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GRADUATE COLLEGE

AN INVESTIGATION OF THE CATALYTIC PROPERTIES OF THE LYTIC PEPTIDASE IN THE STAPHYLOLYTIC PREPARATION LYSOSTAPHIN

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

BY

GARY LEE SLOAN

Norman, Oklahoma

1972

AN INVESTIGATION OF THE CATALYTIC PROPERTIES OF THE LYTIC PEPTIDASE IN THE STAPHYLOLYTIC PREPARATION LYSOSTAPHIN

APPROVED BY

DISSERTATION COMMITTEE

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AN INVESTIGATION OF THE CATALYTIC PROPERTIES OF THE LYTIC PEPTIDASE IN THE STAPHYLOLYTIC PREPARATION LYSOSTAPHIN

CHAPTER I

INTRODUCTION

Lysostaphin is a protein preparation obtained from the culture filtrate of an organism in the genus Staphylococcus. This preparation has been found to be a powerful lytic agent for the cells and cell walls of all strains of Staphylococcus aureus tested, but not those of a large number of other microorganisms (1,2,3).

Commercially prepared lysostaphin contains three active components; a lytic peptidase, a hexosaminidase, and an acetylmuramic acid-L-alanine amidase (4,5). The lytic peptidase and hexosaminidase have been reported to comprise approximately 65% and 5% of lysostaphin by weight, respectively, the remaining 30% containing the amidase (6). These three components can be separated on a Sephadex G-50 column or by isoelectric focusing (5).

The lytic peptidase in lysostaphin is a zinc-metalloenzyme of molecular weight 25,000 (7). The molecule

consists of a single polypeptide chain and is unique in that its optical rotatory dispersion spectrum reveals no evidence of helical structure as normally observed in globular proteins, and in that no half-cystine is found to be present (7).

The lytic peptidase, as the name implies, is the component in lysostaphin which is capable of lysing S. aureus cells. The enzyme has been shown to cleave glycyl-glycine bonds in the polyglycine bridges which form cross-links between glycopeptide chains in the cell wall of S. aureus (4) (see Fig. 1). These bridges appear to be a genus specific characteristic of Staphylococcus, and presumably explain the limited range of bacteriolytic action of the enzyme (8,9). The lytic peptidase has also been reported capable of hydrolyzing artificial substrates such as pentaglycine (4).

Schindler and Schuhardt (1) described a simple and rapid assay for lytic peptidase activity based upon a decrease in turbidity of a standardized suspension of susceptible cells. Since the rate of lysis of a staphylococcal strain is a function of the concentration of the enzyme present, an unknown concentration of the enzyme can be determined by the use of a standard curve.

The hexosaminidase portion of lysostaphin alone has been found to be devoid of lytic activity for S. aureus (4). In contrast to other glycosidases of this type, such as lysozyme, which split the muramic acid-glucosamine bond of the

Figure 1

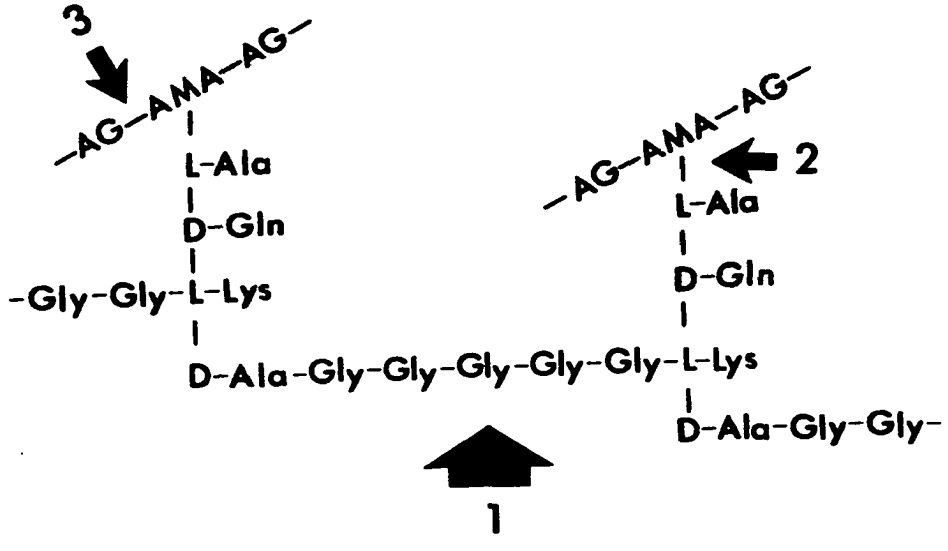
Schematic representation of cell-wall peptidoglycan of S. aureus and the sites of action of the 3 active components in lysostaphin (5,9,10).

1) Site of action of the lytic peptidase

2) Site of action of the amidase

3. Site of action of the hexosaminidase

(AG represents acetyl-glucosamine, AMA represents acetyl-muramic acid.)


3
-AG-AMA-AG-
L-Ala
D-Gln
-Gly-Gly-L-Lys
D-Ala-Gly-Gly-Gly-Gly-Gly-L-Lys
D-Ala-Gly-Gly-
-AG-AMA-AG-
2
L-Ala
D-Gln
1

glycopeptide (9), the hexosaminidase in lysostaphin cleaves the glucosaminyl-muramic acid bond of solubilized staphylococcal cell wall peptidoglycan (4). (See Fig. 1)

The acetylmuramic acid-L-alanine amidase in lysostaphin, which cleaves the bond between the D-lactyl moiety of N-acetylmuramic acid and the N-terminal amino acid (L-alanine) of the peptide attached to it (see Fig. 1), has also been shown to be incapable of lysing whole cells of S. aureus (5).

Many other bacteriolytic enzymes have been isolated by various investigators, and these enzymes have been invaluable as tools for studying bacterial cell wall chemistry. This area has been the subject of several excellent reviews including those by Strominger and Ghuysen (9) and Ghuysen (10).

Important information on bacterial cell walls has also been obtained by the use of inhibitors of cell wall synthesis, such as penicillin. To understand the mechanism of action of penicillin it is first necessary to briefly examine bacterial cell wall synthesis. The synthesis of S. aureus cell walls has been elucidated primarily by Strominger and associates (11,12). They have found three stages in cell wall synthesis:

1) the synthesis of cell wall precursors
2) formation of linear peptidoglycan strands
3) the cross-linking of these linear strands into a two or three dimensional network, the cell wall

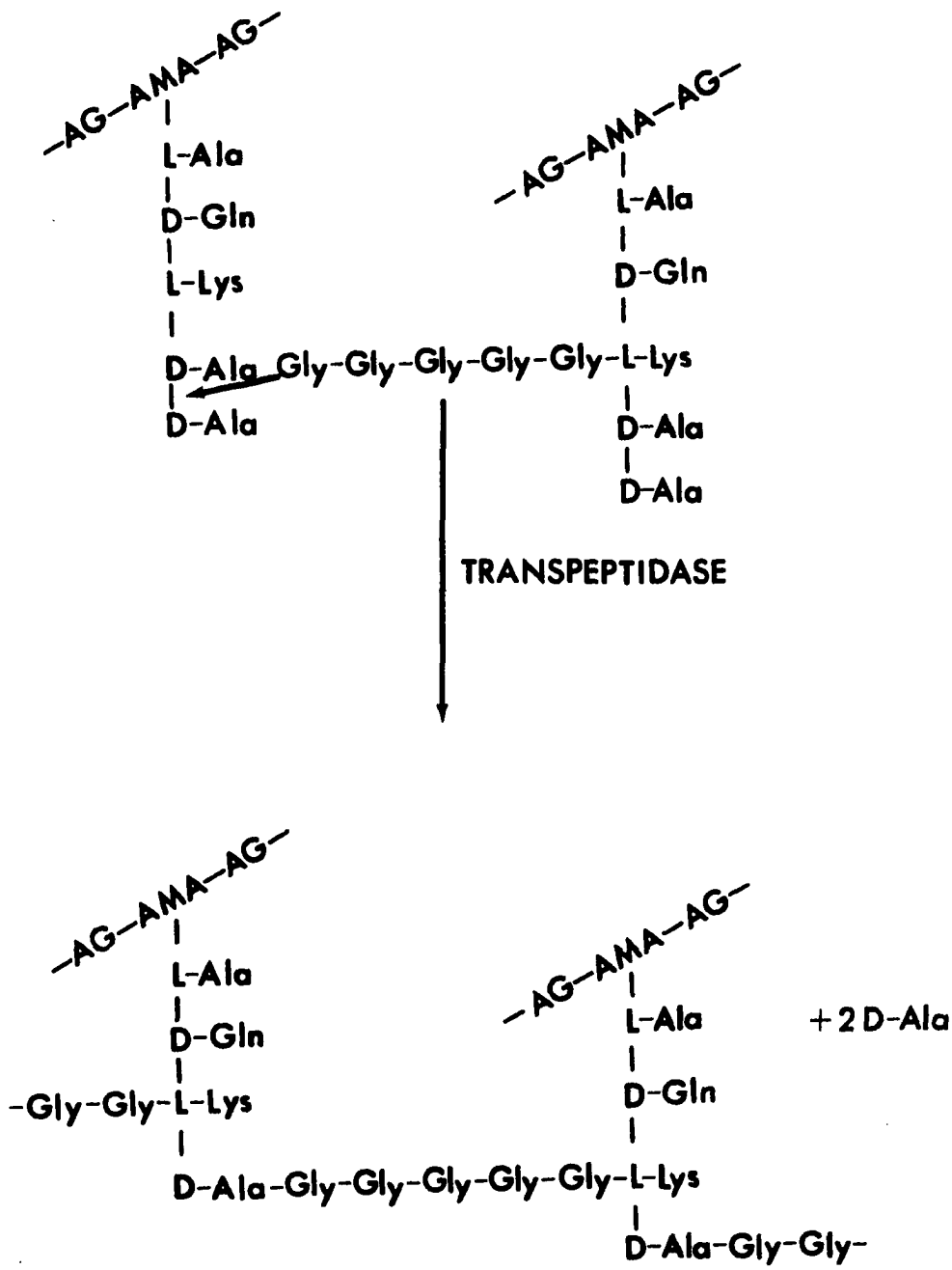
In steps 1 and 2 an acetylglucosamine-acetylmuramic acid-pentapeptide-pentaglycine subunit is made and transported outside the cell where it is accepted by the growing cell wall glycopeptide. The pentapeptide on the acetylmuramic acid has the sequence L-ala-D-gln-L-lys D-ala-D-ala. In the third step of cell wall synthesis a cross-link is formed between adjacent glycopeptide strands by an enzyme mediated transpeptidation reaction. This reaction eliminates D-alanine from the pentapeptide and conserves the energy of the D-alanyl-D-alanine bond broken, via an acyl-enzyme intermediate, to close the cross-bridge by reaction with the free amino end of the pentaglycine chain (see Fig. 2).

The transpeptidation reaction is the step which is inhibited by penicillin, a D-alanyl-D-alanine analog (11,12). When the transpeptidase reacts with penicillin the β-lactam ring of penicillin is broken, forming a penicilloyl-enzyme, thus inactivating the transpeptidase.

The purpose of this investigation was to gain more information on the mechanism of action of the lytic peptidase in lysostaphin. This was to be accomplished by first investigating the possibility of synergism between the active fractions of lysostaphin, next by investigating the action of the lytic peptidase on artificial substrates, and finally by investigating the chemical make-up of the active site itself. Many techniques have been developed to investigate enzyme active sites. These techniques include reagents and

Figure 2

Transpeptidation which forms cross links between adjacent glycopeptide strands in the cell wall of Staphylococcus aureus (11,12).


-AG-AMA-AG-
L-Ala
D-Gln
L-Lys
D-Ala Gly-Gly-Gly-Gly-Gly-L-Lys
D-Ala
-AG-AMA-AG-
L-Ala
D-Gln
D-Ala
D-Ala
TRANSPEPTIDASE
-AG-AMA-AG-
L-Ala
D-Gln
-Gly-Gly-L-Lys
-AG-AMA-AG-
L-Ala
D-Gln
+2 D-Ala
D-Ala-Gly-Gly-Gly-Gly-Gly-L-Lys
D-Ala-Gly-Gly-

procedures which specifically modify amino acid residues and other groups which may be in the active site, and labeling techniques which specifically label a residue in the active site in such a manner that this residue and the surrounding amino acids may be isolated and identified. These techniques have been extensively reviewed (13,14,15).

The best method to study the active site of an enzyme (other than X-ray diffraction studies of enzyme-substrate complexes) is to attach a covalent label to the active site which will be stable to hydrolysis, so that the residue the label has reacted with can be identified. The best label which can be used is a substrate for the enzyme. For example, reaction with radioactive substrate and then isolation of the stable enzyme-substrate intermediate has been used to isolate peptide fragments from the active site of phosphoglucomutase and phosphoglyceromutase (16,17).

In some cases unstable intermediates can be "trapped" in a more stable form. For example, enzymes which form a Schiff-base between the ϵ-NH_2 group of a lysine residue and a carbonyl group of their substrate can be reduced with $NaBH_4$ to secondary amines which are stable (18). This procedure has been utilized to study the active sites of aldolase and transaldolase (19,20). These techniques have the advantage that when a substrate is used to label the active site of an enzyme no ambiguity then exists as to whether or not the label has attached only to the active site.

Another means of studying active sites is through the use of reagents to modify amino acid residues necessary for catalysis or binding. For example, serine residues in the active sites of many enzymes are particularly reactive toward the compound diisopropylfluorophosphate (DFP), a technique introduced by Jansen et al. (21). Radioactively labeled DFP has been used to covalently label unique serine residues in the active sites of many enzymes. Phenylmethanesulfonyl fluoride (PMSF) is another reagent which has been shown to react with a unique serine residue in the active site of chymotrypsin (22).

Photooxidation of amino acid residues in proteins by dyes (usually methylene blue or rose bengal) in the presence of light was introduced by Weil et al. (23). Histidine, tyrosine, tryptophan, methionine, and cystine are the only common amino acids found to be sensitive to this treatment (24). The specificity of methylene blue photooxidation has been found to be partially determined by pH. For example, oxidation of histidine has been found to be the fastest reaction at neutral pH, while tyrosine is the most reactive at high pH and tryptophan and methionine are the only amino acids readily oxidized below pH 4 (24). The anionic dye rose bengal may be more selective for histidine than the cationic dye methylene blue (25).

Another amino acid residue which has been shown capable of reacting specifically with a modifying reagent is the

indole side chain of tryptophan in α-chymotrypsin which has been shown to react specifically with 2-hydroxy-5-nitrobenzyl bromide (HNB-Br) in neutral and acidic solutions (26).

Acetic anhydride has been used to acetylate amino groups relatively specifically (27). N-acetylimidazole has been shown to react with both amino and tyrosyl groups of proteins, but to be more selective for tyrosine under mild reaction conditions (28).

1-Dimethylaminonapthalene-5-sulfonyl chloride (dansyl chloride) has been shown capable of reacting with amino, sulfhydryl, imidazole, and phenolic hydroxyl groups and the serine hydroxyl groups of some serine proteases (22,29). The fluorescence of dansyl derivatives enables their detection in very small amounts. Advantage was taken of this property in the isolation and identification of a lysine residue involved in the active site of rennin by Hill and Laing (30).

The ability of metal-complexing reagents to inhibit the activity of metalloenzymes has been taken as evidence for the involvement of the metal ion in the active site of these enzymes (31).

Another method of active-site labeling with reagents which are not structurally related to the normal enzyme substrate is the technique called differential labeling (14). This technique is based on the protection of the groups in the active site from reaction with a nonspecific reagent by the presence of substrate or competitive inhibitor. For example,

an enzyme may be reacted with a reagent in the presence of substrate in such a manner that all susceptible groups outside the active site are modified. The substrate is then removed and a radioactively labeled form of the reagent is then reacted with the residues in the active site which were formerly protected. The protein may then be degraded to peptide fragments and the peptide(s) with radioactive label isolated and studied.

CHAPTER II

MATERIALS AND METHODS

Enzymes

Lysostaphin (lot no. X3419, 207 units/mg) was purchased from Schwarz/Mann Biochemicals, Orangeburg, New York.

Partially purified enzymes from lysostaphin were the gift of Dr. C. A. Schindler. (These fractions were prepared by Dr. P. A. Tavormina, Mead Johnson and Company by Sephadex gel filtration.)

Enzyme assays

The test organism was Staphylococcus aureus, sv strain (32), grown in Difco Tryptic soy broth for 18 hours at 37 C in a New Brunswick gyrotary incubator. The cells were harvested by centrifugation at 5,000xg for 15 minutes, washed twice with 0.05 M tris (hydroxymethyl) aminomethane buffer (pH 7.5) containing 0.15 M NaCl (Tris-saline buffer), and resuspended in the same buffer to a standard cell concentration. The assays consisted of a 10 minute interaction of lysostaphin and indicator cells at 37 C in a stationary water bath. The turbidity of the samples was read at 0 and 10 minutes on a Bausch and Lomb Spectronic 20 spectrophotometer at a

wavelength of 540 nm. Specific assay conditions and treatment of the enzyme will be indicated for each experiment.

Molecular weight studies

The method of electrophoresis in SDS-polyacrylamide gels, first introduced by Shapiro et al. (33) and extended by Weber and Osborn (34) was used to determine the molecular weights, and relative percentages of the different protein fractions present in lysostaphin preparations.

In a typical experiment 50 µg of each protein standard of known molecular weight, 50 µg of lysostaphin, and 50 µg of each of the three active fractions from lysostaphin were dissolved in 0.1 ml of a 0.01 M sodium phosphate buffer (pH 7.0) that was 1% (w/v) in SDS and 0.1% (v/v) in β-mercaptoethanol. Prior to electrophoresis these samples were placed in a boiling water bath for two minutes to insure denaturation. The gel buffer system of Weber and Osborn (34) was used to make gels that were 10% (w/v) with respect to acrylamide and 0.2% (w/v) with respect N,N' dimethylbisacrylamide. The gels were made 8 cm long in tubes 10 cm long and 0.6 cm in inside diameter. To each gel was added 100 µl of the following mixture:

5 µl 0.05% (w/v) Bromphenol blue

5 µl β-mercaptoethanol

50 µl 0.01 M phosphate buffer, 1% in SDS and 0.1% in β-mercaptoethanol

50 µl protein solution

1 drop glycerine

The gels were run at 8 ma/tube for approximately 4 hours using a Heath-kit IP-17 constant voltage power source. After electrophoresing the gels were removed from the tubes and the length of the gel and the migration distance of the tracking dye (Bromphenol blue) was measured using a vernier calipers. The gels were then fixed in 20% (w/v) sulfosalicylic acid for 16 hours at 37 C, stained with Coomassie Brilliant Blue (0.25% w/v) for 4 hours at 37 C and destained with several changes of 7% (v/v) acetic acid until clear.

The length of the gels and positions of the proteins were then measured.

The mobility (M) of the proteins was calculated according to the method of Weber and Osborn (34) as

$$M = \frac{\text{distance of protein migration}}{\text{length after destaining}} \times \frac{\text{length before staining}}{\text{distance of dye migration}}$$

Proteins used as known molecular weight standards were chymotrypsinogen, trypsin, pepsin, myoglobin and lysozyme. The mobilities of the known standards were plotted against their molecular weights on semi-logarithmic paper giving a standard curve on which the molecular weights of the unknowns could be determined.

The gels were scanned in a Gilford model 240 spectrophotometer equipped with a linear transport mechanism at 562 nm. The areas under the gel scans were calculated manually and these results were used to calculate the fraction of lysostaphin comprised by each component. This technique is

based on the findings of Smith, et al. (35), that the amount of Coomassie Blue bound in gel electrophoresis is proportional to the amount of protein present.

Synergism of lysostaphin components

To determine if the different components in lysostaphin have a synergistic effect on each other an experiment was performed in which all possible combinations of these fractions were combined in a lytic assay.

The following concentrations of the three active fractions in lysostaphin were prepared:

A) 1.0 µg/ml lytic peptidase

B) 42 µg/ml hexosaminidase

C) 48 µg/ml amidase

Three milliliters of indicator cells plus all possible single, double, and triple combinations of these three fractions were combined in a total of 6 ml. One ml of each test component was used each time a component was tested. Readings in the assay were taken at 10 minute intervals for one hour.

Pentaglycine assay

To begin the investigation of the mechanism of action of the lytic peptidase, advantage was taken of the report of Browder et al. (4) that the enzyme would hydrolyze pentaglycine and other synthetic glycyl peptides. This meant a much simpler system was available for studying the mechanism of action of the enzyme, a system in which there would be no

cellular debris to hinder the identification of products.

To demonstrate the hydrolytic activity the lytic peptidase has on pentaglycine an assay procedure was devised in which the enzyme converts the insoluble pentaglycine to more soluble hydrolysis products. In the assay procedure a 1 mg/ml suspension of pentaglycine (International Chemical and Nuclear Corporation) in Tris-saline buffer (pH 7.5) was prepared and then adjusted to give an absorbance of .190 OD units on a Gilford model 240 spectrophotometer. Two and three-tenths ml of this suspension was then added to each of two identical cuvettes. To one cuvette was added 0.2 ml of a 2.5 mg/ml enzyme solution while to the other cuvette was added 0.2 ml of Tris saline buffer. The Gilford spectrophotometer was then used to measure the absorbance change at 540 nm of the two suspensions with readings taken at zero time and at 5 minute intervals until no further changes in absorbance were noticed.

Identification of the products formed by action of lysostaphin on several glycyl peptides

Identification of the hydrolysis products from pentaglycine and other glycyl peptides treated with the lytic peptidase required a chromatography system which could separate the glycyl peptides and any products formed from them by enzymatic action. Initially paper chromatography and ninhydrin detection of the peptides and products was utilized. Whatman 3MM paper and a solvent system of 70:30 isopropanol-water was

the best of many systems tested, but was not sensitive enough to give totally unambiguous results.

A more sensitive method was developed in which the artificial substrates were first reacted with lysostaphin and then with 1-fluoro, 2,4-dinitrobenzene (FDNB), thus producing the yellow dinitrophenyl-derivatives of the substrates and products (36). These DNP-derivatives were then chromatographed on Whatman 3MM paper (previously washed with phthalate buffer) using tert-amyl alcohol saturated with 0.1 M phthalate buffer (pH 6.0) (37) as the solvent system. The hydrolysis products were identified by comparison to chromatographed DNP-standards. This system proved to be more sensitive but still left some doubt as to the ability of some of the glycyl peptides to serve as substrates for the lytic peptidase and as to the products formed from enzymatic hydrolysis.

The system eventually developed for identification of the products of enzymatic activity utilized 1-dimethylaminonaphthalene-5-sulfonyl chloride (dansyl chloride), rather than FDNB, to label the peptides or their hydrolysis products. Dansyl-chloride was first used by Gray and Hartley (38) to label the amino groups of amino acids and peptides. The dansyl-derivatives formed give a strong yellow fluorescence when viewed under UV light. This method has been shown to be 100-fold more sensitive than the FDNB procedure (39).

