1971

Proteinase and Prodigiosin: Study of a Pleiotropic Mutation in Serratia Marcescens.

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Proteinase and Prodigiosin: Study of a Pleiotropic Mutation in *Serratia marcescens*

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Department of Microbiology

by
Charles Joseph Decedue
B. S., Louisiana State University in New Orleans, 1966
January, 1971

Acknowledgment

The author wishes to extend a sincere thank you to Dr. H. D. Braymer who has been both an excellent tutor and a good friend. He also wishes to thank Dr. A. D. Larson for the guidance and encouragement always supplied when it was needed most. The author also appreciates the time generously invested in him by all the members of the Department of Microbiology -- faculty, students and staff.

A very special thank you to Miss Sylvia Schreiner for maintaining the bacterial stocks used in this study and to Miss Amy Tynes for aid in completing the figures used in this dissertation.

Most of all, for her patience, help and understanding, the author wishes to express his gratitude to his wife Donna, to whom this dissertation is dedicated.

This research was supported in part by a traineeship, funded by Public Health Service Training Grant No. GM 00692 from the National Institute of General Medical Sciences, and by grant No. GB 6382 from the National Science Foundation.

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Abstract

A pleiotropic mutant of Serratia marcescens was isolated and identified. This mutant simultaneously loses the ability to synthesize pigment and 90% of the normal proteolytic activity. Revertants arise spontaneously which regain both characteristics found in the wild-type organism. Factors controlling pigmentation in the wild-type organism were studied and the relationship of proteinase and pigment were considered. A possible early precursor of the pigment, prodigiosin, has been isolated and attempts were made to characterize it. The product is extractable into chloroform yet releases threonine upon hydrolysis. The protein responsible for the remaining 10% of proteolytic activity of the mutant has been purified and partially characterized. An associating enzyme is indicated with molecular weight ranging from 8,400 to 32,400. The amino acid composition of the mutant proteinase is drastically different from that of the wild-type proteinase. This difference is clearly manifest in the elution profile and electrophoresis characteristics of the mutant protein. Despite the significant difference of amino acid composition, the mutant protein cross reacts with antiserum to the wild-type giving two bands one of which is caused by a very slow diffusing molecule. The substrate specificity of the mutant enzyme was partially elucidated and is clearly different from that of the wild-type.

Introduction

In 1823 an Italian pharmacist, Bartolomeo Bizio, using experiments similar to those of Spallanzani proved that the spots of blood miraculously appearing on the bread of local peasants were in fact caused by an organic being and not generated spontaneously. Incidental to dispelling fears about "bleeding bread" Bizio named and described the microorganism involved. He called it Serratia marcescens -- Serratia to pay tribute to an Italian physicist Serrafino Serrati and marcescens meaning putrifying or decaying. Thus Serratia is the third oldest named genus among bacteria being preceded only by the names Vibrio and Polyangium (Gaughran, 1969). Prior to 1823 Serratia marcescens had provided the basis of countless "miracles" of "bleeding bread" with recorded incidents going back as far as the time of Alexander the Great (Gaughran, 1969). Interest in the pigment of Serratia, therefore, is at least 2,000 years old. It was not until the mid-1930's however, that the pigment was first identified (Wrede and Rothaas, 1933a) and not until 1960 that the true structure of the pigment was proven (Rapoport and Holden, 1960).

To date only the final two or three steps in the biosynthetic pathway of this interesting pigment have been elucidated. Nothing is known about the biosynthetic origin of the pigment nor is there any acceptable hypothesis as to the function of the pigment.

Recent interest in S. marcescens has revolved around the extracellular

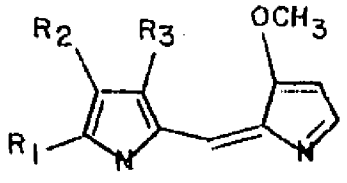
proteinase it produces (Broussard, 1968; Cruz-Camarillo et al., 1968; McQuade and Crewther, 1969; and Murukami et al., 1969). It is known that proteins in the medium stimulate the proteinase production (Murukami et al., 1969); the proteinase has been purified (Broussard, 1968; and Cruz-Camarillo et al., 1968) and its substrate specificity elucidated (Braymer et al., 1968; and McQuade and Crewther, 1969).

In the course of studying the proteinase of S. marcescens ATCC 25419 an apparent relationship between the proteinase and the pigment was observed. It was the purpose of this research to examine this relationship more carefully.

Literature Review

Serratia marcescens is a member of the family Enterobacteriaceae. It is a small Gram-negative rod usually producing a characteristic red, water insoluble pigment and is capable of rapidly liquifying gelatin (Bergey et al., 1939). Synonyms for Serratia marcescens are: Bacillus prodigiosus, Bacterium prodigiosum, Chromobacterium prodigiosum, and Micrococcus prodigiosus (Williams and Hearn, 1967).

