. aeruginosa exonuclease is considerably increased by such treatment and this appears not to be related to the phosphate group but the new ends produced. As Greenberg and Lacks (1967) found with Diplococcus pneumoniae exonuclease, the K_m is not altered by this treatment. They proposed a model in which enzyme is bound to the DNA both at catalytically active areas of the DNA and areas at which no catalysis could occur. Therefore with the introduction of new ends no change in binding affinity occurred but many catalytically active sites were introduced with the formation of new ends, producing the change in V_{max}. This model also appears tenable in the case of P. aeruginosa exonuclease. The reason exonuclease III failed to demonstrate this effect is not clear.

The relative phosphatase and exonuclease rates of exonuclease III are identical until near total release of phosphate has occurred (Richardson et al, 1964b). In addition, the exonuclease shows an initial lag of about 5 minutes during which the rate is less than the phosphatase.

These findings are quite distinct from those found here and in pneumococcal and lambda exonucleases. No lag in P. aeruginosa exonuclease activity is detectable even down to 1 minute and the rate of release of phosphate is well below that of the exonuclease. In view of the fact that phosphate release is likely the first event to occur, it appears that all 3' phosphates are not saturated with enzyme under the conditions used, as a linear release of phosphate occurs over 15 or more minutes. If all such termini were saturated a very rapid burst of phosphate release would be expected to occur followed by no further release.

The *P. aeruginosa* exonuclease also resembles exonuclease III in the well defined requirement for sulfhydryl groups which, however, is not seen in the pneumococcal enzyme (Greenberg and Lacks, 1967). The failure of such a requirement is remarkable indeed in view of the catastrophic effects of deleting mercaptoethanol from purified *P. aeruginosa* preparations. Presumably, the effect of sulfhydryl reagents is to maintain a reduced half-cystine at the active site of the *P. aeruginosa* exonuclease. The sensitivity to inhibition by PCMB, a reagent demonstrating a high but not absolute specificity for SH groups (Boyer, 1959; Smalt *et al*, 1957; Sohler *et al*, 1952), is also suggestive that such a half cystine or half cystines are critical to the maintainence of activity. In interpretation of this data, two reservations are required.

(i) the reactive amino acid(s) may not be a half cystine(s). Such a possibility is unlikely.

(ii) the reactive amino acid(s) might not be located at the active site but at a position which is critical to the conformation of the protein and therefore also to activity.

The favourable role of very high concentrations of mercaptoethanol of up to 0.1 M is suggestive that disulfide bonds do not appear in the molecule, or are not necessary for activity, or are extensively protected from interaction with the solvent by an internal position in the protein.

An interesting and widespread effect among other nucleasesis the inhibition by ionic strength exhibited by P. aeruginosa exonuclease. Very little study as to the origin of this phenomenon has been carried out. Rabin et al (1968) on the basis of inter-relationship of enzyme, thiol and salt concentrations, postulated an association-dissociation equilibrium between subunits of the Neurospora crassa extramitochondrial endonuclease. A specific ionic effect is demonstrated by lambda exonuclease (Little, 1967b) in that K^+ is less effective than Na^+ at equal concentrations. These observations, however, were not demonstrable with the P. aeruginosa exonuclease.

The nature of the inhibition in P. aeruginosa is likely an impairment of the affinity between DNA and the enzyme as the K_m value with 0.07 M NaCl added is almost four times that obtained in the absence of NaCl. The increase of ionic strength decreases the effective radius of electrostatic effects of molecules whether they be attractive or repulsive in nature. It is possible that the reactive groups of the catalytic site have a reduced affinity for the DNA due to a diminished electrostatic attraction which might be necessary itself for binding or for creating the required intermolecular distances to allow covalent or H bonding.

The exonuclease of this work has, as noted, a dual function. The possibility of several other functions was examined for and two were found. A low degree of phosphomonoesterase activity exhibited on p-nitrophenyl phosphate was detected. It seems likely that such activity is a manifestation of the DNA phosphatase activity. Exonuclease III also has a similar low rate of activity on that substrate (Richardson et al, 1964b). The RNase activity detected, for the reasons outlined in section II, subsection G, is felt to be a contaminant. That suggestion is further strengthened by the fact that some preparations are free of RNase activity.

Molecular weight estimation with gel diffusion involves several problems (Ackers, 1964). Error may result from:

(i) electrostatic interaction of protein and gel which can be minimized by increasing the ionic strength or using a pH near the isoelectric point for that protein.

(ii) the extent of hydration of a protein will influence the molecular radius and thus the exclusion from a given pore radius.

(iii) molecular assymetry results in differing molecular radii being presented to a given pore size and thus distorts the elution position. The standard reference proteins normally are ones which are of low frictional ratio.

(iv) diffusion restriction due to steric and frictional resistance of the gel matrix.

(v) dimensional heterogeneity of the gel interstices, which is predicted to be greater in tighter gels.

(vi) inaccuracies in determination of elution volumes.

Thus, an estimation of molecular weight with gel filtration is prone to considerable variation. Even with the elaborate precautions such as low volume elution samples, high ionic strength and the use of large and small pore gels, many of the above objections cannot be overcome.

In view of the objections to gel filtration, an attempt to estimate molecular weight by sedimentation equilibrium was carried out but as indicated in section II, subsection K of the Results, it was inconclusive. A conclusive molecular weight cannot be given at this time, though in view of the close agreement between behavior on G-75 and G-200 gels, 42,500 may not be unreasonable.