The glycyl peptides used were: glycine, diglycine and triglycine (Sigma Chemical Company); tetraglycine and hexaglycine (Schwarz/Mann Chemical Company); pentaglycine (International Chemical and Nuclear Corporation); polyglycine (Miles Laboratories, Inc.).

In a typical experiment 25 μmoles of each peptide was dissolved in 1.0 ml of Tris-saline buffer (pH 7.5). These solutions were then split into two groups by transferring 0.5 ml of each solution into a separate test tube. To one tube of each pair was added 0.2 ml of a 0.5 mg/ml lysostaphin solution in Tris-saline buffer. To the other tubes was added 0.2 ml of buffer. An enzyme control containing only enzyme was also run. The tubes were covered with parafilm and allowed to stand at room temperature for 12-16 hours. The contents of the tubes were then dried *in vacuo* using a Buchler Instruments Company Evapo-Mix. The controls and products were then converted to the dansyl derivative using essentially the same procedure as that suggested by Hartley and Massey (38). In this procedure the contents of the tubes were resuspended on 0.5 ml of 0.2 M $NaHCO_3$ prepared with distilled deionized water. An equal volume of a solution of dansyl chloride (Sigma Chemical Company) in acetone (2.5 mg/ml) was added and the tubes shaken briefly. The tubes were then covered with parafilm and allowed to stand for 2 hours at room temperature. After reaction the contents of the tubes were again dried *in vacuo* and then resuspended in 1.0 ml of Tris-saline buffer.

The dansyl derivatives of the standards and of the hydrolysis products were chromatographed on Gelman ITLC type S thin layer chromatography media using tert-amyl alcohol saturated with 0.1 M phthalate buffer (pH 6.0) as the solvent. Equal amounts of the standards and experimental solutions were added to the chromatograms using 10 µl disposable pipettes (Microcaps, Drummond Scientific Company). After chromatography the chromatograms were viewed using a Mineralight (Ultra-Violet Products Inc.) equipped with a long-wave filter. The movement of the dansyl derivatives and the solvent front migration were measured with a vernier calipers and the R_f values calculated for each standard and product.

Action of lysostaphin on dansylated glycyl peptides

In this procedure 0.5 ml of Tris-saline solutions of the dansylated glycyl peptide standards were reacted with 0.2 ml of a 0.5 mg/ml lysostaphin solution. After a 12 hour reaction period chromatography was run on 10 µliter aliquots from each mixture utilizing the standard chromatography system. The products were then identified by their R_f values.

Action of lysostaphin on other artificial substrates

The ability of the lytic peptidase to catalyze transpeptidation reactions suggested the enzyme might possibly at one time have been involved in cell wall synthesis in the

organism which produces it. This hypothesis requires that the enzyme be capable of acting on D-alanyl-D-alanine. To test this hypothesis and also to investigate the range of activity the enzyme has for other peptides the following peptides were tested for their ability to act as substrates for lysostaphin:

Di-D-alanine and poly-D-alanine (Cyclo Chemical Company)

Di-, tri-, tetra-, and penta-L-alanine, D-alanylglycine, L-alanyl glycine, glycyl-L-alanine, L-alanylglycylglycine and glycylglycyl-L-alanine (Sigma Chemical Company)

The procedure for the investigations with the above peptides was as described for the glycyl peptides.

Inhibition of lytic peptidase activity by penicillin, cylcoserine, and artificial substrates

The ability of the lytic peptidase to utilize di-D-alanine as a substrate further substantiated the possibility that the lytic peptidase may have originally been involved in cell wall synthesis. If this was correct it was possible that penicillin might inhibit the lytic peptidase just as it does the transpeptidase involved in cell wall synthesis. To investigate this possibility an enzyme assay was run in which different concentrations of penicillin G (lot # 31C-0110, 1650 units/mg - a gift from Sigma Chemical Company) were used

in an attempt to inhibit the lysis of the test organism by the lytic peptidase. A 4.2 µg/ml suspension of lysostaphin in Tris-saline buffer was prepared and the following protocol was followed:

Tube	
A	3 ml cells + 1 ml enzyme + 1 ml buffer
B	3 ml cells + 1 ml enzyme + 250,000 units penicillin in 1 ml buffer
C	3 ml cells + 1 ml enzyme + 100,000 units penicillin in 1 ml buffer
D	3 ml cells + 1 ml enzyme + 50,000 units penicillin in 1 ml buffer
E	3 ml cells + 1 ml enzyme + 25,000 units penicillin in 1 ml buffer

The percent transmittance of the tubes was read at zero time and at 5 minute intervals for 30 minutes.

In order to identify the type of inhibiting effect that penicillin has on the lytic peptidase an experiment was devised which would give data appropriate for a Lineweaver-Burk plot (40). Several dilutions of a known cell concentration (determined by direct count with a Petroff-Hauser counting chamber) were prepared from a standard cell suspension. Three cuvettes for each dilution were run; one containing 4.0 ml of the cells + 1.0 ml lysostaphin (4 µg/ml), another containing 4.0 ml cells + 1.0 ml lysostaphin + 100,000 units penicillin, and the third containing 4.0 ml cells + 1.0 ml

lysostaphin + 20,000 units penicillin. Readings were made at zero and 10 minutes. From these results the reciprocal of the velocity ($\frac{1}{\text{decrease in \% T}}$) was plotted against the reciprocal of the substrate concentration ($\frac{1}{\text{\# cells/ml}}$).

D-cycloserine is another antibiotic that inhibits cell wall synthesis (11). This compound inhibits the formation of di-D-alanine by inhibiting two enzymes; alanine racemase, which converts L-alanine to D-alanine, and D-alanyl-D-alanine synthetase, which synthesizes the dipeptide from D-alanine (11). To determine if this antibiotic has any inhibitory effect on lytic peptidase activity an enzyme assay was performed including D-cycloserine in 10^{-1}, 10^{-2}, and 10^{-3} M concentration in tubes containing 4.0 ml indicator organism. To each tube 1.0 ml of an 8 µg/ml lysostaphin solution was added and readings taken at zero time and at 5 minute intervals for 20 minutes.

To compare the inhibition of lytic peptidase by penicillin to the inhibition of the enzyme by other substrate analogs the following compounds were investigated as reversible inhibitors:

1) glycine
2) diglycine
3) triglycine
4) di-L-alanine
5) di-D-alanine

These compounds were used in a concentration equivalent to 50,000 units of penicillin/ml (8.5×10^{-2} M). In the

experiment 1.0 ml of a 4.4 µg/ml enzyme solution was added to cuvettes containing 4.0 ml of indicator cells plus one of the inhibitors in the previously mentioned concentration. Readings were taken at zero time and at 5 minute intervals for 30 minutes.

Mechanism of transpeptidation

Transpeptidation reactions are reactions in which a peptide bond is broken and then reformed by the transfer of a portion of the peptide to another peptide. There are two possible mechanisms for this type of reaction: (1) acyl transfer (carboxyl transfer), in which the enzyme intermediate involves the carbonyl group of the broken peptide bond and: (2) imino transfer (amine transfer) in which the enzyme intermediate involves the amino group of the broken peptide bond (41).

To identify the mechanism of transpeptidation by which the lytic peptidase acts an experiment was performed to see if dansyl diglycine would act as an acceptor in the reaction, even though it is not susceptible to hydrolysis by the enzyme. In the procedure 0.2 ml of a 25 µmole/ml dansyl diglycine solution in Tris-saline buffer was added to each of two tubes containing 0.2 ml of buffer plus 5 µmoles of either triglycine or tetraglycine. Then 0.1 mg of lysostaphin was added to the tube containing dansyl diglycine plus triglycine, and also the tube containing dansyl diglycine plus tetraglycine. A 10 µl aliquot was spotted on a chromagram from each tube at zero

time, one-half hour, and thereafter at hourly intervals for 8 hours. The chromatograms were then developed using the standard solvent system. The presence of any fluorescent transpeptidation products could only be explained by an amine type transpeptidation reaction.

Ability of glycine to serve as an acceptor in transpeptidation reactions

In one experiment the following procedure was followed: 1.0 μmoles of diglycine and 1.0 μmoles of 1-^{14}C-glycine (Calatomic, Inc., 33mCi/mmole) were dissolved in 1.0 ml of Tris-saline buffer. To this mixture 0.2 ml of a 0.5 mg/ml lysostaphin solution was added and the mixture incubated for 8 hours. After incubation the contents of the tube were dried in vacuo and reacted with dansyl chloride as before. The reaction mixture was dried again in vacuo and resuspended in 1.0 ml Tris-buffer. Ten μl of this mixture was then chromatographed on ChromAR 1000 (Mallinckrodt Laboratory Products) thin-layer chromatography media. After chromatography using tert-amyl alcohol saturated with 0.1 M phthalate buffer as the solvent the chromatogram was traced and then cut into 14-1 cm strips. These strips were put into individual scintillation vials and eluted with 1.0 ml of water. Ten ml of scintillant (2,5 diphenyloxalole, naphthalene, and 1,4 dioxane) was added to each vial and the samples were counted in a DPM-100 Beckman liquid scintillation counter in order to determine if the diglycine was now radioactively labeled.

In another experiment dansyl-glycine (0.2 ml of a 25 μmole/ml solution) was added to each of two small tubes containing 25 μmole of diglycine and triglycine respectively. Two-tenths of a ml of a .5 mg/ml lysostaphin solution was then added to each tube and the mixtures allowed to react at room temperature for 8 hours. Controls of dansyl glycine plus diglycine and triglycine and dansyl glycine plus enzyme were also run. After the 8 hour reaction time 10 μl amounts from each tube were chromatographed using the standard chromatography system in order to detect any incorporation of the fluorescent dansylglycine into transpeptidation products.

Penicillinase activity of the lytic peptidase

The possibility that the lytic peptidase may have the ability to hydrolyze penicillin (i.e. have penicillinase activity) was investigated by reacting the enzyme and a known amount of penicillin together and determining the loss in penicillin activity over a period of time. A variation of the penicillin assay of Foster and Wilker (42) was used to assay for penicillin activity through the use of a standard curve. In the assay procedure Penicillin G (Sigma Chemical Company) was diluted with Tris-saline buffer to give concentrations of 1,2,4,6,8, and 10 units/ml. Two hundred ml of sterile Difco nutrient broth was then inoculated with 2.0 ml of an overnight Bacillus cereus culture in nutrient broth and 9.0 ml of this inoculated broth was distributed into sterile cotton

plugged 16 x 180 mm pyrex culture tubes. One ml of each of the penicillin dilutions was then added to the inoculated broth tubes, the contents mixed vigorously, and incubated for 4.5 hours at 37 C in a New Brunswick gyrotary incubator. After incubation the percent transmittance at 540 nm was read directly from the tubes on a Bausch and Lomb Spectronic 20 spectrophotometer.

In the penicillin assay to determine if lysostaphin could act as a penicillinase the following reaction mixtures were prepared:

Tube	Contents
A	8.0 ml buffer + 1.6 mg lysostaphin + 0.8 ml penicillin (.24 mg/ml) = 45 units/ml final penicillin concentration
B	8.0 ml buffer + 0.8 ml penicillin (.24 mg/ml) = 45 units/ml final penicillin concentration
C	8.8 ml buffer + 1.6 mg lysostaphin

The above mixtures were reacted together at room temperature and assayed for penicillin activity at zero time and every hour for 4 hours. In the assay 1.0 ml of each undiluted test suspension was added to an assay tube as was 1.0 ml of 1:5, 1:10, and 1:20 dilutions of the reaction mixtures. Lytic peptidase activity was also assayed at zero time and every hour using the standard assay technique.

Effect of penicillinase on S. aureus

In order to determine if a penicillinase from an other

source has staphylolytic activity 1 mg of penicillinase (Calbiochem, 40,000 units/mg) was added to a suspension of S. aureus in Tris-saline buffer. Readings were taken at zero time and at 10 minute intervals for 30 minutes.

Inhibition of lytic peptidase activity by metal chelation

1,10-Phenanthroline and 8-hydroxyquinoline sulfonate are both strong chelating agents. The inhibition of enzymes by such metal complexing agents has served as a classical means of establishing the essentiality of a metal in the catalytic process of an enzyme (31). To determine if the zinc atom present in the lytic peptidase of lysostaphin was important in either substrate binding or catalysis (or both) the above two complexing agents were tested for their ability to inhibit the enzyme.

1,10-Phenanthroline and 8-hydroxyquinoline sulfonate (Sigma Chemical Company) were reacted at room temperature with 10 ml of 4.1 and 4.8 µg/ml lysostaphin concentration respectively in the following concentration of the chelating agents: 10^{-2}, 9×10^{-3}, 5×10^{-3}, 10^{-3}, 9×10^{-4}, 5×10^{-4}, and 10^{-4}M. A cell control of buffer plus the chelating agent and an enzyme control of enzyme alone were also run. At zero time, 10 minutes and at 20 minute intervals thereafter for 90 minutes, 1.0 ml was removed from the reaction mixtures, added to 3.0 ml of indicator cells, and assayed for lytic activity.

Photooxidation with methylene blue

To determine if the lytic peptidase has one or more histidines involved in its active site a procedure utilizing methylene blue as a photoxidizing agent was utilized. In this procedure 9.9 ml of a 4.2 µg/ml solution of lysostaphin in 0.1 M sodium phosphate buffer (pH 7.0) was dispensed into each of three 30 ml pyrex beakers. To two of the beakers (kept in the dark) 0.1 ml of a 1% (w/v) solution of methylene blue (National Aniline Division of Allied Chemical Corp.) was added. The final concentration of methylene blue was 0.01% (2.7×10^{-4} M). The enzyme control and one beaker with enzyme plus methylene blue were put on a Bell Co. Glass, Inc. 4-place magnetic stirrer under a 200 W, 120 V Kenrad lamp positioned 15 cm above the stirrer. The solutions were stirred at maximum speed to insure adequate oxygenation. The light was turned on and 0.5 ml samples were removed at zero time and at 5 minute intervals for the first 30 minutes. Samples were then removed at 10 minute intervals for an additional hour. Samples were kept in the dark in foil lined and covered test tubes to protect them from further photoxidation. The third beaker, containing enzyme plus methylene blue, was stirred in the dark and samples were taken as for the solutions in the light. A fourth beaker containing 0.01% methylene blue and buffer was also kept in the light and sampled as a cell control. After all samples were collected the enzymatic activity remaining was determined by assay. In the assay 0.1 ml from

each sample was added to 4.0 ml of the test organism in a darkened room.

Photooxidation with rose bengal

Rose bengal has also been used extensively as a photo-activated oxidant. Its anionic nature makes rose bengal even more specific for histidine residues than the cationic methylene blue (25). In the procedure utilizing rose bengal the conditions were similar to those for oxidation by methylene blue except for the dye concentration. It was found that the enzyme was much more sensitive to the rose bengal (National Aniline Division of Allied Chemical Corp.), therefore the dye was used in a 0.001% (w/v) (1×10^{-5}M) concentration. The enzyme solution used was 4.0 µg/ml. In the rose bengal photooxidation an attempt was also made to protect the active site by addition of a substrate. In this experiment a test solution which was 10^{-2}M with respect to triglycine in addition to the enzyme and rose bengal was also subjected to photooxidation.

Effect of N-acetylimidazole on lytic peptidase activity

N-acetylimidazole specifically acylates tyrosine residues under mild conditions (28). In an attempt to determine if tyrosine plays a part in its active site this reagent was reacted with the lytic peptidase utilizing a procedure similar to that of Riordan et al. (28). In the procedure 5.0 ml of a 50 µg/ml lysostaphin solution was

dispensed into 4 test tubes. A 60, 600, and 6000 M excess of N-acetylimidazole was added to three of the tubes, the other tube being used as an enzyme control. A 0.1 ml aliquot was removed and added to 4.0 ml of indicator organism at 10 minutes and thereafter at 20 minute intervals for 90 minutes.

Effect of HNB-Br on lytic peptidase activity

A procedure similar to that described by Koshland et al. (26) utilizing 2-hydroxy-5-nitrobenzyl bromide (HNB-Br) was used to determine what effect the modification of accessible tryptophan residues would have on activity of the lytic peptidase. In the procedure the HNB-Br was dissolved in acetone at 0.2 M concentration and then rapidly mixed with a 50 µg/ml lysostaphin solution in 0.1 M potassium hydrogen phthalate-NaOH buffer (pH 5.0) so that the final concentration of acetone was 5% with a reagent concentration of 0.01 M. Controls of enzyme plus acetone and enzyme alone were also run. After reaction for 10 minutes (HNB-Br has a half-life of one minute) the lytic activity remaining was assayed by adding 0.1 ml of each reaction mixture to 4.0 ml indicator cells and measuring the percent reduction in turbidity at 5 minute intervals.

Inhibition of lytic peptidase activity by PMSF

Phenylmethanesulfonyl fluoride (PMSF) was used to investigate the possibility that the lytic peptidase may have a

serine in its active site. In the experiment 9.0 ml of a 4.0 µg/ml lysostaphin solution in Tris-saline buffer was mixed with 1.0 ml of an appropriate concentration of PMSF in isopropanol to give the desired final concentration of inhibitor and enzyme. The final enzyme concentration was 3.6 µg/ml in a solution that was 10% (v/v) in isopropanol. The PMSF concentrations investigated were 2×10^{-4}, 10^{-3}, and 3×10^{-2} M. After mixing, standard assays were run at zero and 10 minutes, and thereafter at 20 minute intervals for 120 minutes.

Effect of acetic anhydride on lytic peptidase activity

To test the effect of amino group modification on the activity of the lytic peptidase the acetylation method of Fraenkel-Conrat (27) using acetic anhydride was performed. In this procedure 0.5 ml of a 100 µg/ml solution of lysostaphin in Tris buffer (pH 7.5) was added to two 10 ml beakers in an ice bath. An equal volume of a saturated solution of sodium acetate was added to both beakers. To one beaker 2 µl of acetic anhydride was added over the course of 1 hour. The reaction was allowed to proceed for 2 hours at 0 C with continuous stirring of the contents of both beakers. At the end of the reaction 0.1 ml from each beaker was added to 4.0 ml test organism in a lytic assay.

Reaction of lytic peptidase with $NaBH_4$ in the presence of substrate

Enzymes which form a Schiff-base intermediate between the ε- amino group of a lysine and a carbonyl group of the substrate can be irreversibly reduced with $NaBH_4$ in the presence of substrate, causing an inactivation of the enzyme. The procedure of Grazi et al. (20) was utilized to determine if the lytic peptidase forms such an intermediate. In the procedure the following protocol was followed using 152 µg/ml lysostaphin in a 0.1 M phosphate buffer (pH 6.0), 1216 µg/ml pentaglycine in the same buffer, 1 M $NaBH_4$ and 2 M acetic acid:

Tube	Contents
A	1.0 ml enzyme + 1.8 ml buffer
B	1.0 ml enzyme + 1.0 ml pentaglycine + 0.4 ml $NaBH_4$ + 0.4 ml acetic acid
C	1.0 ml enzyme + 1.0 ml buffer + 0.4 ml $NaBH_4$ + 0.4 ml acetic acid
D	1.0 ml enzyme + 1.0 ml pentaglycine + 0.4 ml $NaBH_4$ + 0.4 ml buffer
E	1.0 ml enzyme + 1.0 ml pentaglycine + 0.8 ml buffer
F	2.0 ml buffer + 0.4 ml $NaBH_4$ + 0.4 ml acetic acid

The reactions were carried out over a period of 60 minutes at 4 C. The borohydride and acetic acid were added

in small aliquots several times during the first 30 minutes of reaction. The mixtures were allowed to react an additional 30 minutes and were then assayed for lytic activity by adding 0.1 ml of the above mixtures to 4.0 ml indicator organism. Readings were taken at zero time and at 5 minute intervals for 30 minutes.

Inactivation of lytic peptidase by dansyl chloride

The method of Hill and Laing (30) was used to determine if dansyl chloride would inactivate the lytic peptidase. In the procedure 3.0 ml of a 0.33 mg/ml lysostaphin solution in 0.1 M phosphate buffer (pH 8.2) was reacted at 4 C for 12 hours with 1:1, 2:1, 3:1, 5:1, and 10:1 molar ratios of dansyl chloride (Sigma Chemical Company) diluted in acetone. A control of enzyme plus acetone was also run. After reaction 0.1 ml from each tube was removed and diluted 1:10 with Tris-saline buffer. Then 0.1 ml of these dilutions was added to 4.0 ml of indicator cells and the amount of enzymatic activity remaining was assayed by determining the percent reduction in turbidity of the cells over a 40 minute interval.

The contents of each tube was then dialyzed in the cold against phosphate buffer until no fluorescence was observed in the dialysis buffer. These samples were adjusted to a volume of 3.0 ml and the amount of dansyl bound to the enzyme was determined by UV difference spectroscopy using the extinction coefficient calculated by Hartley and Massey (29) of 3.3×10^6 cm^2/mole at 340 nm.

CHAPTER III

RESULTS

Molecular weight studies

The SDS-polyacrylamide gel electrophoresis showed lysostaphin to contain 5 different protein fractions after denaturation. The gel scans for the three active purified components of lysostaphin and for lysostaphin are shown in Figure 3. The relative amount of each component which is present in lysostaphin is also indicated. Figure 4 is a standard curve for determining molecular weights from the known protein standards. Table 1 lists the mobilities (M) of the different fractions in lysostaphin and Table 2 lists the molecular weights of the lysostaphin components.

Purified lytic peptidase is shown to contain a small amount of protein A. The purified hexosaminidase also has a small amount of protein A and the amidase contains a small amount of protein E.

Figure 3

Optical density profiles of lysostaphin and its components. The gels were scanned at 562 nm. Full scale is equivalent to 2.95 optical density units. Electrophoresis was from left to right. The numbers in parenthesis indicate the percent of lysostaphin comprised by each component.

1 Lytic peptidase

2 Hexosaminidase

3 Amidase

4 Lysostaphin

A Protein A (1.3%)

B Hexosaminidase (2.1%)

C Lytic peptidase (76.0%)

D Amidase (8.1%)

E Protein E (12.4%)

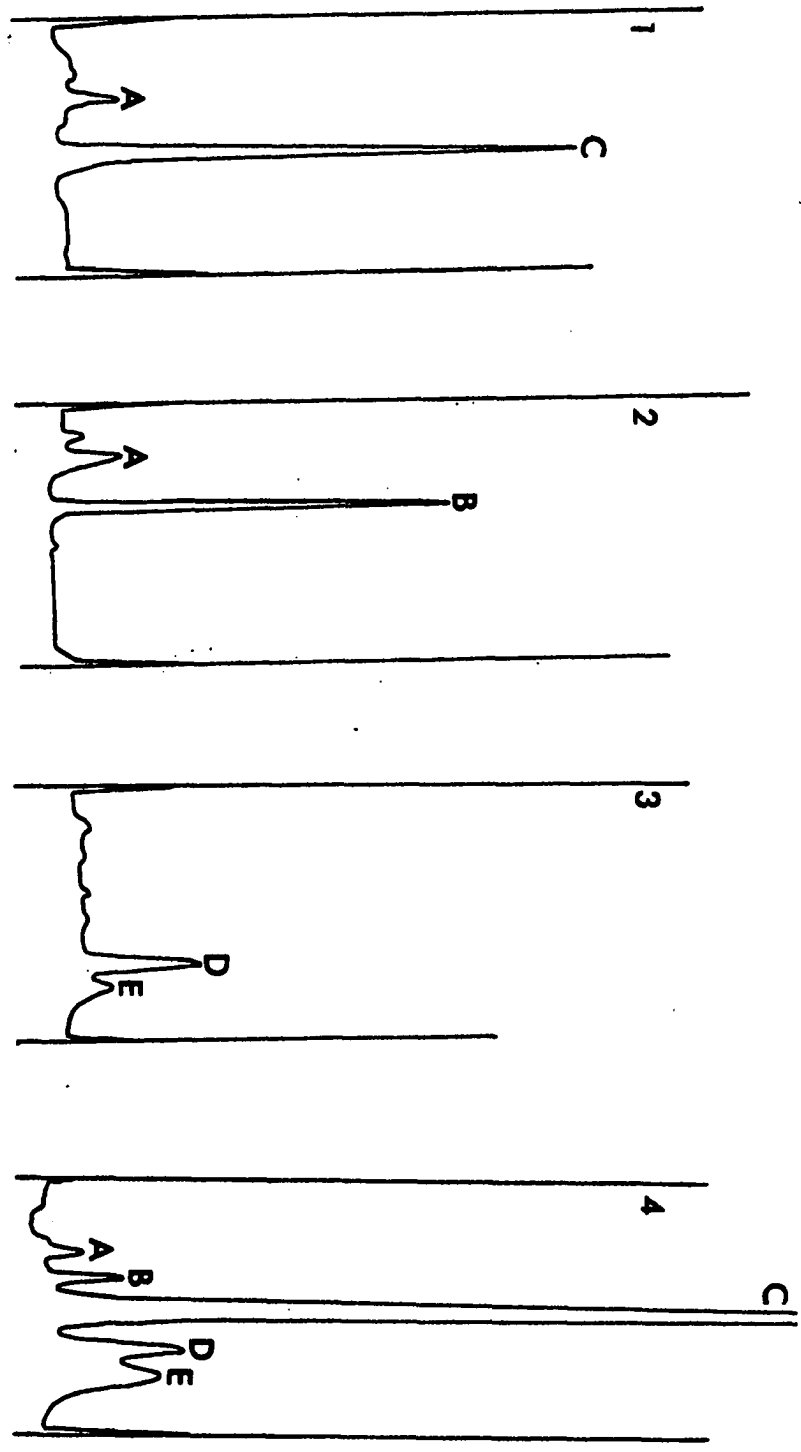

1
A
C
2
A
B
3
D
E
4
A
B
C
D
E

Figure 4

Standard curve for molecular weight determinations

● Protein standards

1 Pepsin

2 Chymotrpsinogen

3 Trypsin

4 Myoglobin

5 Lysozyme

■ Lysostaphin and its components

A Protein A

B Hexosaminidase

C Lytic peptidase

D Amidase

E Protein E

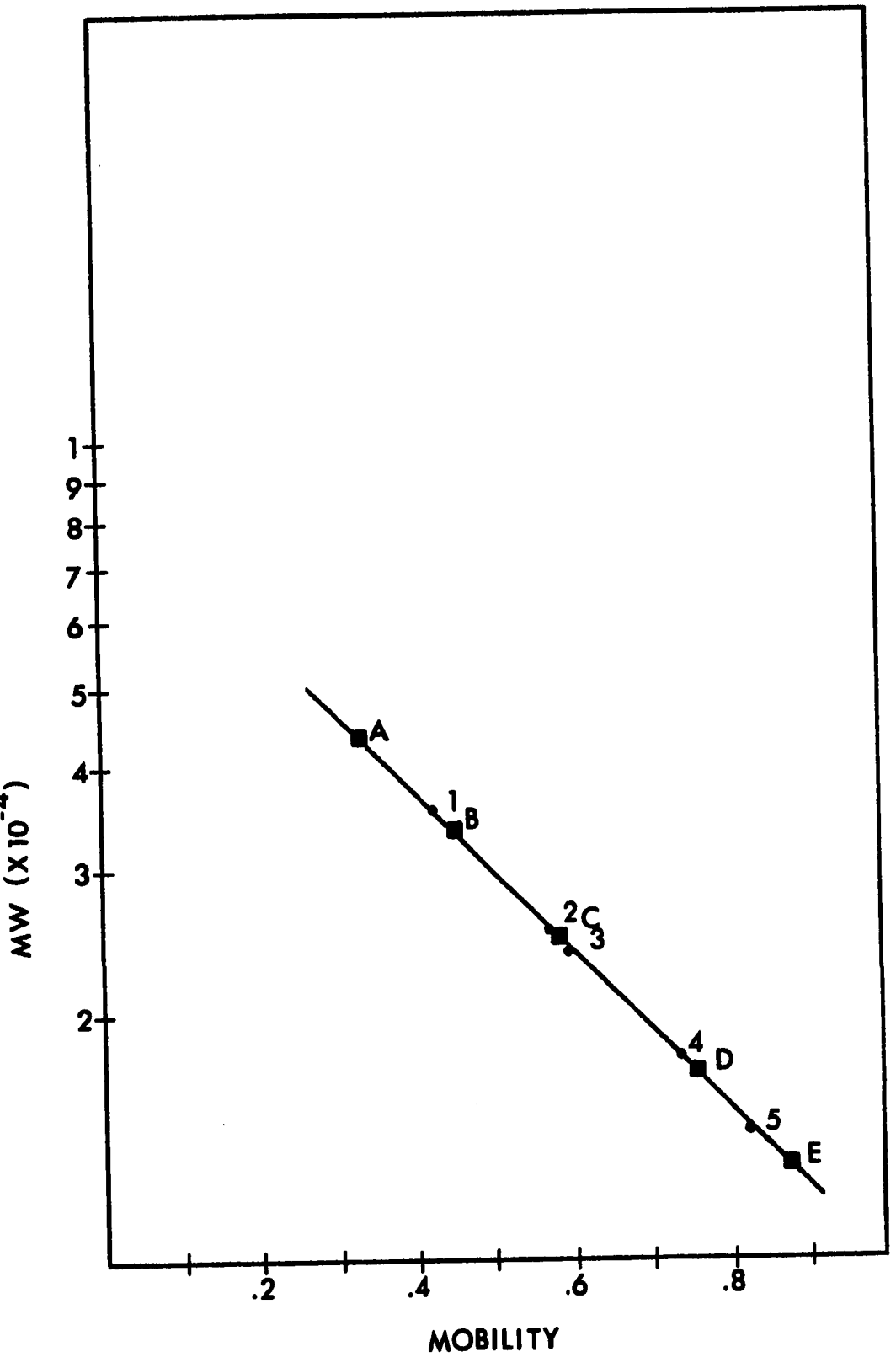

MW (X10^{-4})
1
9
8
7
6
5
4
3
2
A
1
B
2
C
3
4
D
5
E
.2
.4
.6
.8
MOBILITY

TABLE 1.--Mobilities (M) of major and minor components in lysostaphin and lysostaphin fractions on SDS acrylamide gel electrophoresis

Sample Electrophoresed	M (Major Components)	M (Minor Components)
Hexosminidase	.447	.329
Lytic peptidase	.579	.333
Amidase	.755	.872
Lysostaphin	.580	.331
		.448
		.755
		.874

TABLE 2.--Mobilities and molecular weights of protein standards and of lysostaphin components

	Mobility (M)	Molecular Weight
Standards		
Pepsin	.423	35,000
Chymotrypsinogen	.576	25,000
Trypsin	.591	23,300
Myoglobin	.741	17,800
Lysozyme	.823	14,300
Lysostaphin Components		
Protein A	.331	43,000
Hexosaminidase	.447	33,000
Lytic peptidase	.579	24,800
Amidase	.755	17,000
Protein E	.874	12,800

Synergism

Figure 5 shows that the hexosaminidase and the amidase in lysostaphin have no lytic effect by themselves on whole S. aureus cells. There also appears to be no enhancement of lytic peptidase activity by the presence of either of these two enzymes, or by both in conjunction.

Pentaglycine assay

Figure 6 shows the decrease in absorbance of a pentaglycine suspension treated with lysostaphin over a period of 80 minutes. Attempts were made to develop assays similar to the one developed for pentaglycine on other glycyl peptides, but these attempts were unsuccessful due to the different solubility properties of the other potential artificial substrates.

Identification of the products formed by action of lysostaphin on several glycyl peptides

Figure 7 shows tracings of thin-layer chromatograms of the dansyl derivative of several glycyl peptides and the dansyl derivatives of the products formed by reaction of these peptides with lysostaphin. Table 3 gives the R_f values of the dansyl derivatives of the glycyl peptides. Table 4 gives the R_f values and identification of the products formed from each of these peptides after treatment.

These results show that not only are the peptides hydrolyzed by the enzyme, but that there is also a production

Figure 5

Synergism of lysostaphin components. The upper curve shows the range of values obtained for the lytic peptidase (A); alone, in combination with the hexosaminidase (A + B), in combination with the amidase (A + C), and with both of these fractions (A + B + C). The bottom curve shows the range of values of the hexosaminidase (B), the amidase (C), combined hexosaminidase and amidase (B + C) and the cell control.

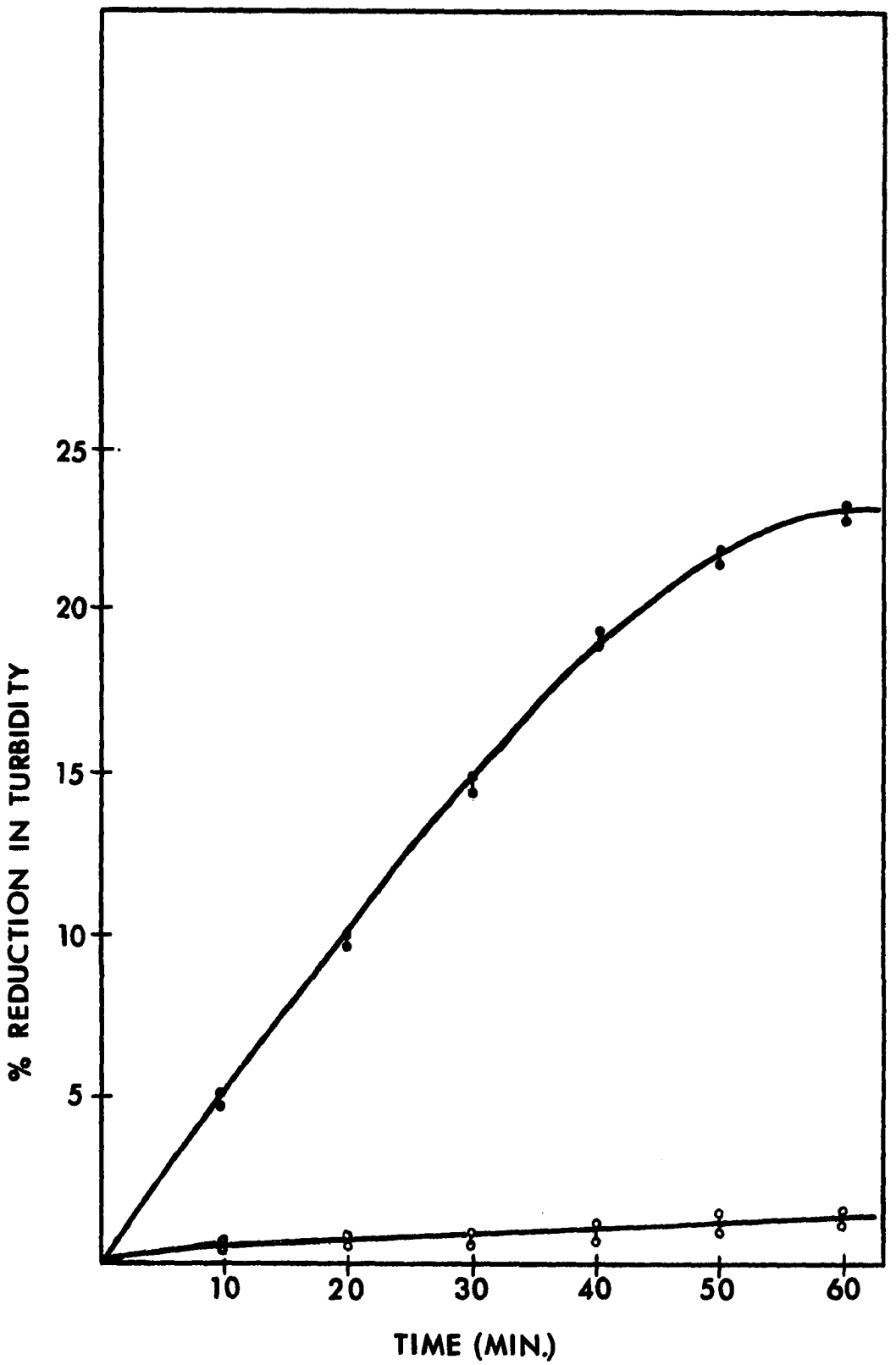

% REDUCTION IN TURBIDITY
25
20
15
10
5
10
20
30
40
50
60
TIME (MIN.)

Figure 6

Hydrolysis of pentaglycine by lysostaphin.

Wavelength 540 nm.

Temperature 24 C.

● Pentaglycine + lysostaphin

○ Pentaglycine without lysostaphin

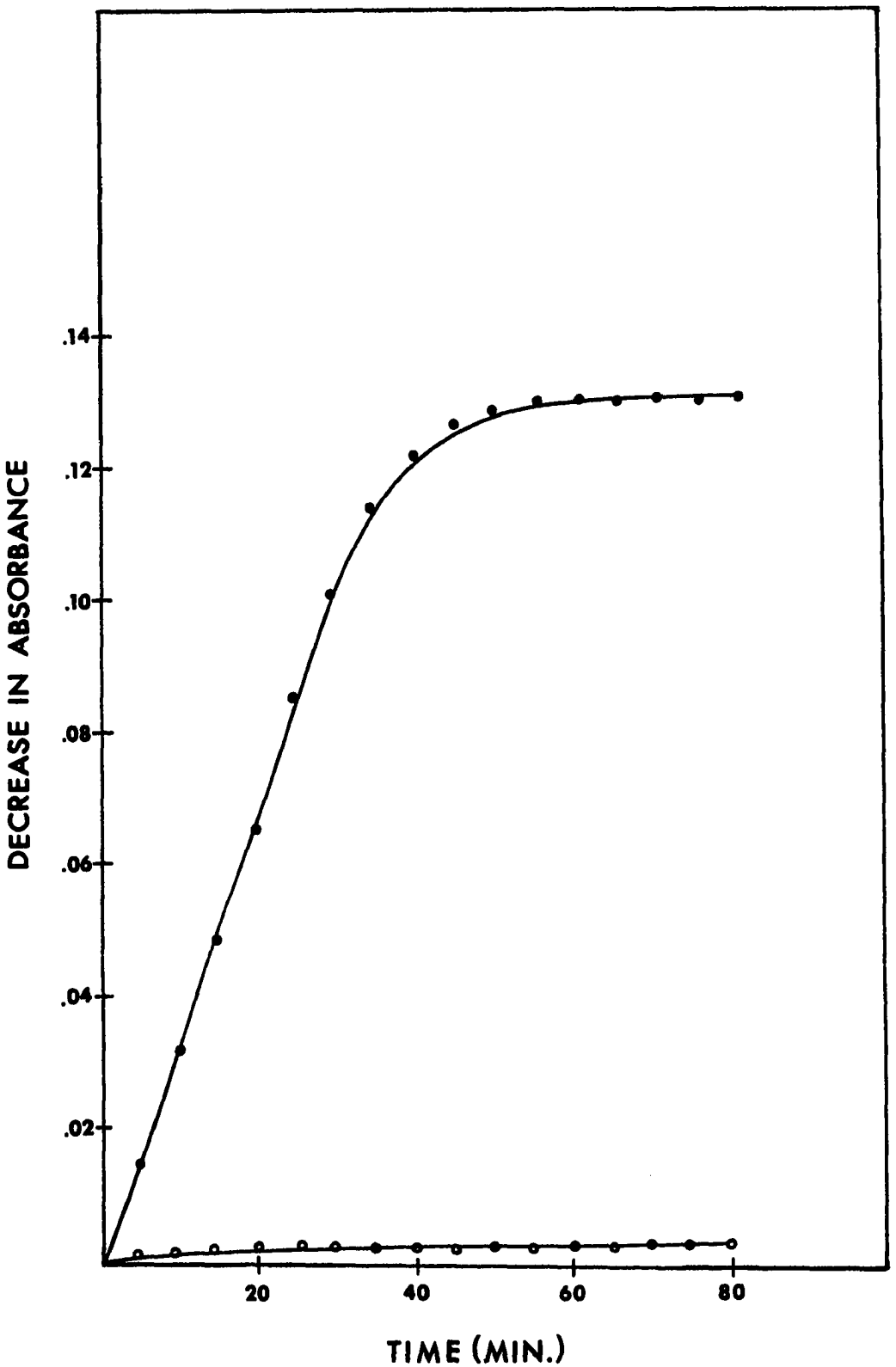

DECREASE IN ABSORBANCE
.14
.12
.10
.08
.06
.04
.02
20
40
60
80
TIME (MIN.)

Figure 7

Tracings of thin-layer chromatograms of dansyl-derivatives of several glycyl peptides and the products formed from these peptides by reaction with lysostaphin. TLC media-Gelman ITLC type S. Solvent-tert-amyl alcohol saturated with 0.1 M phthalate buffer (pH 6.0).

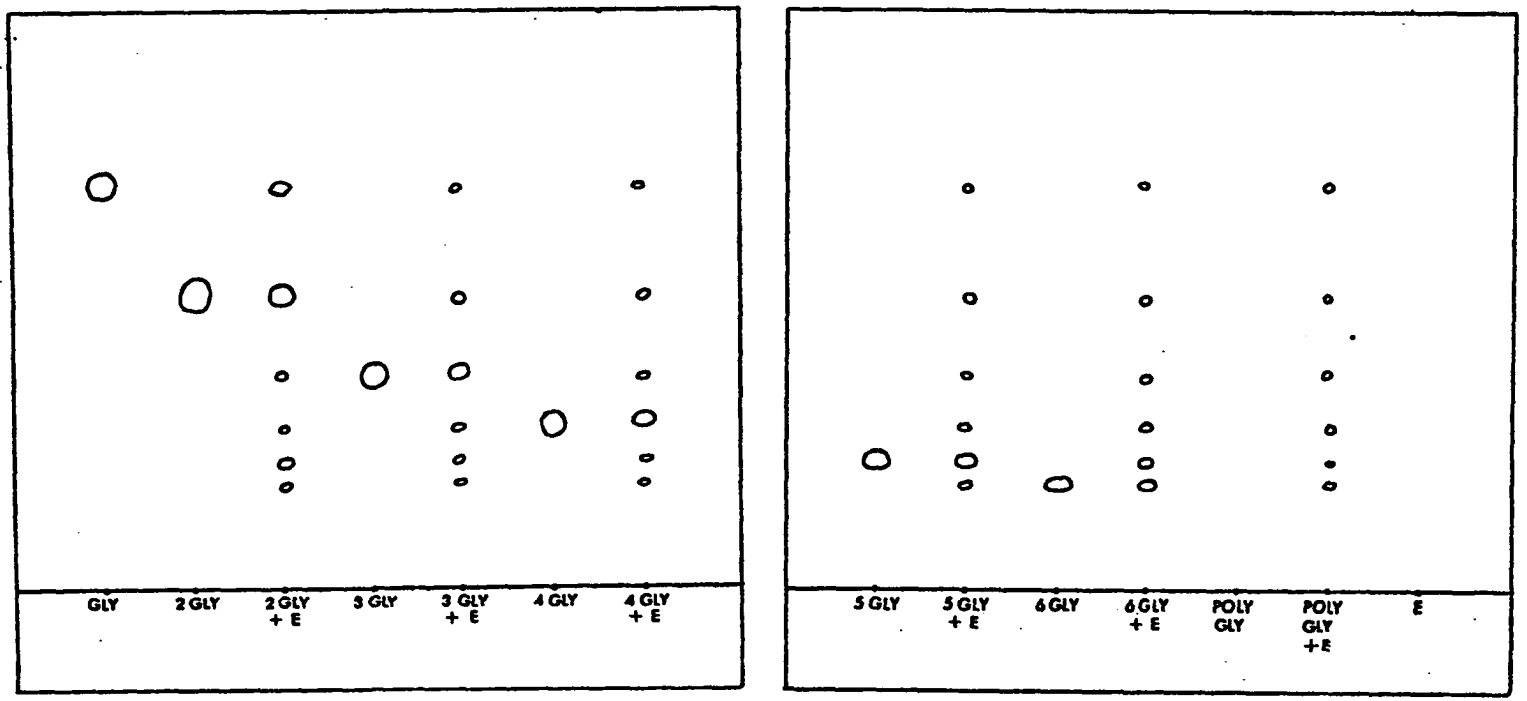

GLY
2 GLY
2 GLY + E
3 GLY
3 GLY + E
4 GLY
4 GLY + E
5 GLY
5 GLY + E
6 GLY
6 GLY + E
POLY GLY
POLY GLY +E
E

TABLE 3.--R_f values from thin-layer chromatography of dansyl derivatives of glycyl peptide standards on Gelman ITLC type S thin-layer chromatography media Solvent = tert-amyl alcohol - 0.1 M Phthalate buffer

Compound	R_f
DNS-glycine	.84
DNS-diglycine	.59
DNS-triglycine	.42
DNS-tetraglycine	.33
DNS-pentaglycine	.26
DNS-hexaglycine	.22
DNS-polyglycine	No movement

TABLE 4.--R_f values and identification of products from glycyl peptides reacted with lysostaphin and labeled with dansyl chloride. Thin-layer chromatography media--Gelman ITLC type S. Solvent--tert-amyl alcohol -0.1 M phthalate buffer

Compound	R_f Value and Identification
DNS-(diglycine + enzyme)	.83(gly); .60(digly); .44 (trigly); .33(tetragly); .25 (pentagly); .22(hexagly)
DNS-(triglycine + enzyme)	.84(gly); .59(digly); .43 (trigly); .33(tetragly); .26 (pentagly); .21(hexagly)
DNS-(tetraglycine + enzyme)	.84(gly); .60(digly); .42 (trigly); .32(tetragly); .25 (pentagly); .22(hexagly)
DNS-(pentaglycine + enzyme)	.83(gly); .61(digly); .43 (trigly); .33(tetragly); .26 (pentagly); .22(hexagly)
DNS-(hexaglycine + enzyme)	.84(gly); .61(digly); .42 (trigly); .33(tetragly); .26 (pentagly); .22(hexagly)
DNS-(polyglycine + enzyme)	.84(gly); .61(digly); .42 (trigly); .33(tetragly); .25 (pentagly); .21(hexagly)
DNS-enzyme	----------

of new, larger peptides from smaller ones. This process is called transpeptidation and has been demonstrated in several other proteolytic enzymes (41).