It is not possible to compare the molecular weight with those for exonuclease III or the pneumococcal enzyme as they have not been determined. Indeed due to low protein recoveries no estimation of purity other than freedom from several contaminating enzymes (exonuclease III is contaminated with RNase activity) has been expressed for either enzyme.

The P. aeruginosa system of DNases detected by the conventional type of assays used in this work is quite unique. E. coli contains several detectable DNases, the most significant of which in terms of quantity is endonuclease I (Lehman, Roussos and Pratt 1962a, b). Diplococcus pneumoniae contains an endonuclease, in addition to the enzyme previously discussed (Greenberg and Lacks, 1967). It is present in much greater relative quantity than the second enzyme of the system examined in this work. In view of the finding of an exonuclease - phosphatase in several bacterial systems, the possibility of a significant role in DNA metabolism is suggested.

The failure to detect other deoxyribonuclease activities in P. aeruginosa does not exclude their presence. Special requirements such as ATP (Oishi, 1969), ATP and S-adenosyl methionine (Braun and Behrens, 1969), DNA with a particular modification such as UV treatment (Kaplan et al, 1969), alkylation (Freidberg, Hadi and Goldthwait, 1969), unmodified DNA in terms of the host specific modifications (presumably methylations) (Braun and Behrens, 1969), specific activation, for example by heat (Nester and McCarthy, 1969), or specific stabilization (for example glycerol in this system) and other possibilities could be necessary for their demonstration. Special assays, for example those capable of detecting very limited hydrolysis such as the one measuring comparative sedimentation coefficient values devised by Braun and Behrens (1969) may be required.

An extensive characterization of the second enzyme detected in this system was not done due to the very low quantities present and the apparent lability as indicated by its disappearance after the 37° incubation.

The physiological function of the *P. aeruginosa* exonuclease is not clear from the results of the present study, nor from analogy with similar enzymes. The well known relationship between enhanced DNA polymerase activity and removal of 3' phosphates may indicate a function for this enzyme. However, how 3' phosphates would be introduced into the DNA remains a mystery here and in general(Lehman, 1967). The possibility that the exonuclease acts in association with an endonuclease ("nickase"), DNA polymerase and polynucleotide ligase to repair areas of DNA might also be possible. Lambda exonuclease has been identified to take part in recombination (Signer *et al*, 1968) and the possibility that exonuclease III could also be involved in this process is suggested by Richardson (1969). Similarly, it is possible that *P. aeruginosa* could be involved in recombination. Obviously, this is essentially an area of speculation at the present level of knowledge.

The exonuclease described in this system demonstrates apparently negligible resemblance to the intracellular deoxyribonuclease activity described by Guschlbauer and Halleck (1961). Those workers found on very rudimentary characterization a distinct pH optimum (pH 6) and a strange requirement for both citrate and Mg^{++}. When they examined another Pseudomonas species they were unable to detect this activity. No description of the growth phase of the organism at the time of harvest, no details of the assay, and no attempt to characterize the type of DNA attack, requirement for sulfhydryl reagents, etc. were given. It is impossible on the rudimentary information available from that work to compare the activity of this system to that.

The work carried out in this study on P. aeruginosa DNases has opened several areas of future research. The unique problems of stability, the maintainence of activity after proteolytic cleavage, and the relationship of this enzyme to other similar exonucleases make this enzyme an interesting protein to pursue from a structural standpoint, i.e., amino acid analysis, extensive study of sedimentation behavior and perhaps crystallographic structure. All of these objectives require large scale purification techniques, however, in the region of several hundred litres of growth medium.

A close examination for highly specific DNases as outlined in this discussion would be most interesting in view of the low number of DNases present. This is particularly interesting because of the high radiation sensitivity (Holloway, 1969) of P. aeruginosa which could be related to the relative paucity of DNases. It is also of interest in defining, if possible, the minimal DNase complement a bacterium might require for normal cellular function.

The second DNase also could be characterized particularly in any study undertaken on a large scale.

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APPENDIX

LIST OF OFFICIAL ENZYME NOMENCLATURE

Trivial name	E. C. Number	Systematic name
Micrococcal nuclease	3.1.4.7	-----
Pancreatic deoxyribonuclease	3.1.4.5	Deoxyribonucleate oligonucleotido-hydrolase
Pancreatic ribonuclease	2.7.7.16	Polynucleotide 2-oligonucleotido-transferase (cyclizing)
Phosphodiesterase*	3.1.4.1	Orthophosphoric diesterphosphohydrolase
Polynucleotide phosphorylase	2.7.7.8	Nucleosidediphosphate: polynucleotide nucleotidyl transferase.
Alkaline phosphatase	3.1.3.1	Orthophosphoric monoester
DNA polymerase	2.7.7.7	Deoxynucleoside triphosphate: DNA deoxynucleotidyl transferase
5' nucleotidase	3.1.3.5	5'-ribonucleotide phosphohydrolase
Trypsin	3.4.4.4	----- General class - peptide peptidohydrolases
Hexokinase	2.7.1.1	ATP: D-hexose 6-phosphotransferase
Phosphoglyceric acid mutase	5.4.2.1	D-phosphoglycerate 2,3-phosphomutase
Pyruvate decarboxylase	4.1.1.1	2-oxo-acid carboxy-lyase

*Source varies - for example, snake venom, _Micrococcus sodonensis_