Action of lysostaphin on dansylated glycyl peptides

Figure 8 shows tracings of chromatograms of the products from dansylated glycyl peptides after reaction with lysostaphin. Table 5 gives a summary of the results obtained based on R_f values and comparison to standards run on the same chromatograms. These results show the enzyme capable of action on all dansylated peptides larger than dansyl-diglycine.

Action of lysostaphin on other artificial substrates

Tables 6 and 7 give the R_f values and identification of products from thin-layer chromatography of the dansyl derivatives of several potential artificial substrates for lysostaphin, and for the products formed after reaction with the enzyme. Again both hydrolysis and transpeptidation reactions are seen to be catalyzed by this enzyme.

Inhibition of lytic peptidase activity by penicillin, cycloserine, and artificial substrates

Figure 9 shows the inhibiting effect of several concentrations of penicillin on the lytic activity of lysostaphin. Similar results were also obtained in a pentaglycine assay.

Figure 8

Tracing of thin-layer chromatogram of dansyl-glycyl peptides after reaction with lysostaphin. TLC media--Gelman ITLC type S. Solvent--tert-amyl alcohol saturated with 0.1 M phthalate buffer (pH 6.0).

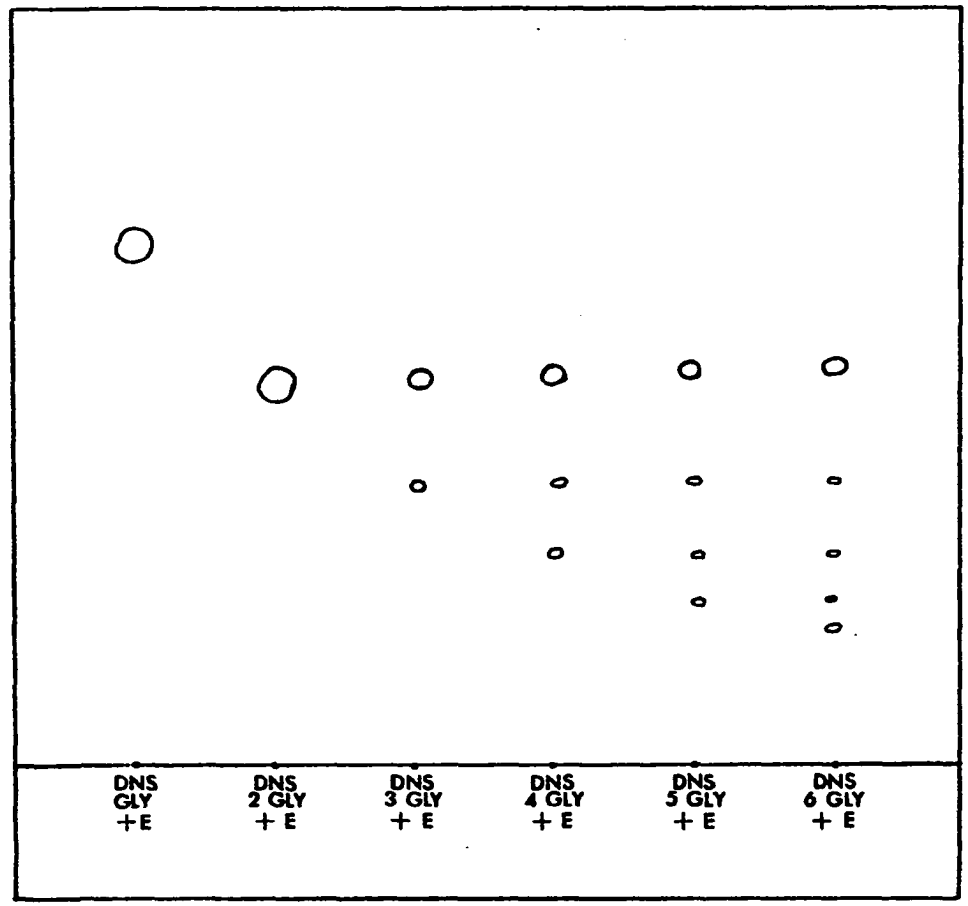

DNS GLY +E
DNS 2 GLY +E
DNS 3 GLY +E
DNS 4 GLY +E
DNS 5 GLY +E
DNS 6 GLY +E

TABLE 5.--R_f values and identification of products from dansylated glycyl peptides reacted with lysostaphin. Thin-layer chromatography media--Gelman ITLC type S. Solvent--tert-amyl alcohol -0.1 M phthalate buffer (pH 6.0)

Compound Reacted with Lysostaphin	R_f Value and Identification of Products
DNS-diglycine	No reaction
DNS-triglycine	.59(digly); .43(trigly)
DNS-tetraglycine	.59(digly); .43(trigly); .32 (tetragly)
DNS-pentaglycine	.59(digly); .43(trigly); .33 (tetragly); .26(pentagly)
DNS-hexaglycine	.59(digly); .42(trigly); .32 (tetragly); .25(pentagly); .22(hexagly)

TABLE 6.--R_f values of dansyl derivatives of several artificial substrates for the lytic peptidase. Thin-layer chromatography media was Gelman ITLC type S. Solvent system was tert-amyl alcohol -0.1 M phthalate buffer (pH 6.0)

Compound	R_f
Alanine	.86
Di-D-alanine	.79
Di-L-alanine	.79
Tri-L-alanine	.70
Tetra-L-alanine	.60
Penta-L-alanine	.50
Poly-D-alanine	No movement
D-alanylglycine	.64
L-alanylglycine	.64
Glycyl-L-alanine	.61
L-alanylgylcylglycine	.41
Glycylglycyl-L-alanine	.50

TABLE 7.--R_f values and identification of dansyl derivatives of several alanyl peptides after reaction with lysostaphin. Thin-layer chromatography media was Gelman ITLC Type S. Solvent system was tert-amyl alcohol -0.1 M phthalate buffer (pH 6.0)

Peptide Reacted with Lysostaphin	R_f Value and Identification of Products
Di-D-ala	.85(ala); .78(diala); .70 (triala); .60(pentalala); .44, .36, .33 (possibly hexa-, hepta-, and octa-ala)
Di-L-ala	.86(ala); .77(diala); .69 (triala); .59(tetraala)
Tri-L-ala	.85(ala); .77(diala); .69 (triala); .59(tetraala); .51(pentaala)
Tetra-L-ala	.86(ala); .78(diala); .68 (triala); .60(tetraala); .51(pentaala)
Penta-L-ala	.85(ala); .78(diala); .69 (triala); .59(tetraala); .50 (pentaala)
Poly-D-ala	.86(ala); .78(diala); .70 (triala); .60(tetraala); .51 (pentaala); .44, .36, .33 (possibly hexa, hepta-, and octa-ala)

TABLE 8.--R_f values and identification of dansyl derivatives of several mixed alanyl, glycyl peptides after reaction with lysostaphin. Thin-layer chromatography media was Gelman ITLC Type S. Solvent system was tert-amyl alcohol -0.1 M phthalate buffer (pH 6.0)

Peptide Reacted with Lysostaphin	R_f Value and Identification of Products
D-alagly	.85(ala); .64(alagly); .60 (glyala or digly or both); .54(unknown); .41(alaglygly or trigly or both)
L-alagly	Same as D-alagly
Gly-L-ala	.83(gly) .76(diala?) .59 (glyala and possibly glygly)
L-alaglygly	.81(gly); .64(alagly); .60 (digly or glyala or both); .54(unknown); .40(ala-glygly)
Glygly-L-ala	.85(ala); .59(glyala or digly or both); .54(unknown); .50 glyglyala)

Figure 9

Effect of several concentrations of penicillin on lytic peptidase activity.

A Enzyme control

B Enzyme + 50,000 units/ml penicillin

C Enzyme + 20,000 units/ml penicillin

D Enzyme + 10,000 units/ml penicillin

E Enzyme + 5,000 units/ml penicillin

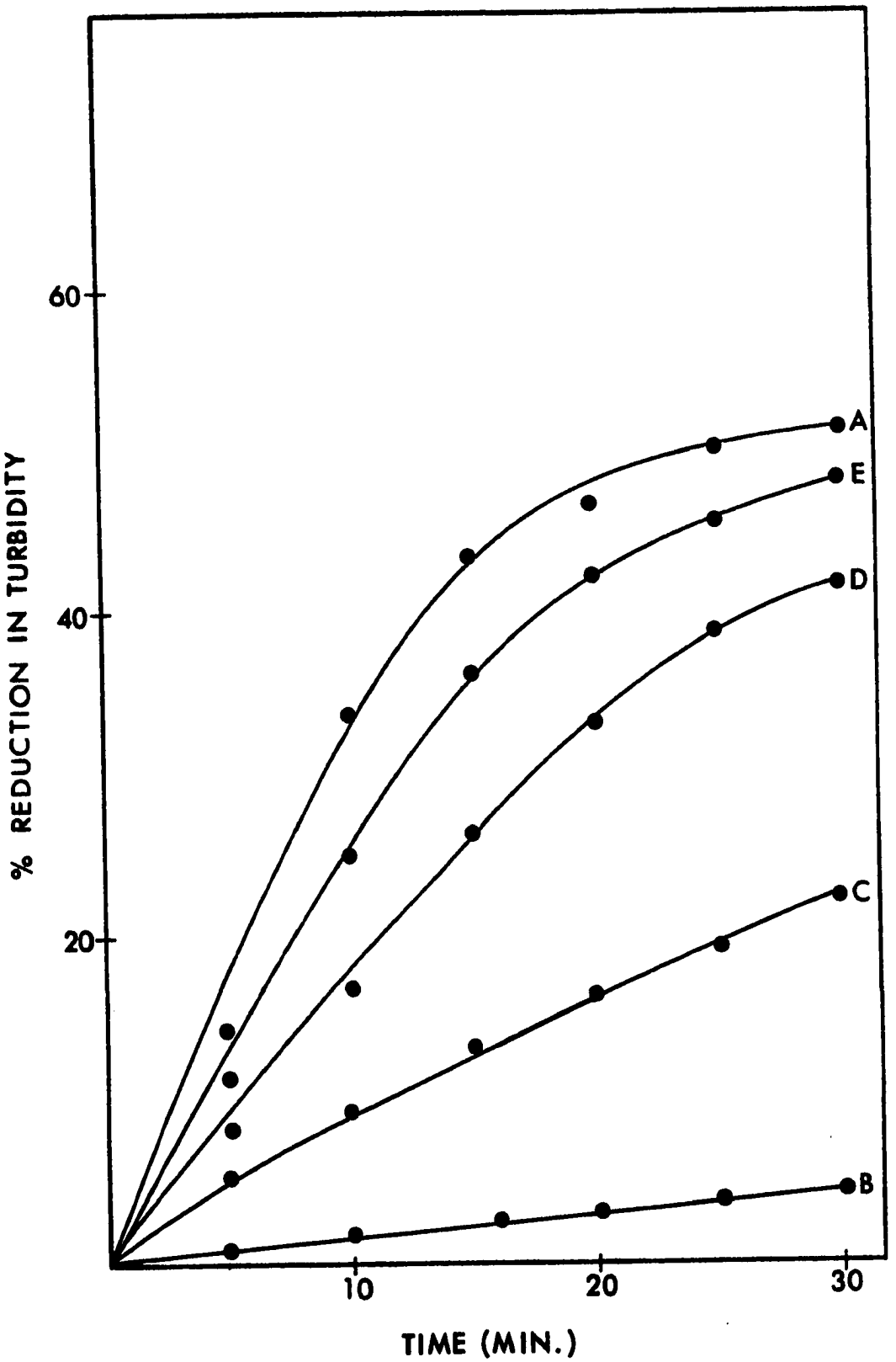

60
40
20
% REDUCTION IN TURBIDITY
A
E
D
C
B
10
20
30
TIME (MIN.)

Figure 10 is a Lineweaver-Burk plot showing that penicillin gives results that are consistent with a reversible competitive type of inhibition, as would be expected from the similarity in structure of penicillin and di-D-alanine.

Figure 11 shows that D-cycloserine has no inhibitory effect on lytic peptidase activity.

Figure 12 shows the results of a lytic assay in which several different artificial substrates were used as competitive substrates to inhibit lytic peptidase activity. These results indicate di-D-alanine to be the strongest competitive substrate, but much less potent than an equimolar concentration of penicillin, as seen in Figure 9 previously.

Mechanism of transpeptidation

Figure 13 shows tracings of the thin-layer chromatograms from the reactions between dansyl diglycine, triglycine and lysostaphin, and between dansyl diglycine, tetraglycine and lysostaphin. These results show the appearance of fluorescent transpeptidation products as time progresses, meaning the enzyme must have the amine type intermediate in its reaction mechanism.

Ability of glycine to serve as an acceptor in transpeptidation reactions

Figure 14 shows the results from the experiments using ^{14}C-labeled glycine in attempts to obtain radioactively labeled diglycine through transpeptidation. These results

Figure 10

Lineweaver-Burk plot for the inhibition of lytic peptidase activity by penicillin.

- ● Enzyme control
- ▲ Enzyme + 20,000 units/ml penicillin
- ■ Enzyme + 4,000 units/ml penicillin

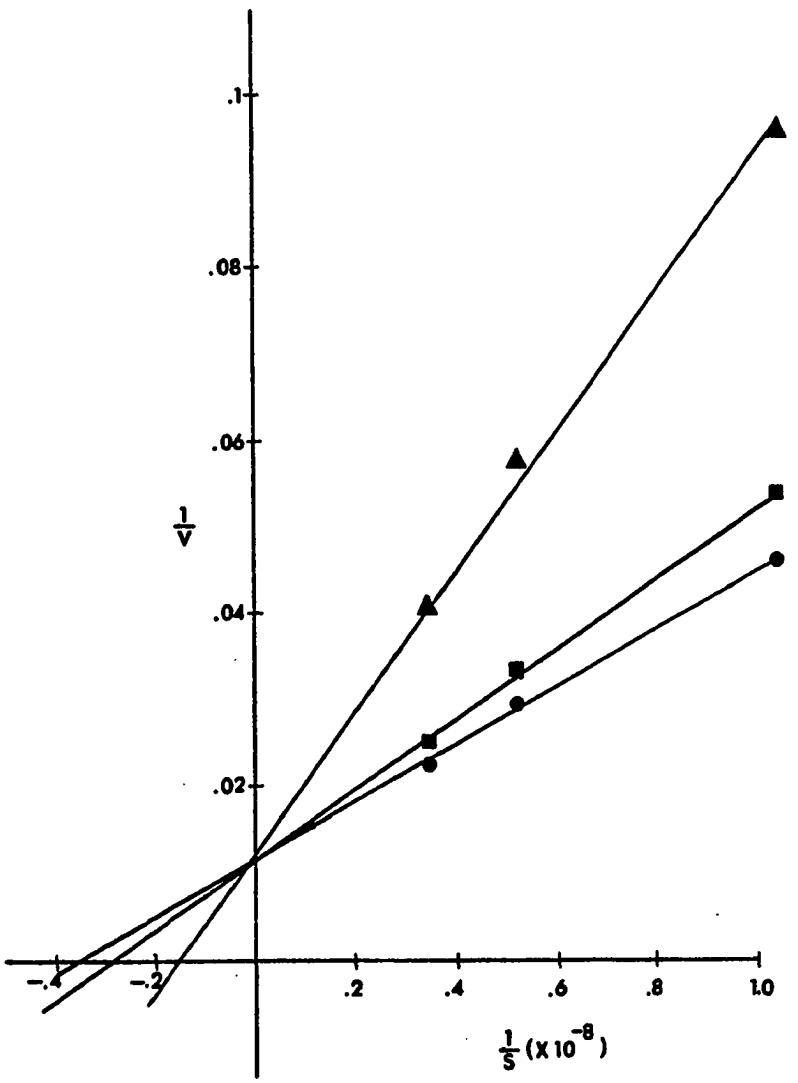

.1
.08
.06
.04
.02
1/V
-.4
-.2
.2
.4
.6
.8
1.0
1/S (X 10^-8)

Figure 11

Effect of D-cycloserine on lytic peptidase activity.

● Enzyme control and enzyme +10^{-1}, 10^{-2}, or 10^{-3} M concentrations of cycloserine

o Cell controls

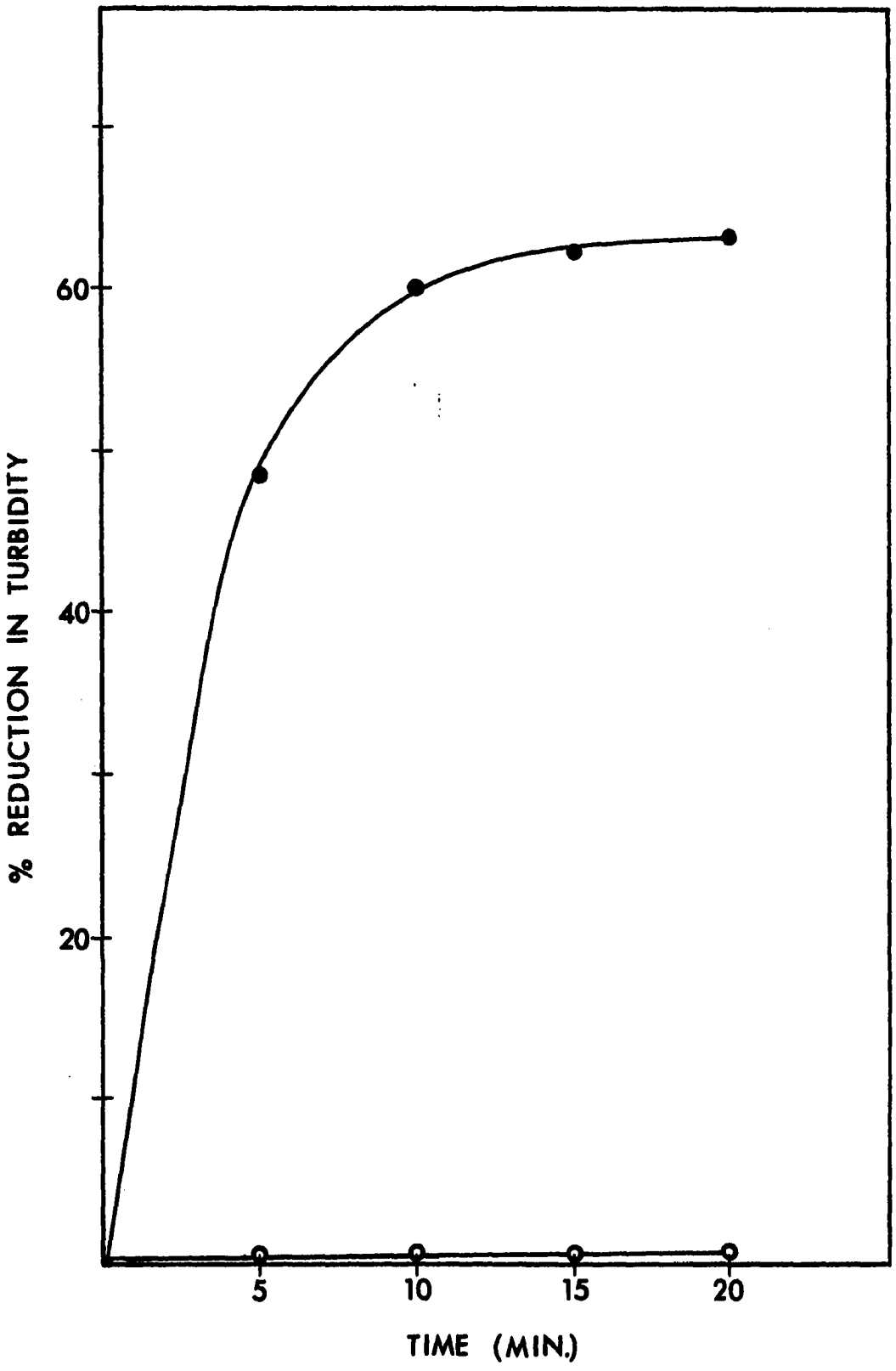

% REDUCTION IN TURBIDITY
60
40
20
5
10
15
20
TIME (MIN.)

Figure 12

Inhibition of lytic peptidase actavity by artificial substrates. Inhibitor concentration was 8.5×10^{-2} M.

A Enzyme control and enzyme + glycine

B Enzyme + diglycine

C Enzyme + triglycine

D Enzyme + di-L-alanine

E Enzyme + di-D-alanine

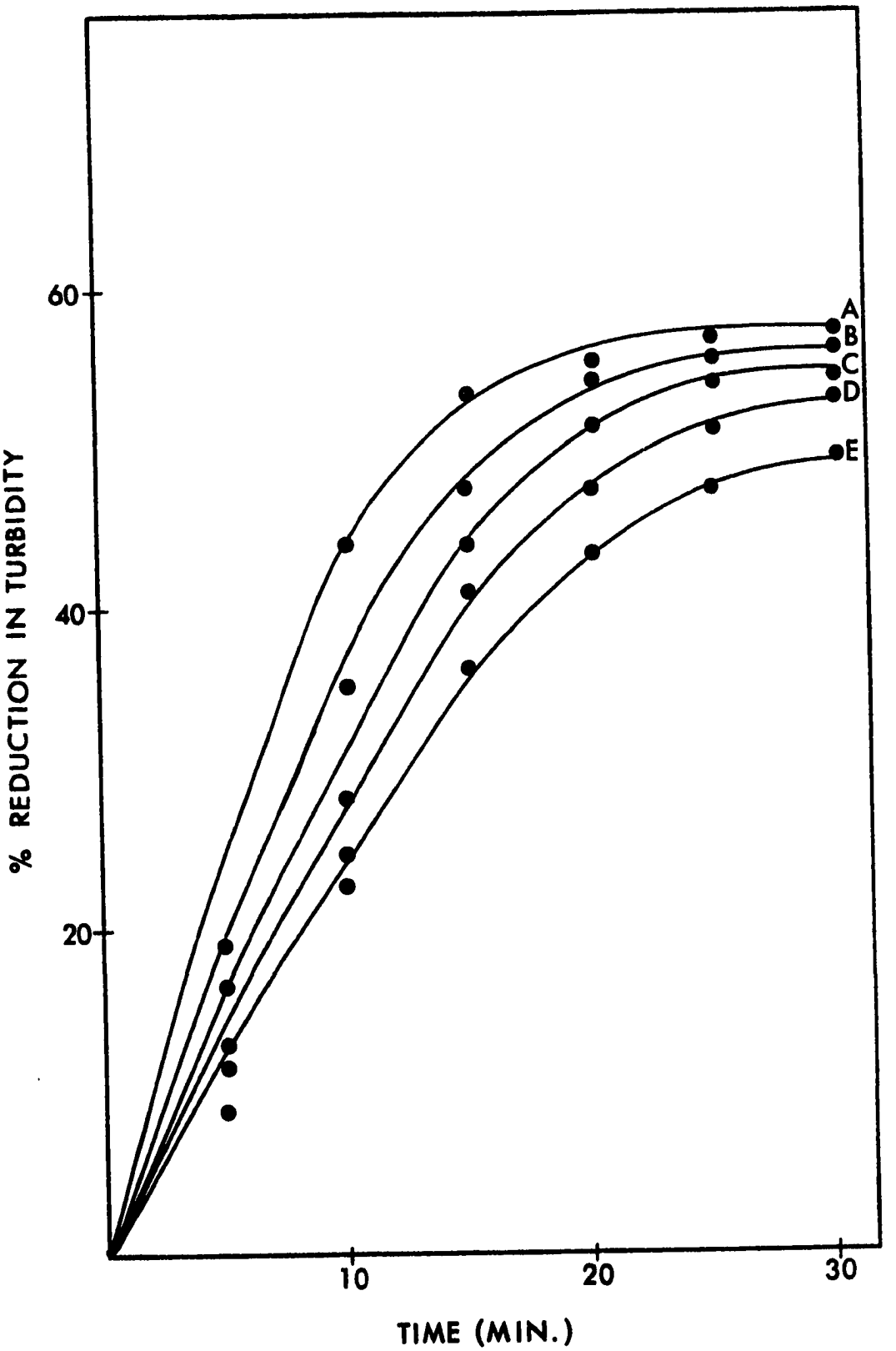

60
40
20
% REDUCTION IN TURBIDITY
A
B
C
D
E
10
20
30
TIME (MIN.)

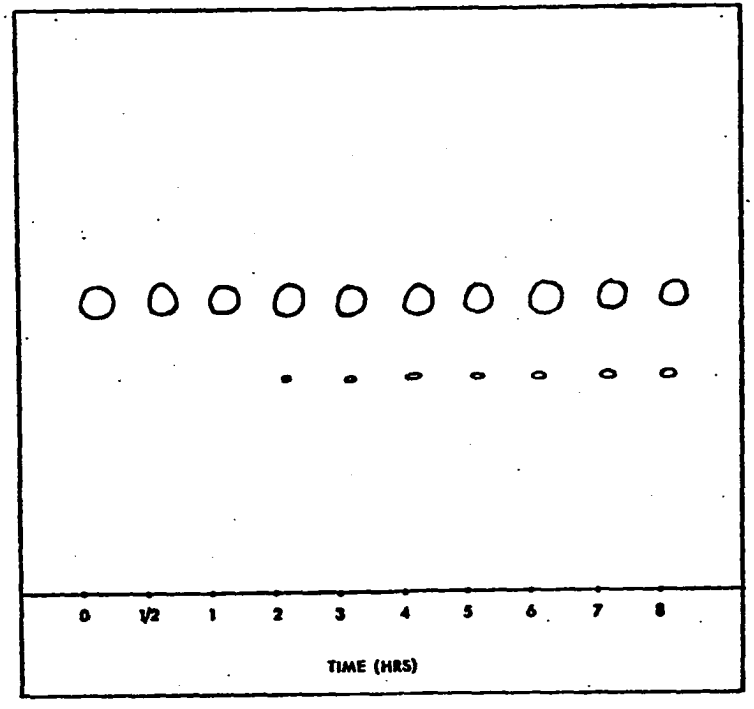
Figure 13

Utilization of dansyl diglycine as the acceptor in transpeptidation reactions.

A. Results from reaction of dansyl diglycine, triglycine and lysostaphin over an 8 hour period.

B. Results from the reaction of dansyl diglycine, tetraglycine and lysostaphin over an 8 hour period.

Chromatography on Gelman ITLC type S media. Solvent system was tert-amyl alcohol saturated with 0.1 M phthalate buffer pH 6.0.


0
1/2
1
2
3
4
5
6
7
8
TIME (HRS)

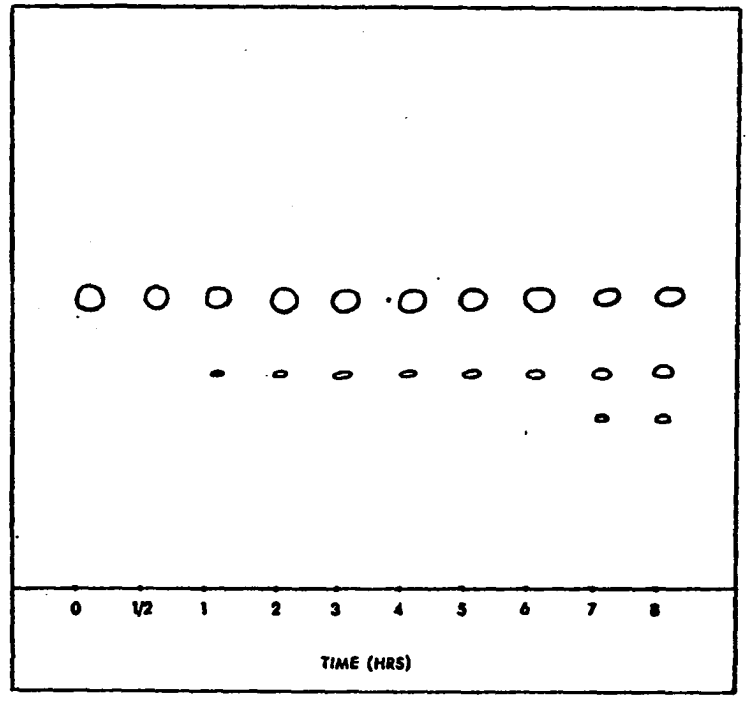
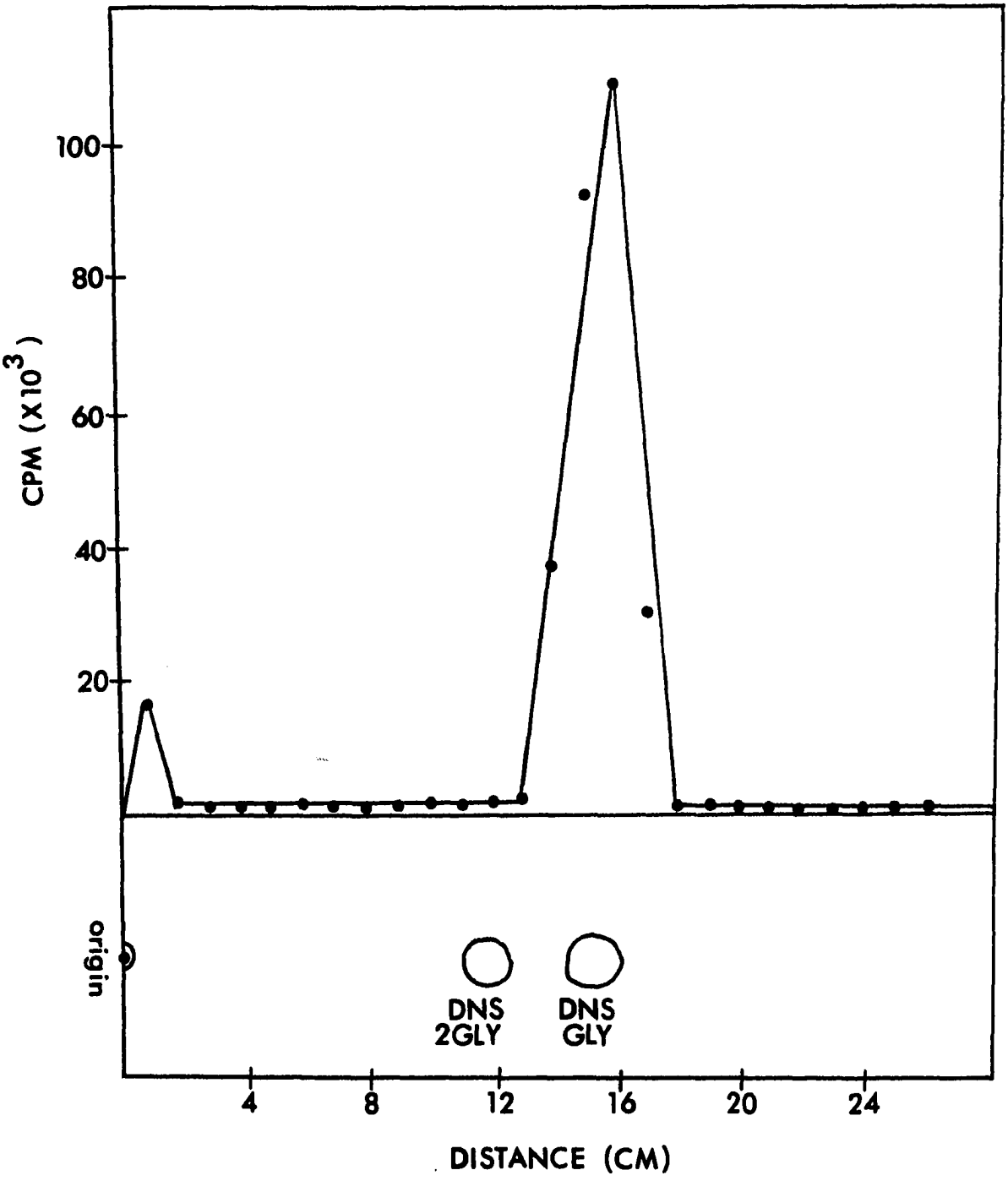

0
1/2
1
2
3
4
5
6
7
8
TIME (HRS)

Figure 14

Tracing of thin-layer chromatogram and corresponding radioactivity from experiment involving transpeptidation of ^{14}C-labeled glycine.


CPM (X10^3)
100
80
60
40
20
origin
DNS 2GLY
DNS GLY
4
8
12
16
20
24
DISTANCE (CM)

seem to indicate that glycine is not capable of reacting in the transpeptidation reactions since no significant radioactivity is apparent in the area of the chromatogram containing the dansyl-diglycine. To further substantiate this the dansyl-diglycine was eluted from several chromatograms and re-chromatographed. The spots from these chromatograms were then eluted and counted in the scintillation counter. No significant counts were found in this case.

The radioactive peak corresponding to the origin of the chromatogram probably indicates a binding of the radioactive glycine to the enzyme, perhaps at the active site. (See Discussion.)

The chromatograms from the experiment attempting to incorporate dansyl-glycine into the transpeptidation products from diglycine and triglycine showed only one fluorescent spot. The fluorescent spot had an R_f value of .82 indicating it was dansyl-glycine and that dansyl-glycine will not act as an acceptor for transpeptidation reactions.

Penicillinase activity of the lytic peptidase

Figure 15 is a standard curve for the assay of penicillin concentrations between 1 and 10 units using Bacillus cereus as the test organism.

Figure 16 shows the loss in activity of penicillin incubated with lysostaphin. Approximately 20 units/hour penicillin activity was lost in this experiment. The enzyme

Figure 15

Standard curve for penicillin. Test organism was Bacillus cereus. Assay time was 4.5 hours. Spectrophotometer readings were made at 540 nm.

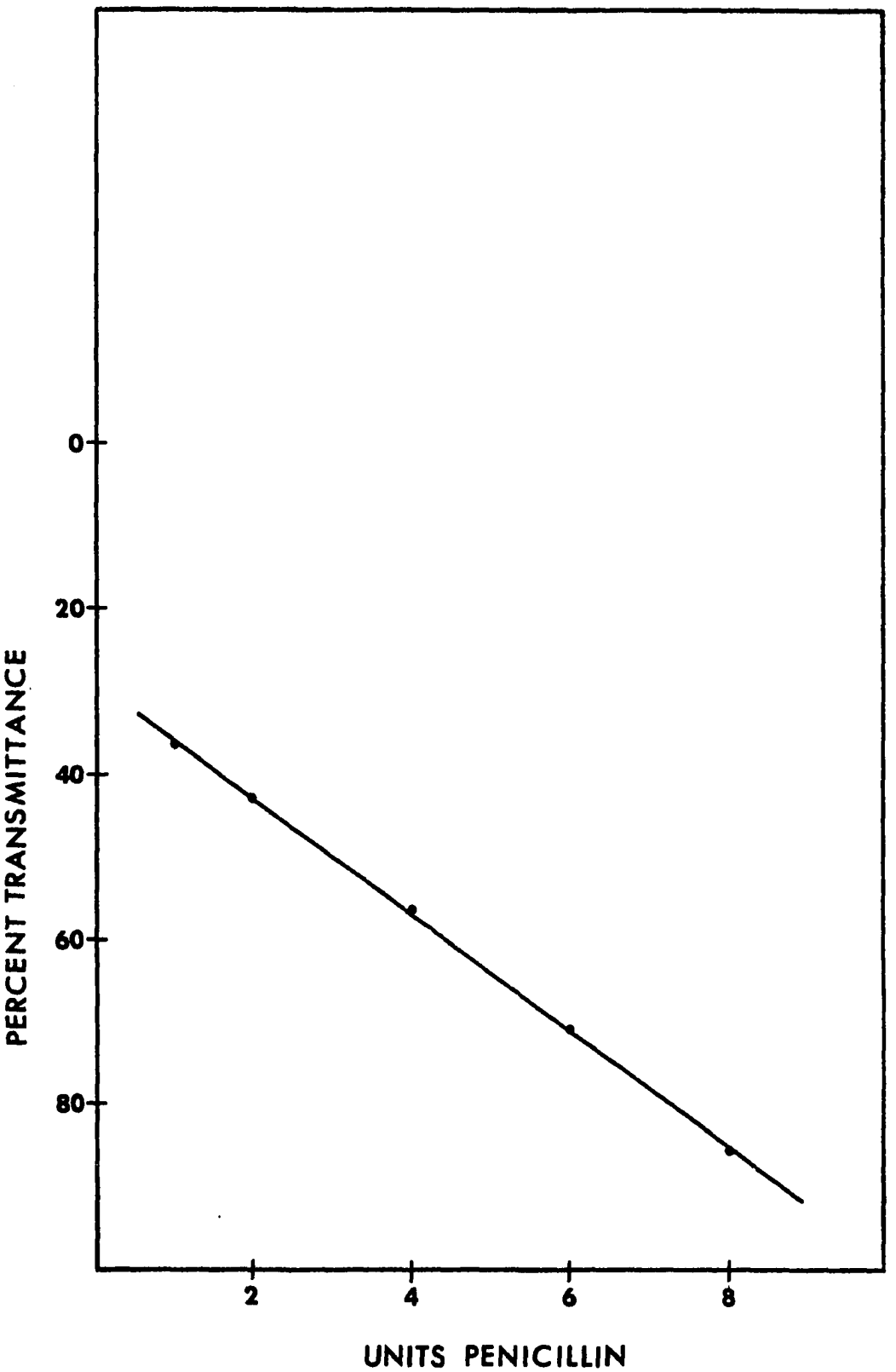

PERCENT TRANSMITTANCE
0
20
40
60
80
2
4
6
8
UNITS PENICILLIN

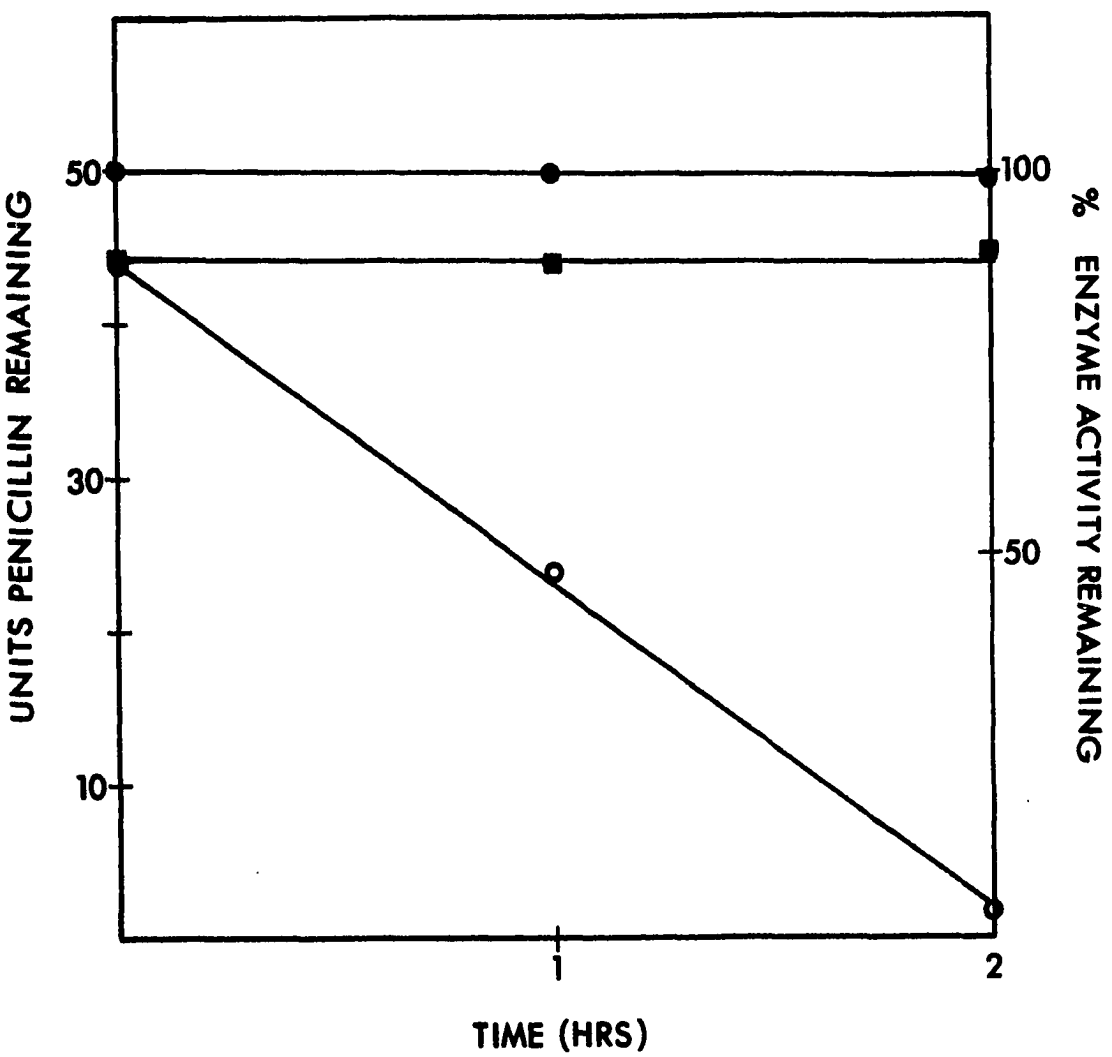
Figure 16

Penicillinase activity of lysostaphin.

● % Enzyme activity remaining

■ Units penicillin remaining in control

O Units penicillin remaining in penicillin reacted with lysostaphin


UNITS PENICILLIN REMAINING
50
30
10
100
50
% ENZYME ACTIVITY REMAINING
1
2
TIME (HRS)

activity was seen to stay constant over the course of the experiment, as did the penicillin concentration in the control without lysostaphin. Addition of lysostaphin to the B. cereus cultures had no effect on the organism.

Effect of penicillinase of S. aureus

Table 9 shows the commercial penicillinase had no lytic effect on a suspension of S. aureus cells.

Inhibition of lytic peptidase activity by metal chelation

Figure 17 shows the effect of several concentrations of 1,10-phenanthroline on lytic peptidase activity. Figure 18 shows similar results for 8-hydroxyquinoline sulfonate.

Figure 19 shows a comparison of the inhibitory effect of the two chelating agents by plotting the percent of lytic activity remaining after 30 minutes reaction with the inhibitors vs. the log of inhibitor concentration. Both chelating agents strongly inhibit the enzymes activity, 1,10-phenanthroline to a greater extent than 8-hydroxyquinoline sulfonate.

Photooxidation with methylene blue

Figure 20 shows the results from the enzyme assays after treatment of the lytic peptidase with methylene blue both in the light and in the dark. The enzyme was not affected by the methylene blue in the dark, however, in the

TABLE 9.--Effect of penicillinase on S. aureus

Time (min.)	% T
0	25.0
10	25.0
20	24.9
30	25.0

Figure 17

Inhibition of lytic peptidase activity by 1,10-phenanthroline.

Enzyme concentration = 4.1 µg/ml.

A 1×10^{-2} M

B 9×10^{-3} M

C 5×10^{-3} M

D 1×10^{-3} M

E 9×10^{-4} M

F 5×10^{-4} M

G 1×10^{-4} M

H Cell control

I Enzyme control

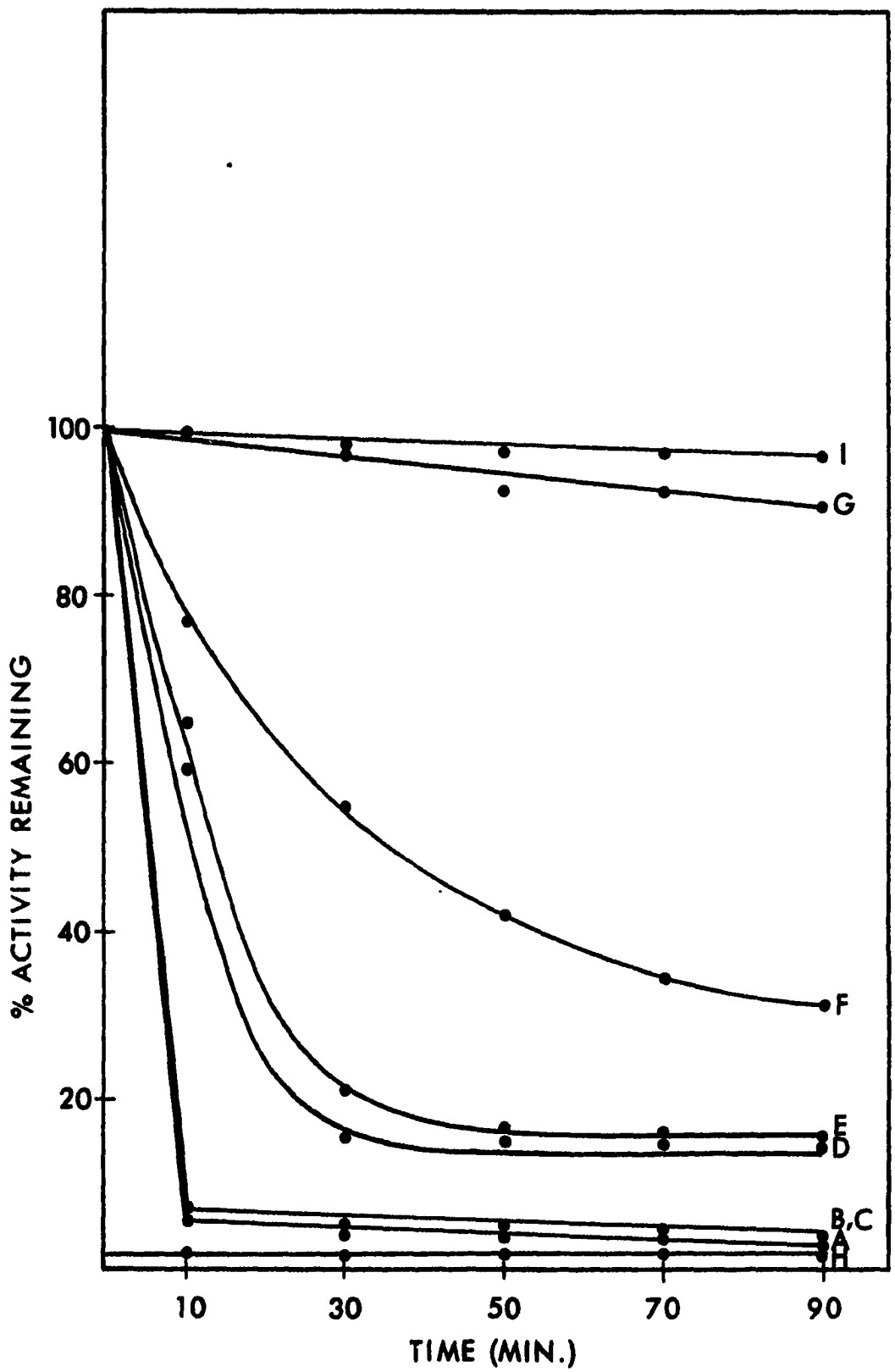

100
80
60
40
20
% ACTIVITY REMAINING
10
30
50
70
90
TIME (MIN.)
I
G
F
E
D
B,C
A
H

Figure 18

Inhibition of lytic peptidase activity by 8-hydroxyquinoline sulfonate. Enzyme concentration = 4.8 µg/ml.

A 1×10^{-2} M

B 9×10^{-3} M

C 5×10^{-3} M

D 1×10^{-3} M

E 9×10^{-4} M

F 5×10^{-4} M

G 1×10^{-4} M

H Cell control

I Enzyme control

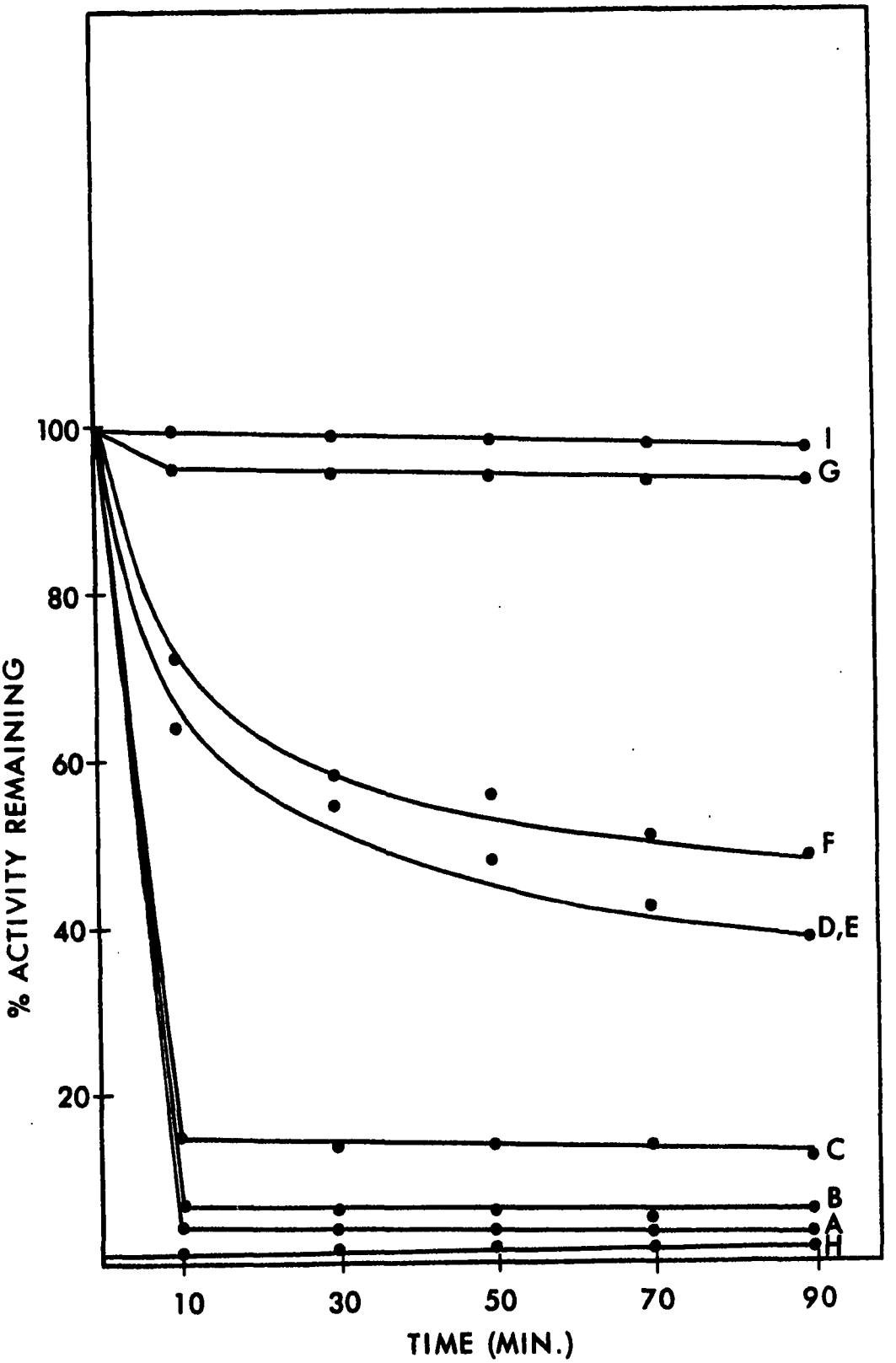

100
80
60
40
20
% ACTIVITY REMAINING
10
30
50
70
90
TIME (MIN.)
I
G
F
D,E
C
B
A
H

Figure 19

Comparison of the inhibition of lytic peptidase activity at 30 minutes by 1,10-phenanthroline and 8-hydroxyquinoline sulfonate.

o 8-hydroxyquinoline sulfonate

• 1,10-phenanthroline

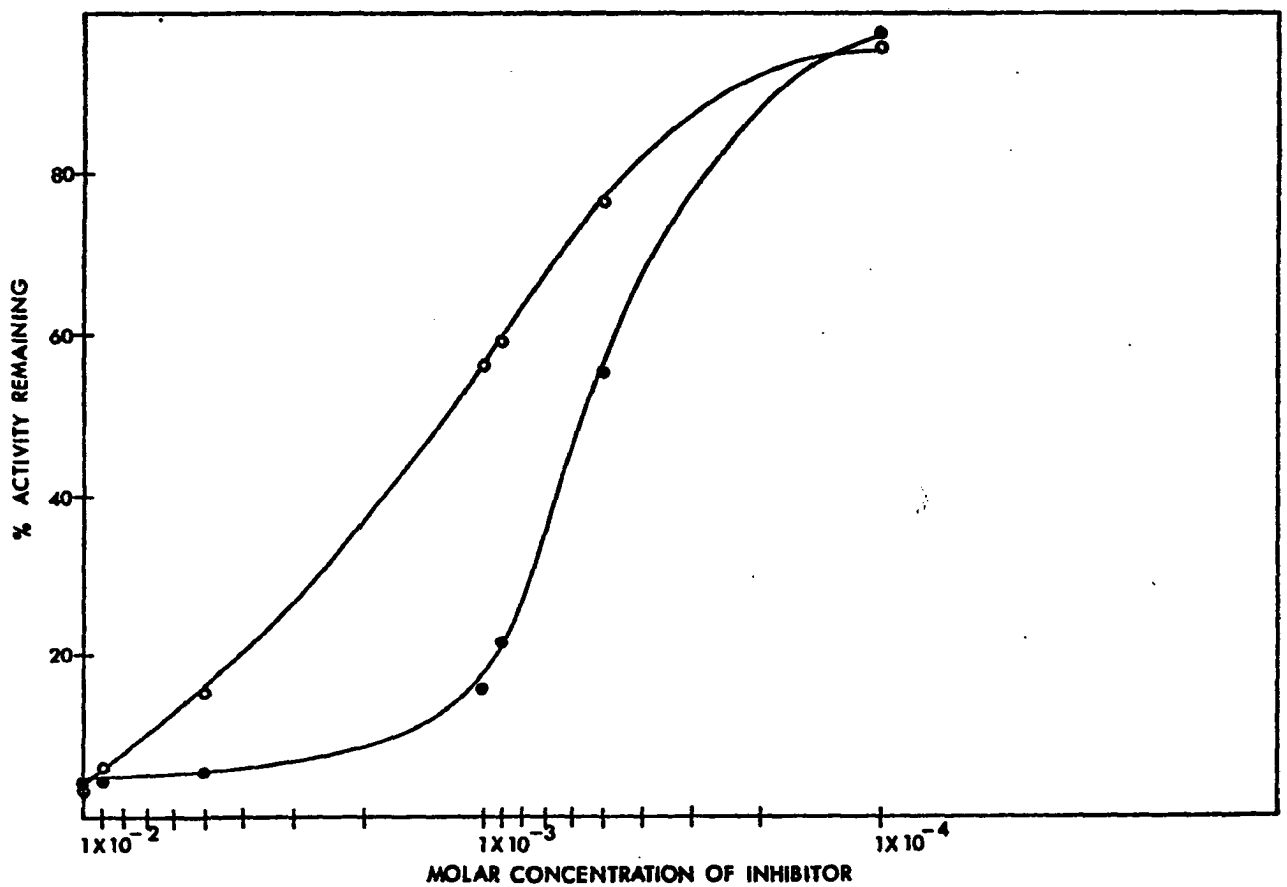

% ACTIVITY REMAINING
80
60
40
20
1X10⁻²
1X10⁻³
1X10⁻⁴
MOLAR CONCENTRATION OF INHIBITOR

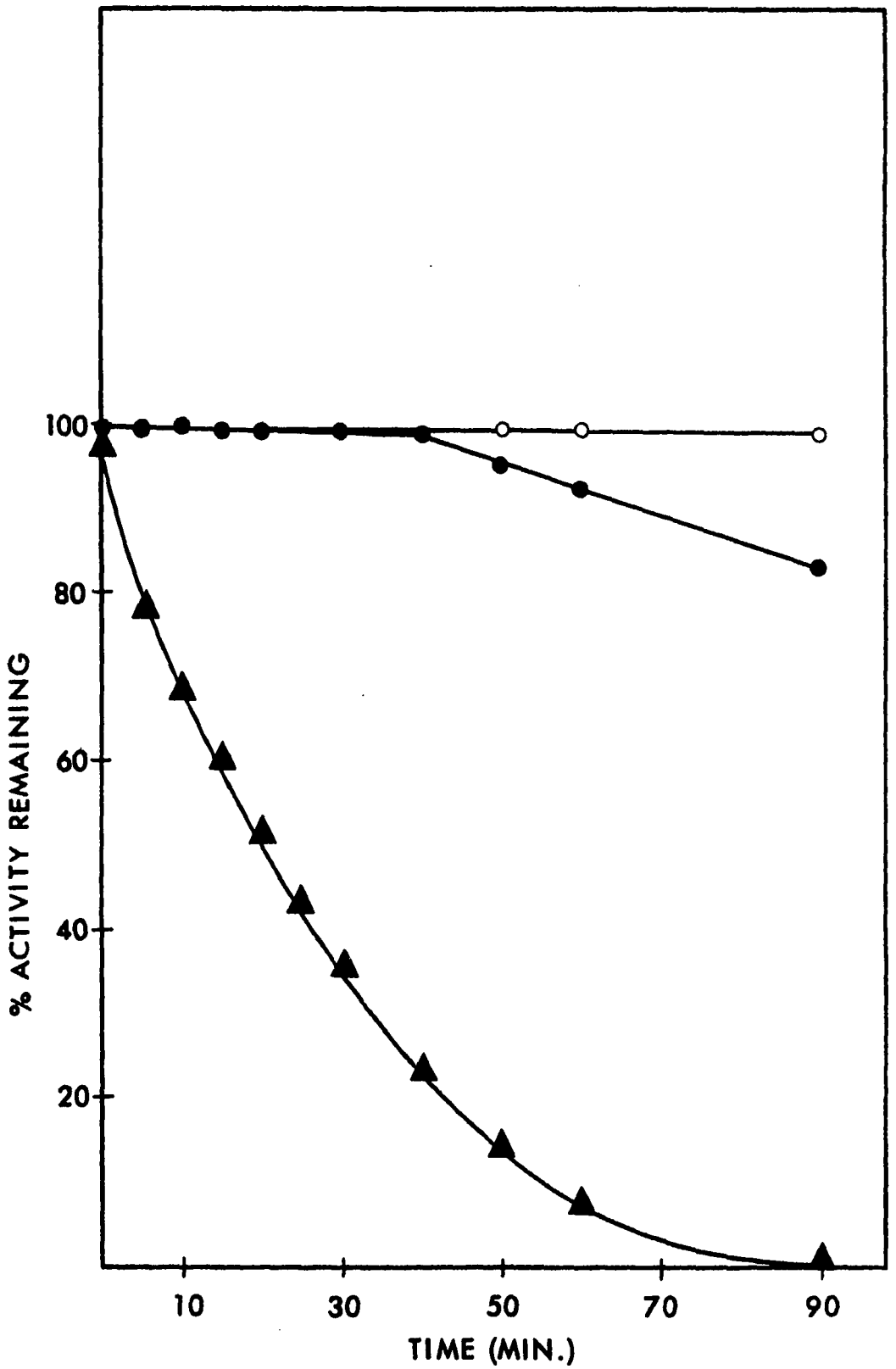
Figure 20

Effect of methylene blue photooxidation on lytic peptidase activity.

● Enzyme in the light

o Enzyme plus methylene blue in the dark

▲ Enzyme plus methylene blue in the light


100
80
60
40
20
% ACTIVITY REMAINING
10
30
50
70
90
TIME (MIN.)

light the methylene blue was shown to oxidize some important group(s) in the enzyme, gradually destroying the enzymes activity. This group is most likely histidine, but could also be tyrosine, trytophan, or methionine.

The enzyme control began losing activity after 40 minutes, presumably due to denaturation by the heat produced by the light source, and possibly the rapid stirring.

The cell control showed no lysis of the indicator cells was caused by the methylene blue.

Photooxidation with rose bengal

The lytic peptidase was found to be much more susceptible to oxidation by rose bengal, perhaps indicating the residue(s) affected is (are) histidine. Figure 21 shows the results of the assay for enzymatic activity after treatment with rose bengal both in the light and in the dark. The enzyme was not affected by the rose bengal in the dark but its activity was drastically reduced by the rose bengal in the light. The presence of a substrate gave some protection to the enzyme. Again the enzyme control in the light began losing activity after approximately 40 minutes and no lysis of indicator cells due to the dye was demonstrated.

Effect of N-acetylimidazole on lytic peptidase activity

Figure 22 shows the percent activity remaining after treatment with N-acetylimidazole for 90 minutes. No effect

Figure 21

Effect of rose bengal photooxidation on the lytic peptidase.

● Enzyme in the light

o Enzyme plus rose bengal in the dark

□ Enzyme plus rose bengal plus 10^{-2} M triglycine in the light

■ Enzyme plus rose bengal in the light

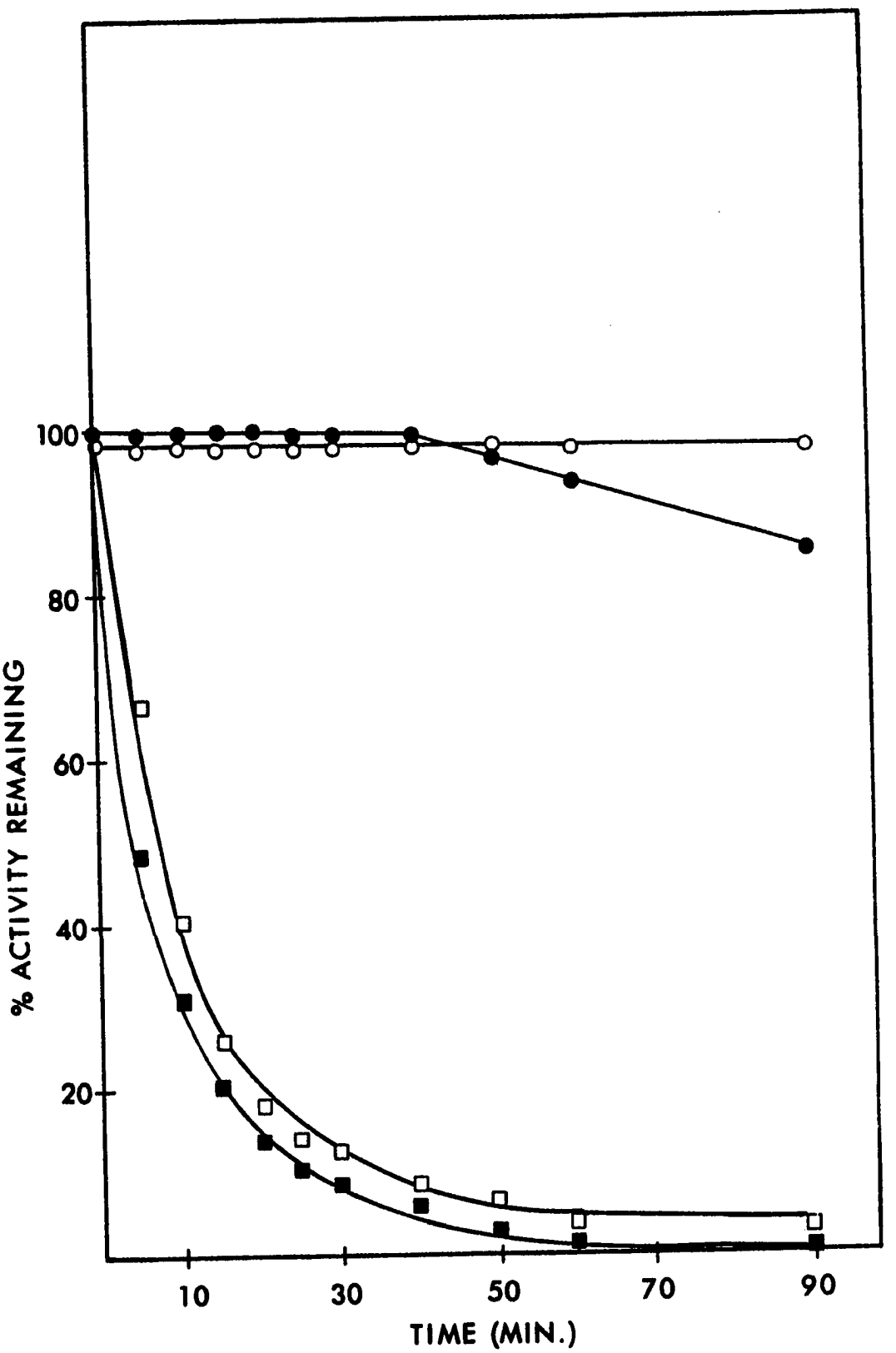

100
80
60
40
20
% ACTIVITY REMAINING
10
30
50
70
90
TIME (MIN.)

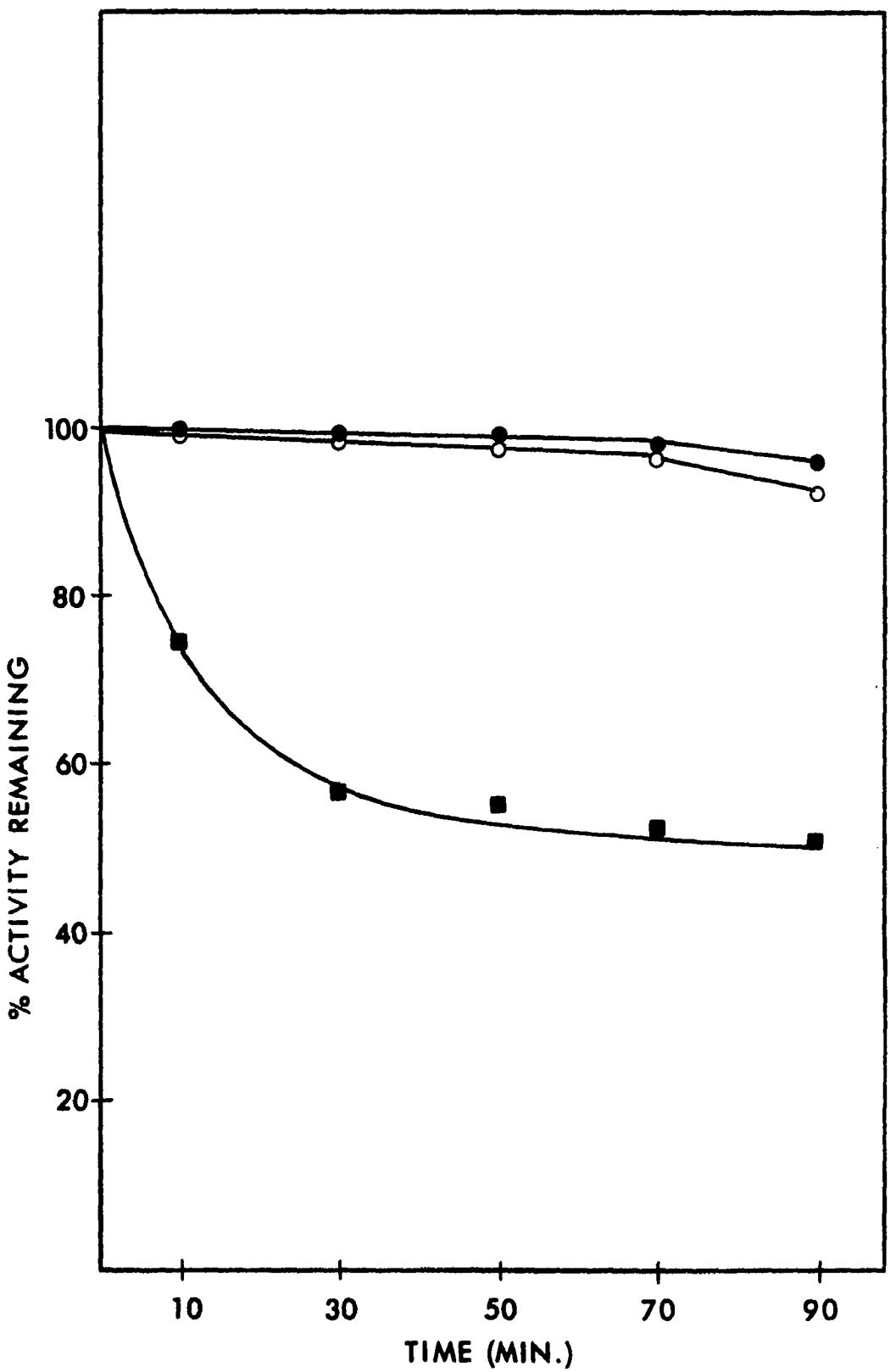
Figure 22

Effect of N-actylimidazole on lytic peptidase activity.

● Enzyme control and enzyme plus 60 M excess of reagent

o Enzyme plus 600 M excess of reagent

■ Enzyme plus 6000 M excess of reagent


100
80
60
40
20
% ACTIVITY REMAINING
10
30
50
70
90
TIME (MIN.)

on the enzyme is seen with a 60 M excess of the reagent. A small amount of inactivation appears to have taken place with a 600 M excess, and almost 50% inactivation results from reaction with a 6000 M excess.

Effect of HNB-Br on lytic peptidase activity

Treatment with HNB-Br, a reagent specific for tryptophan residues, is seen in Figure 23 to inactivate the lytic peptidase slightly. This suggests there may be a tryptophan residue in or near the active site of the enzyme which, if altered, may affect enzymatic activity, but not completely inhibit activity.

Inhibition of lytic peptidase activity by PMSF

Figure 24 shows the percent activity remaining in the lytic peptidase after treatment with PMSF. The three concentrations of PMSF shown in this experiment (2×10^{-4}, 10^{-3}, and 3×10^{-2} M) were in approximately a 120:1, 600:1, and 2000:1 molar ratio to the enzyme. Molar ratios of less than 100:1 seemed to give little or no inhibition of the enzyme. Concentrations above 2000:1 were not obtainable due to the insolubility of the PMSF in solutions that were 10% (v/v) in isopropanol. If solutions greater than 10% (v/v) in isopropanol were used loss in enzymatic activity due to denaturation of the enzyme began to occur.

Figure 23

Effect of HNB-Br on lytic peptidase activity.

● Enzyme control and enzyme plus acetone

⊙ Enzyme plus HNB-Br

● Cell control

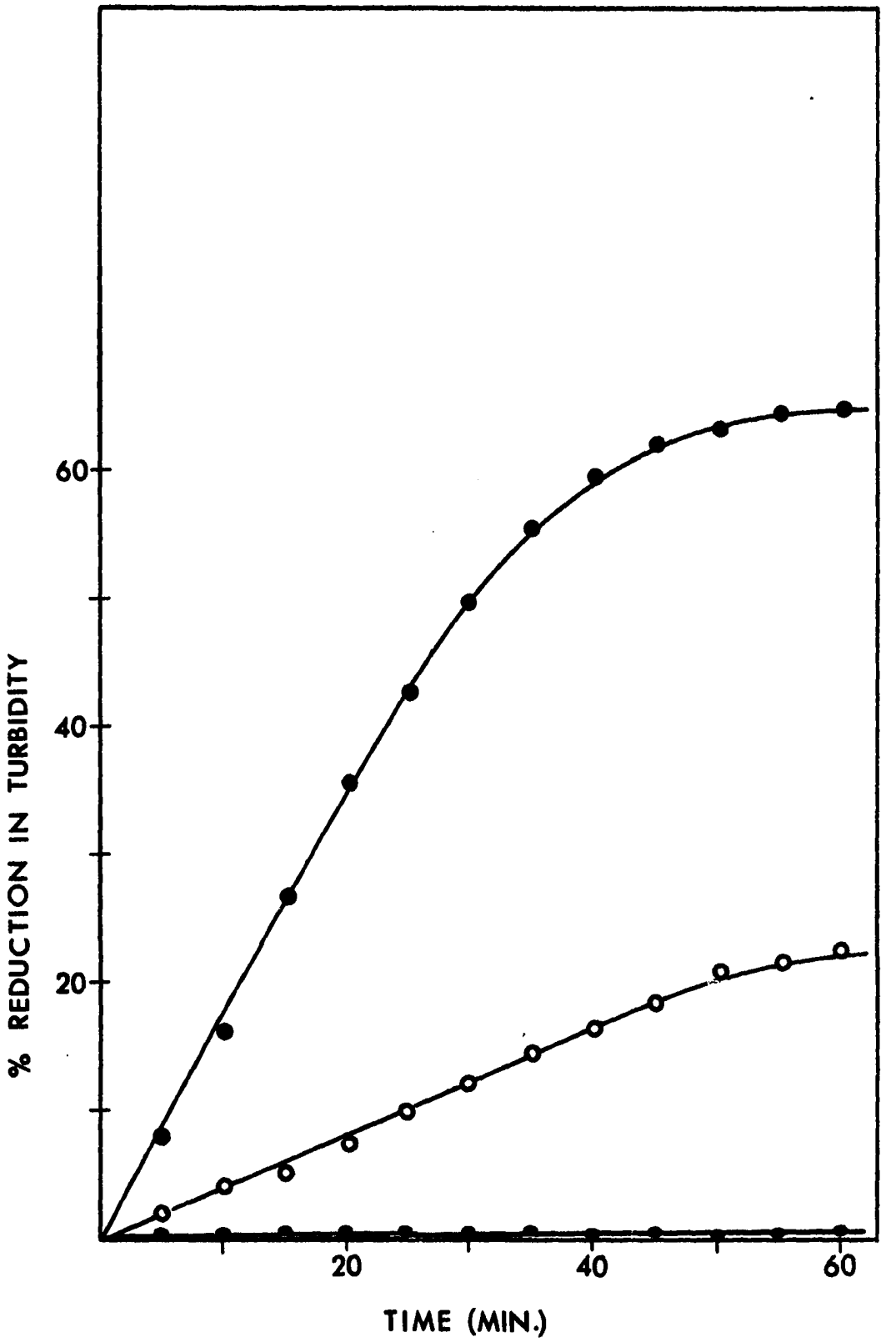
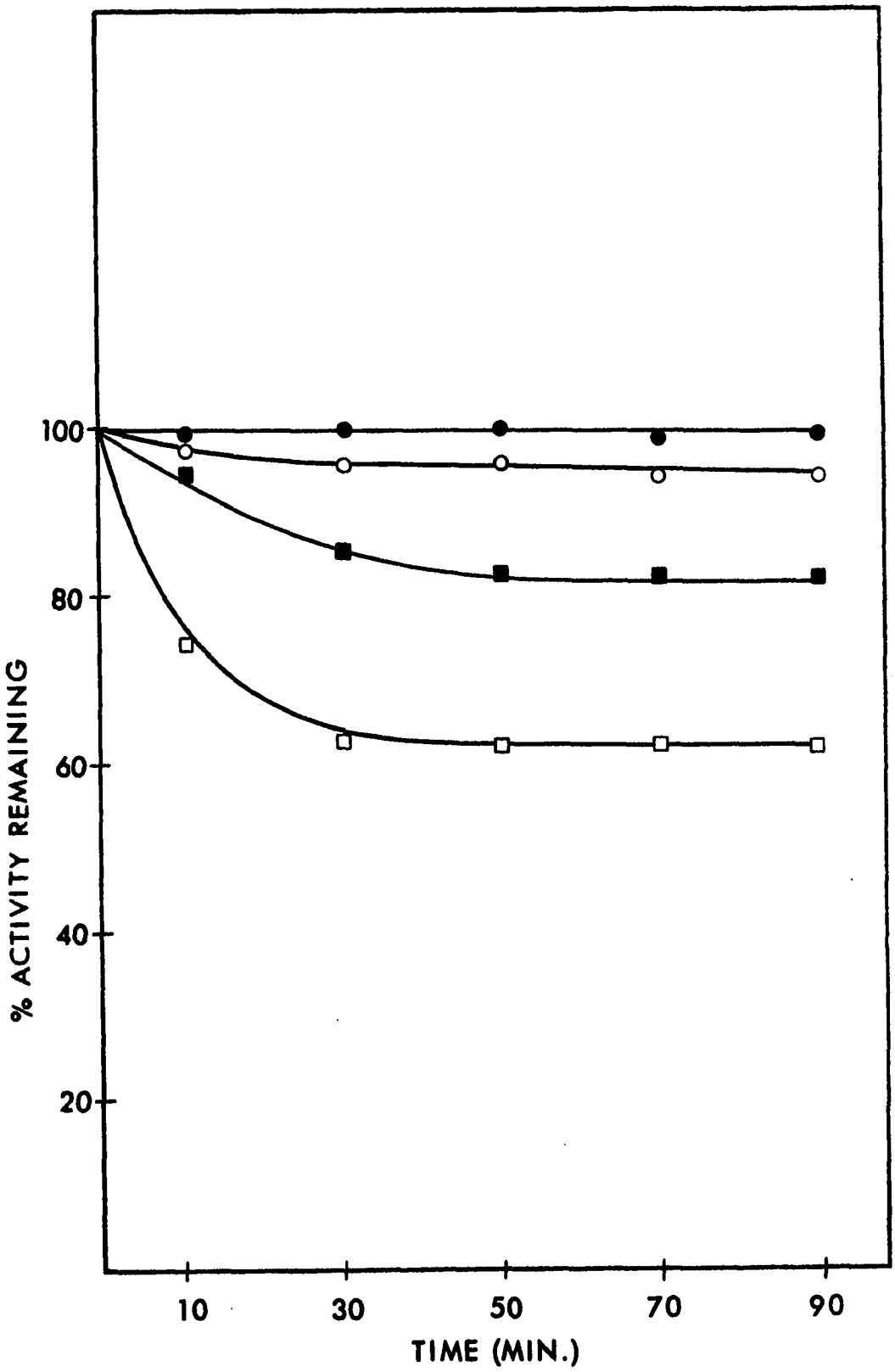

% REDUCTION IN TURBIDITY
60
40
20
20
40
60
TIME (MIN.)

Figure 24

Percent lytic peptidase activity remaining after treatment of lysostaphin with PMSF.

- ● Control (with and without isopropanol)
- ◎ 2×10^{-4} M PMSF
- ■ 10^{-3} M PMSF
- □ 3×10^{-2} M PMSF


100
80
60
40
20
% ACTIVITY REMAINING
10
30
50
70
90
TIME (MIN.)

Effect of acetic anhydride on lytic peptidase activity

Figure 25 shows that acetylation with acetic anhydride gives a 100% inactivation of lytic peptidase activity.

Reaction of lytic peptidase with $NaBH_4$ in the presence of substrate

Figure 26 shows the results from the lytic assay after treatment with $NaBH_4$ in the presence of pentaglycine. Tube B would be expected to have a great loss in activity if the enzyme had a Schiff-base intermediate in its catalytic reaction. Tube C shows a greater loss in activity due to the $NaBH_4$ treatment without substrate being present, suggesting the enzyme inactivation taking place is non-specific.

Inactivation of lytic peptidase by dansyl chloride

Figure 27 shows the results from the lytic assays after treatment of the enzyme with dansyl chloride. Table 10 gives the number of moles of dansyl bound per mole enzyme and the percent inactivation of the enzyme which corresponds to these values (the percent inactivation was calculated from 10 minute readings in the assay). These results indicate there may be a group in the active site which will react with dansyl chloride preferentially under certain conditions.

Figure 25

lytic assay of acetic anhydride treated lysostaphin.

● Enzyme control

o Enzyme plus acetic anhydride and cell control

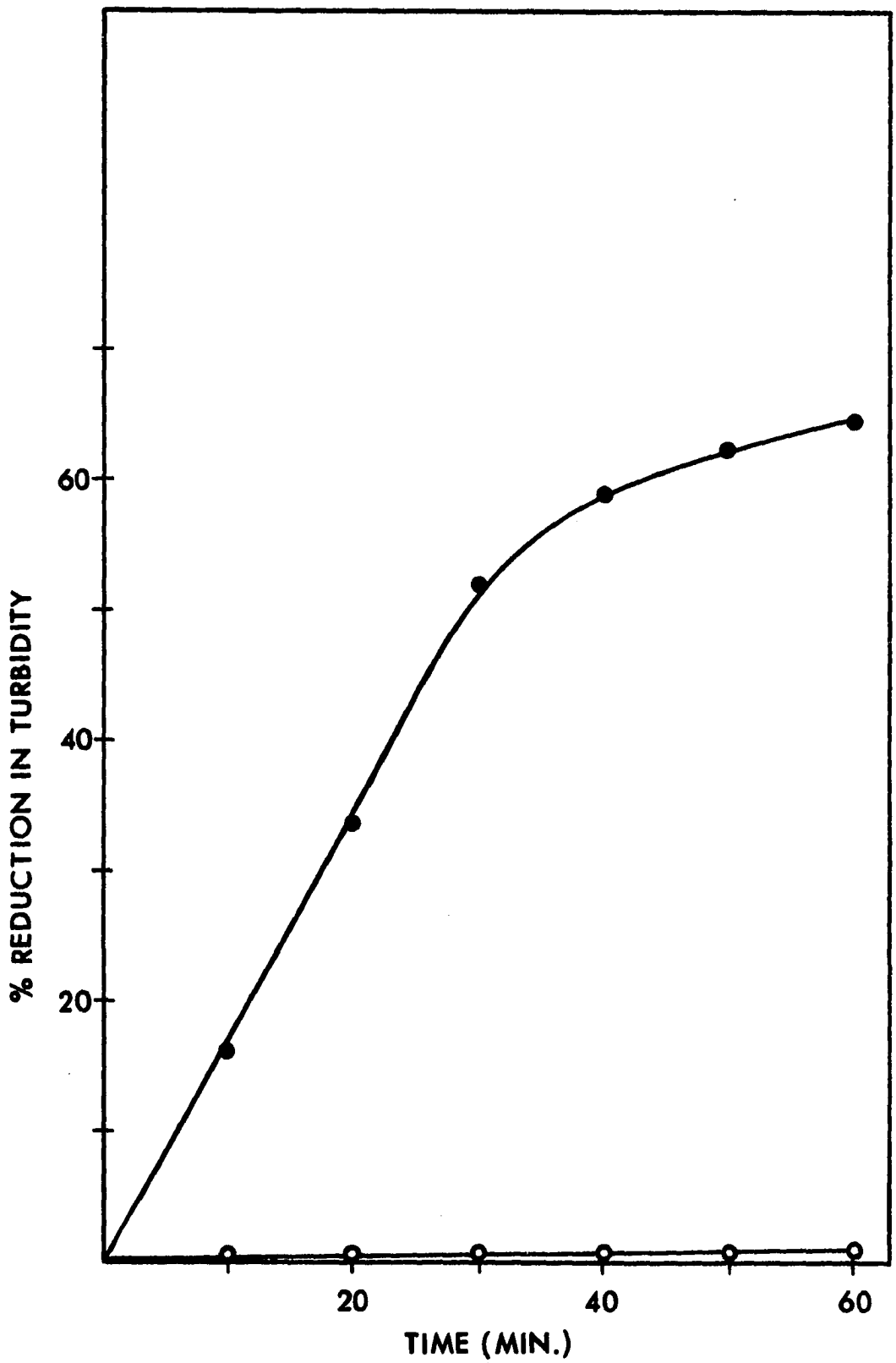

% REDUCTION IN TURBIDITY
60
40
20
20
40
60
TIME (MIN.)

Figure 26

$NaBH_4$ treatment of lysostaphin in the presence and absence of substrate.

● Tubes A + E

⭘ Tube B

■ Tubes C + D

□ Tube F

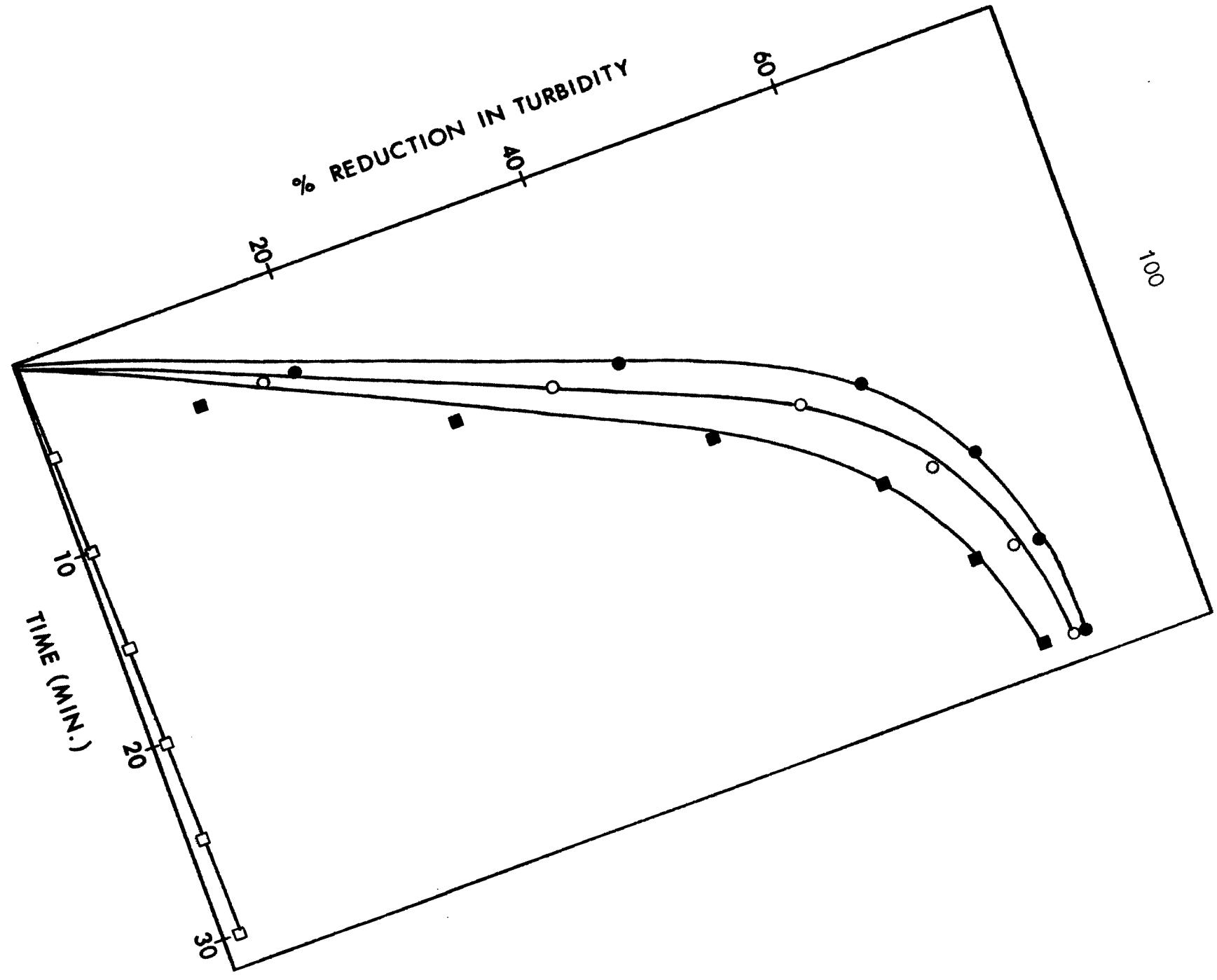

% REDUCTION IN TURBIDITY
20
40
60
100
TIME (MIN.)
10
20
30

Figure 27

Inactivation of lytic peptidase by reaction with dansyl chloride.

A Enzyme control and enzyme + acetone

B 1:1 molar ratio dansyl chloride/enzyme

C 2:1 molar ratio dansyl chloride/enzyme

D 3:1 molar ratio dansyl chloride/enzyme

E 5:1 molar ratio dansyl chloride/enzyme

F 10:1 molar ratio dansyl chloride/enzyme

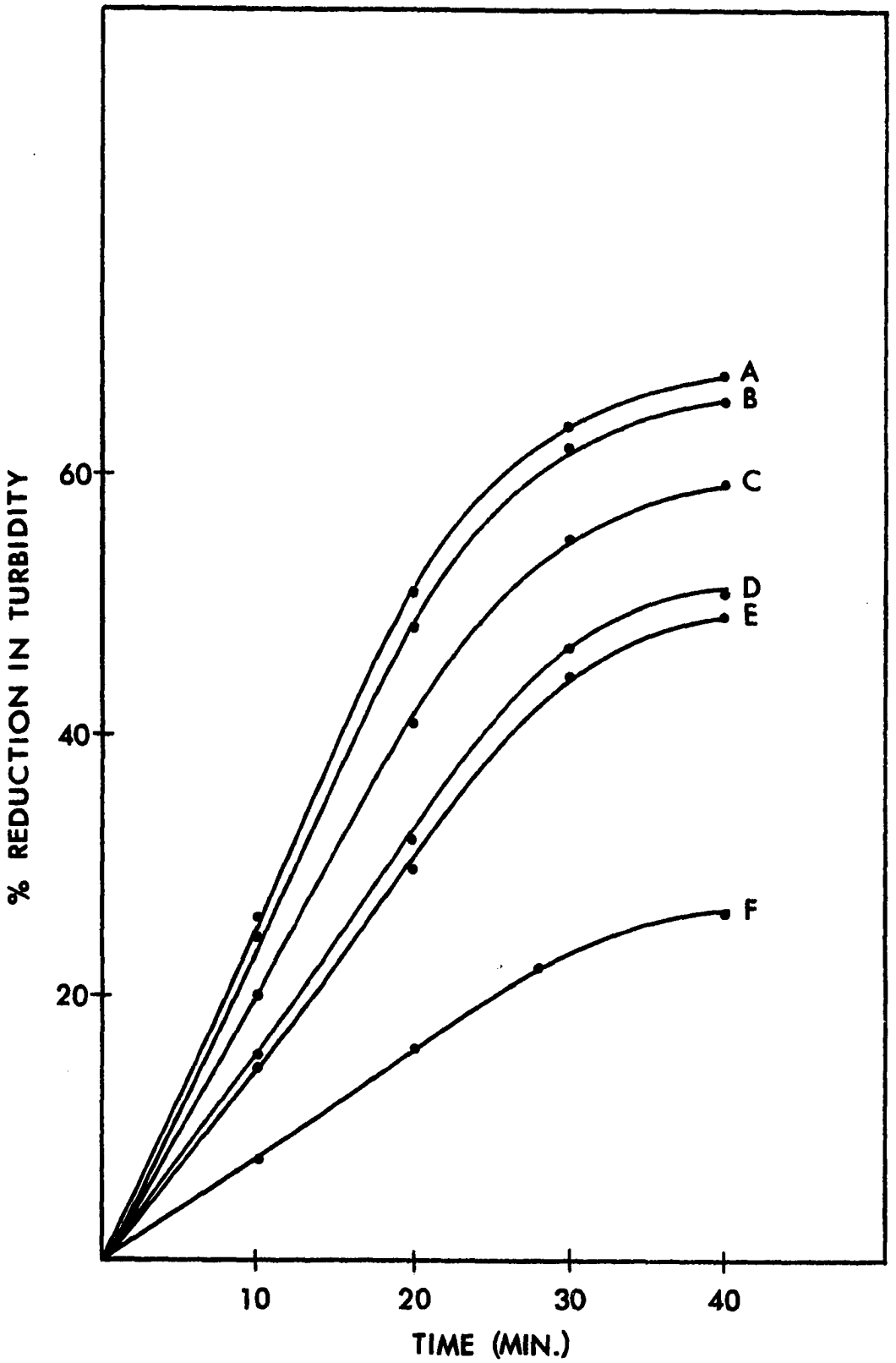

% REDUCTION IN TURBIDITY
60
40
20
A
B
C
D
E
F
10
20
30
40
TIME (MIN.)

TABLE 10.--The moles of dansyl bound per mole of enzyme and the resulting inactivation of the enzyme

Ratio DNS-Cl: Enz	Moles Bound DNS: Enz	Percent Inactivation
1:1	.05	4.6
2:1	.30	26.0
3:1	.70	57.5
5:1	.96	60.0
10:1	4.0	75.2

CHAPTER IV

DISCUSSION

The molecular weight studies indicate the lytic peptidase in lysostaphin has a molecular weight of approximately 24,800. This figure is in very good agreement with the data of Trayer and Buckley (7) (obtained by several different techniques) which indicates the molecular weight of the lytic peptidase is between 24,500 and 25,500. Wadstrom and Vesterberg (5) report the following molecular weights for the active components in lysostaphin (determined by Sephadex chromatography):

Lytic peptidase	30,000
Hexosaminidase	55,000
Amidase	26,000

The results in this study indicate the hexosaminidase molecular weight to be approximately 33,000 and the amidase approximately 17,000, in their denatured state. It is possible the hexosaminidase and the amidase exist as dimers in their active form, which would make the results in the two studies agree more closely. However, this possibility was not investigated in this study. The close agreement between the lytic

peptidase value for SDS gel studies and its molecular weight by amino acid analysis (7) (the most accurate molecular weight determination procedure) suggests the values in this investigation may be more nearly correct than those determined by Sephadex filtration.

The percent composition of lysostaphin determined in this paper varies slightly from the results obtained by Watanakunakorn and Browder (6). However, their results were on a lysostaphin preparation having an activity of only 165 units/mg. The results in this paper are on a lysostaphin preparation with an activity of 207 units/mg.

The lack of synergism between lysostaphin components substantiates earlier reports that the hexosaminidase and amidase have no lytic activity by themselves (4,5) and that no synergism exists between the amidase and lytic peptidase produced by S. aureus strain M18, which produces three very similar, if not identical, enzymes to those in lysostaphin (5). This lack of synergism raises the question as to what is the function of the other components in lysostaphin, since they apparently do not give the organism survival value by their ability to destroy neighboring cells. At the present time only speculation is possible.

The ability of the lytic peptidase to utilize di-D-alanine as a substrate and to catalyze transpeptidation reactions suggested a possible role in cell wall biosynthesis for the enzyme. Transpeptidase activity has been reported in

many proteolytic enzymes, including trypsin, chymotrypsin, papain, pepsin and others (41). Only one of the enzymes catalyzing transpeptidations studied so far (pepsin) has been shown to have the amine type mechanism (41). (See Figure 28.) The results in this study indicate the lytic peptidase also has this type of mechanism since fluorescent transpeptidation products can be demonstrated from the reaction of dansyl diglycine with either triglycine or tetraglycine in the presence of lysostaphin. Only the amine type mechanism could give these results since the dansyl diglycine is not susceptible to hydrolysis and since it can only act as an acceptor in transpeptidation reactions through its carboxyl group due to the binding of its amino terminus with the dansyl group (see Figure 29).

The presence of an amine type enzyme intermediate in the mechanism of the lytic peptidase apparently rules out the possibility that this enzyme is capable of closing the crossbridges in cell wall synthesis, since this reaction requires an acyl type intermediate in the transpeptidation reaction.

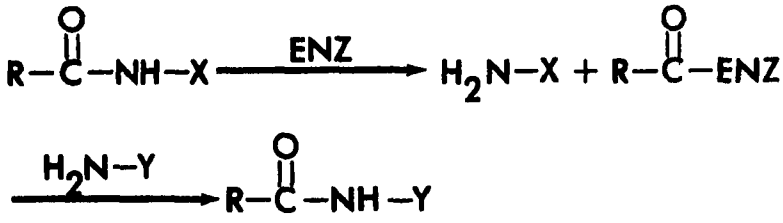
The ability of the lytic peptidase to hydrolyze peptide bonds other than glycyl-glycine bonds agrees with the results of Krulwich *et al.* (43) on isolated cell walls of *Arthrobacter crystallopoietes*. They reported the hydrolysis of D-alanyl-L-alanine, L-alanyl-L-lysine and L-alanyl-glycine bonds in the crossbridges of the *Arthrobacter* cell wall by this enzyme. These results plus the results presented in

Figure 28

1) Acyl type mechanism for transpeptidation reactions. The enzyme in this case interacts with the carbonyl group of the peptide bond that is broken.

2) Amino type mechanism for transpeptidation reactions. The enzyme in this case interacts with the amino group of the peptide bond that is broken.

1) ACYL (CARBOXYL) TRANSFER



2) IMINO (AMINE) TRANSFER

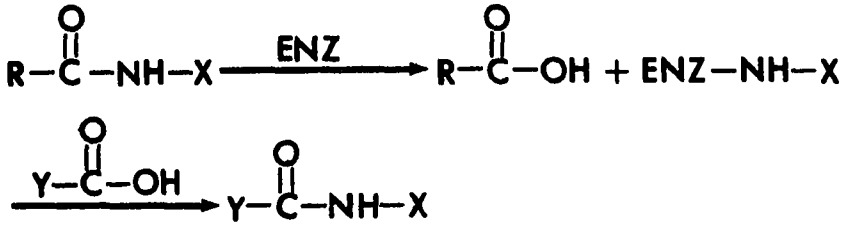
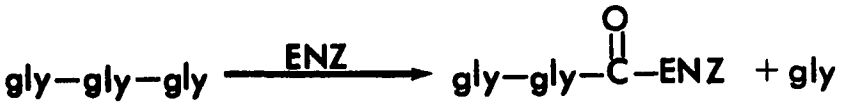


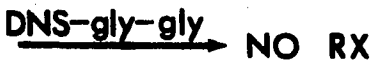
Figure 29

1) Reaction of dansyl diglycine with triglycine plus enzyme would not be capable of producing fluorescent transpeptidation products.

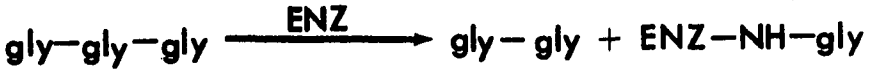
2) This same reaction via the imino mechanism would be capable of producing fluorescent transpeptidation products.

1) ACYL TRANSFER





2) IMINO TRANSFER



this paper indicate that the lytic peptidase has a broader specificity of action than was previously suspected.

The penicillinase activity of the lytic peptidase and the ability of penicillin to act as a competitive inhibitor of the enzyme apparently contra-indicates their combined use in chemo-therapy. It cannot be ascertained if the lytic peptidase is a penicillinase which has been modified to be a powerful bacteriolytic enzyme, or if it is a bacteriolytic enzyme which also has a specificity broad enough to allow the hydrolysis of penicillin. The second possibility is most likely due to the weak penicillinase activity of the enzyme. That this dual activity is not a widespread phenomenon is perhaps substantiated by the fact that another penicillinase (Calibiochem) has no lytic effect on S. aureus.

The fact that penicillin is a more powerful inhibitor of the enzyme than other substrate analogs, including D-alanyl-D-alanine may be due to the rigidity of the peptide bond in the β-lactam ring of penicillin in comparison to the less rigid bonds in other substrate analogs. This rigidity would perhaps most closely approximate the rigidity of the natural pentaglycine substrate in cell walls.

The transpeptidation reaction appears to require at least a dipeptide for correct alignment of the substrates in order for transfer reactions to occur, since neither radioactive glycine nor dansyl-glycine would act as an acceptor for transpeptidation reactions.

A preliminary model for the interactions of substrate with the active site of the enzyme may be postulated from the results of this investigation. Figure 30 shows this proposed model. The active site apparently has a binding site (I) which interacts with a carbonyl group in the peptide substrate, thus positioning and stabilizing the peptide for catalysis which occurs at the catalytic site (C). The evidence also suggests there may be another binding site (II) which is important in stabilizing and positioning the peptide.

Figure 31 shows the postulated binding of glycine, diglycine and triglycine according to this model. The model explains why glycine cannot be involved in transpeptidation reactions since when it binds, its carboxyl group is too far removed from the catalytic site. Diglycine is seen to have two binding possibilities. In the binding shown in 2a the diglycine would act as an acceptor in transpeptidation reactions, but could not be hydrolyzed itself. In the binding shown in 2b the diglycine could be hydrolyzed and one-half of the molecule released (the amino terminal glycine) while the other one-half of the molecule (carboxyl terminal glycine) could then either be released (hydrolysis) or transferred to another peptide in an amine type transpeptidation reaction.

Triglycine is seen to be most likely cleaved in this model at the second peptide bond from the amino end. Larger peptides would have several binding schemes and could produce several products.

Figure 30

Proposed model for the active site of the lytic peptidase in lysostaphin.

I = Binding Site

II = Possible Second Binding Site

C = Catalytic Site

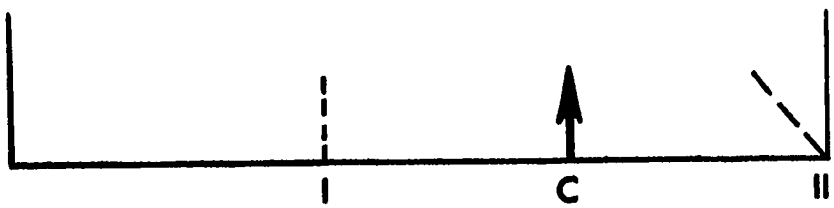

I
C
II

Figure 31

Postulated binding of several glycyl peptides and glycine to active site in lytic peptidase.

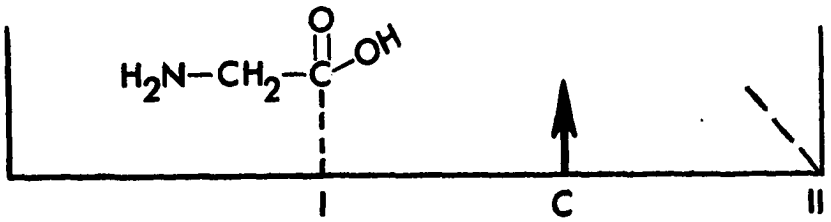
1) glycine--may bind but will not be involved in transpeptidation reactions due to distance from catalytic site

2a) diglycine--may be involved in transpeptidation reactions as acceptor but will not be hydrolyzed

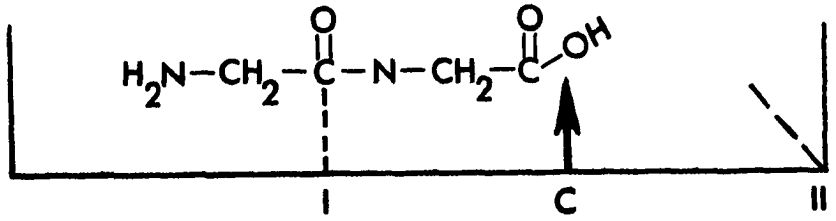
2b) diglycine--in this type binding may be hydrolyzed

3) triglycine (or other glycyl peptides)--longer peptides will have several possible binding schemes

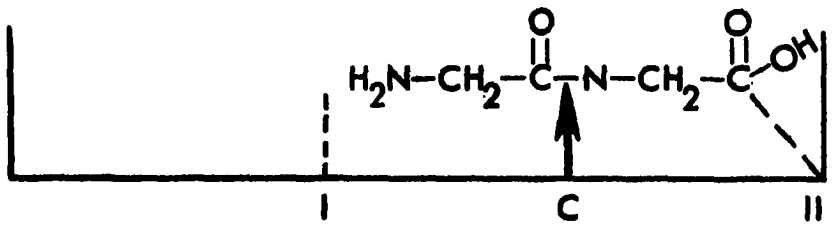
1)


H2N–CH2–C(=O)–OH
I
C
II

2a)


H2N–CH2–C(=O)–N–CH2–C(=O)–OH
I
C
II

2b)


H2N–CH2–C(=O)–N–CH2–C(=O)–OH
I
C
II

3)

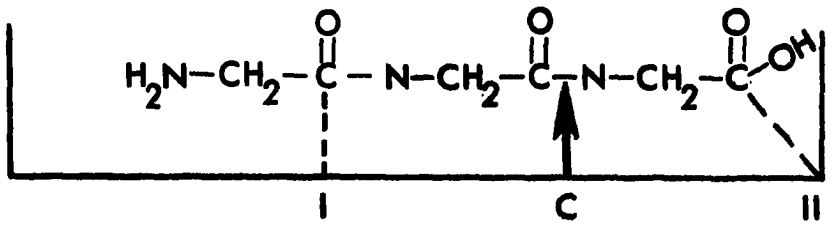

H2N–CH2–C(=O)–N–CH2–C(=O)–N–CH2–C(=O)–OH
I
C
II

Figure 32 shows the situation with dansyl-peptides. Dansyl-glycine is seen to be capable of binding, but incapable of further reaction. Dansyl diglycine has two binding possibilities. In 2a the dansyl diglycine binds and can be involved as the acceptor molecule in transpeptidation reactions. The binding in 2b, according to the model, is not allowed due to steric hinderance of the bulky dansyl group which would have to be incorporated into the active site for hydrolytic action on dansyl diglycine to take place. This would explain the inability of the enzyme to act on dansyl diglycine while it can act on unlabeled diglycine. Any other dansyl peptide (for example, dansyl triglycine) would be acceptable since the dansyl group could be excluded from the active site. This would explain why the only fluorescent product formed from dansyl triglycine is dansyl diglycine.

Table 11 gives the products predicted by the model for action of the lytic peptidase on dansyl glycyl peptides, and the actual products obtained. The model indicates why dansyl diglycine is the major product seen from these reactions since it is the final fluorescent breakdown product possible from all other dansyl glycyl peptides.

This model may also explain why some radioactivity was found at the origin of the chromatograms (with lysostaphin) in the attempt at utilizing ^{14}C-glycine in transpeptidation reactions. The model shows glycine could bind at the active site and still not be involved in any reactions there.

Figure 32

Postulated binding of dansyl glycyl peptides to active site of lytic peptidase.

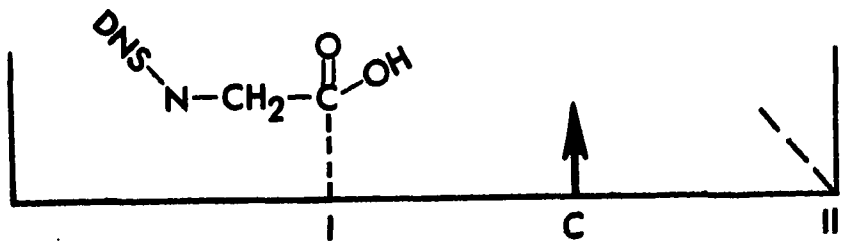
1) DNS-glycine--may bind but no involvement in reactions

2a) DNS-diglycine--may be involved in transpeptidation reactions as acceptor

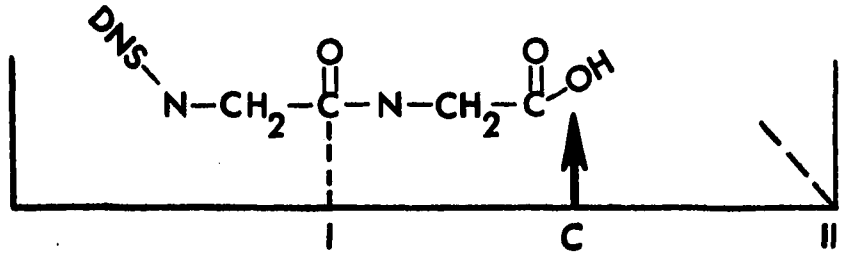
2b) DNS-diglycine--this type of binding not allowed due to steric hinderance of bulky dansyl group

3) DNS-triglycine--allowed binding since bulky dansyl group can be excluded from the active site.

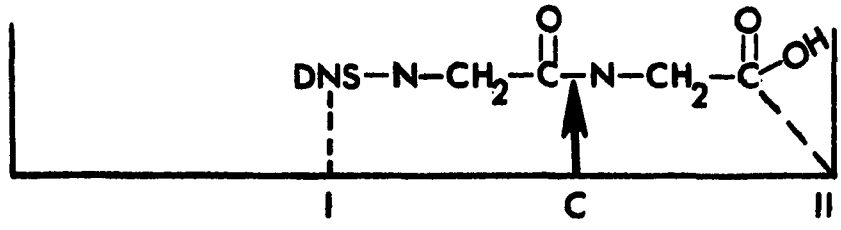
1)


DNS
N–CH2–C
O
OH
I
C
II

2a)


DNS
N–CH2–C–N–CH2–C
O
O
OH
I
C
II

2b)


DNS–N–CH2–C–N–CH2–C
O
O
OH
I
C
II

3)

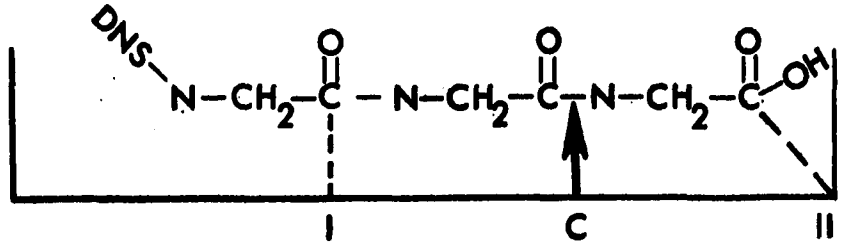

DNS
N–CH2–C – N–CH2–C–N–CH2–C
O
O
O
OH
I
C
II

TABLE 11.--A comparison of actual reaction products from hydrolysis of dansyl-glycyl peptides by the lytic peptidase and those predicted by the model

Compounds	Predicted	Found
DNS-gly	No reaction	No reaction
DNS-digly	Will work as acceptor in transpeptidation reactions only	Will work as acceptor in transpeptidation reactions only
DNS-trigly	DNS-digly	DNS-digly
DNS-tetragly	DNS-digly DNS-trigly⟶DNS-digly	DNS-digly (major product) and a small amount of DNS-trigly
DNS-pentagly	DNS-digly DNS-trigly⟶DNS-digly DNS-tetragly⟶DNS-digly	DNS-digly (major product) DNS-trigly (small amount) DNS-tetragly (small amount)
DNS-hexagly	DNS-digly DNS-trigly⟶DNS-digly DNS-tetragly⟶DNS-digly DNS-pentagly⟶DNS-digly	DNS-digly (major product) DNS-trigly (small amount) DNS-tetragly (small amount) DNS-pentagly (small amount)

The active site studies on the lytic peptidase give some preliminary evidence concerning the chemical make-up of the active site, and also perhaps to the mechanism of action of the enzyme.

The ability of metal-complexing reagents to inhibit lytic peptidase activity indicates the zinc atom in each molecule of the enzyme is essential for activity, being involved in either substrate binding, catalysis, or perhaps both. Zinc has been suggested as capable of binding to either carbonyl groups or amino nitrogen (44). At this time the group bound in the lytic peptidase is unknown.

The photooxidation studies seem to indicate that one or most histidines may be involved in the active site of the enzyme. Although other amino acid residues (such as tryptophan, tyrosine, and methionine) which are present in the lytic peptidase (7) have been reported to also be susceptible to this treatment, the increased sensitivity of the enzyme to photooxidation with the anionic dye rose bengal has been shown to indicate a histidine residue is being affected in other, similar, studies (25). This is further substantiated by the fact that a reagent specific for tyrosine residues (N-acetylimidazole) gave no inactivation of the enzyme at a concentration shown in other enzymes to acylate tyrosines important in their activity. Even at very high concentrations this reagent only destroyed 50% of the lytic peptidase activity. Similarly a reagent specific for

tryptophan (HNB-Br) was shown only to destroy approximately one-third of the lytic peptidase activity, indicating the alteration of the accessible tryptophans in the enzyme molecule will only partially affect catalytic activity.

Inhibition of enzyme activity by PMSF, an inhibitor of serine proteases, indicates a serine residue may be important in the active site of the lytic peptidase. The inability of the PMSF to completely inactivate the enzyme cannot be explained at this time. Whether the sulfonation of a serine in or near the active site gives only a partial loss in activity or if only a fraction of the serines are being blocked remains unanswered. Another possibility is that a residue other than serine is being modified by this reagent, and this modification results in a partial loss in activity. Other groups, such as the sulfhydryl group at the active site of papain, have also been reported capable of reacting with this reagent (45).

Treatment of the enzyme with $NaBH_4$ in the presence of substrate gives no inactivation of the enzyme, indicating there is no Schiff-base intermediate formed between the enzyme and its substrate. This does not necessarily mean there is not an ϵ-amino group from lysine involved in the active site, only that no such group is involved in a covalent, Schiff-base type intermediate.

The stoichiometric inactivation of lytic peptidase activity under the proper conditions by dansyl chloride

suggests this may be the best method of investigation for further studies on the active site of the lytic peptidase. This method has been used on several enzymes, reacting with a lysine, serine, or histidine residue, depending on the enzyme being studied (22,29,30). A differential labeling technique using radioactive dansyl chloride might also be utilized if the active site does not prove capable of preferentially reacting with dansyl chloride. The use of one of these techniques to label the active site so that a peptide from the active site may be isolated and studied as to the sequence of the amino acids present there is probably the next step necessary in order to discover the mechanism of action of this enzyme.

CHAPTER V

SUMMARY

Lysostaphin is a protein preparation obtained from the culture filtrate of an organism in the genus Staphylococcus. This preparation contains three enzymes, one of which, the lytic peptidase, is a very powerful lytic agent for other organisms in this same genus.

It was originally reported that the lytic peptidase specifically cleaved only glycyl-glycine bonds in the cell wall of staphylococci. Results from this investigation show the enzyme can also hydrolyze alanyl-alanine and glycyl-alanine bonds. The enzyme was also shown to be capable of catalyzing transpeptidation reactions in which a new peptide bond is formed by the transfer of a part of a peptide to another peptide, instead of to water as in hydrolysis.

There are two possible mechanisms of transpeptidation, one involving an acyl type enzyme intermediate, and the second involving an amine type intermediate. The lytic peptidase appears to catalyze the amine type reaction. This is only the second proteolytic enzyme so far reported to demonstrate this mechanism.

The lytic peptidase was also found to have penicillinase activity, being capable of hydrolyzing and thus inactivating penicillin. Penicillin was also shown to be a reversible competitive inhibitor of the lytic peptidase.

Metal complexing reagents were shown to inhibit the lytic activity of the lytic peptidase, which is a zinc metalloenzyme. This suggests that the zinc atom is at the active site of the enzyme and is required either for substrate binding or catalysis (or both).

The lytic peptidase was also found to be sensitive to photooxidation by rose bengal and methylene blue, suggesting there may be a histidine residue important in the active site of the enzyme.

Reagents specific for serine (phenylmethanesulfonyl fluoride), and tryptophan (2-hydroxy-5-nitrobenzyl bromide) gave only a partial inactivation of lytic peptidase activity. A reagent specific for tyrosine (N-acetylimidazole) gave no inactivation of lytic peptidase activity , except in very high concentration, indicating no tyrosine residues are involved in the active site.

Treatment with acetic anhydride gave a 100% inactivation of enzyme activity, suggesting one or more amino groups may be involved in the active site. Treatment of the enzyme with sodium borohydride in the presence of substrate gave no inactivation of enzyme activity, indicating the enzyme does not have a Schiff-base type intermediate in its mechanism of action.

The fluorescent compound dansyl chloride was shown to be a stoichiometric inhibitor of enzyme activity under certain reaction conditions, suggesting its use as a covalent label of the active site for further studies.

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