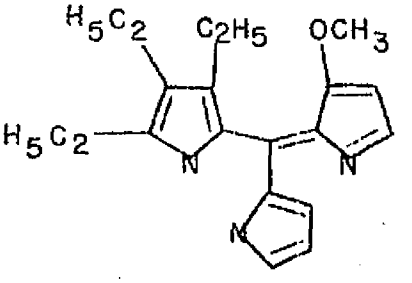
The characteristic red pigment is prodigiosin ($C_{20}H_{25}N_3O$) which was first extracted and named by Kroft (1902). It was first purified by Wrede and Hettche in 1929. In 1933 Wrede and Rothaas (1933a) began characterization of the pigment. By distillation of prodigiosin with soda lime, they obtained a product with the formula $C_{10}H_{17}N$. They were also able to isolate maleimide and methoxymaleimide from the pigment. By various oxidations and reductions they were able to determine that the $C_{10}H_{17}N$ product was a substituted pyrrole. The pigment was not cleaved by hydrolytic agents and was only slowly reduced with platinum hydroxide. They felt that the pigment could fit the formula below:



I

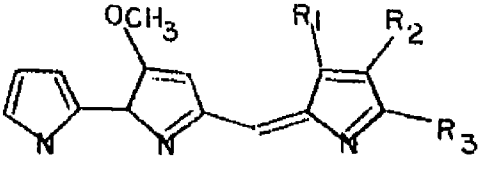
$$R_1 + R_2 + R_3 = C_6H_{12}$$

They hypothesized that the remainder of the molecule (C_4H_5N) was in the form of a third ring, probably a pyridine derivative. After this article appeared Raudnitz (1933) proposed that the remaining C_4H_5N did not constitute a pyridine derivative as proposed by Wrede and Rothaas (1933a) but rather was a third, unsubstituted pyrrole ring. He proposed the following structure:

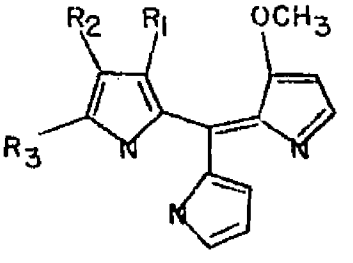


II

Shortly after this Wrede and Rothaas (1933b) confirmed that the third ring was an unsubstituted pyrrole. They proposed the following two models for prodigiosin:



III

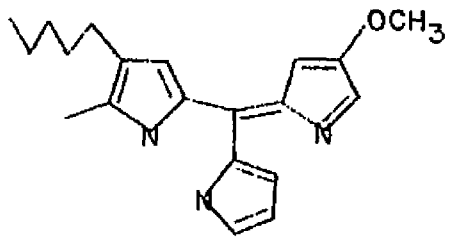


IV

Where $R_1 + R_2 + R_3 = C_6H_{15}$

On the basis of the color characteristics Wrede and Rothaas preferred structure IV (1933b). Finally in 1934 Wrede and Rothaas by comparing melting point and boiling point observations of synthetic compounds with those of prodigiosin identified the substituents of ring I as α-methyl- and β-n-amyl (Wrede and Rothaas, 1934). They then assigned the following structure to prodigiosin:

V

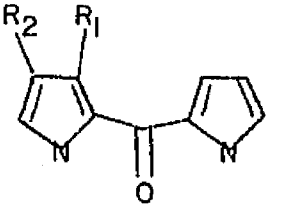


For the next quarter-century this structure remained unchallenged even though no attempt seems to have been made to synthesize the compound. One report was published in 1950 which seemingly lent support to the tripyrrylmethene structure (Hubbard and Rimington, 1950).

A chance observation in 1954 provided a clue which would ultimately result in the undoing of the Wrede and Rothaas model. In that year Rizki (1954a) observed cross-feeding by different white mutants of Serratia. Later (1954b) Rizki showed that the induced pigment was prodigiosin as judged by spectrophotometric analysis. Williams and Green (1954) also reported cross-feeding in Serratia colorless mutants.

The reports of successful cross-feeding experiments prompted Santer and Vogel (1956) to search for the accumulation of intermediates of

prodigiosin by white mutants capable of cross-feeding. In 1956 they isolated a stable prodigiosin precursor from mutant 9-3-3. This precursor was extracted from spent culture fluid with chloroform and purified on an alumina column. After recrystallization they obtained colorless needles which did not melt up to 250 C. Elemental analysis agreed with the formula $C_{10}H_{10}O_2N_2$. The precursor was shown to contain a pyrrole ring. This compound was proven to be a precursor of prodigiosin when radioactively labeled material purified from strain 9-3-3 was used to cross-feed another strain (WF); the pigment derived from this cross-feeding had the same radiospecific activity as did the C_{10} product which was added. Since this precursor differed from prodigiosin Rapoport and Willson reasoned that for the tripyrrylmethene structure to be correct for prodigiosin, the C_{10} precursor of Santer and Vogel had to be a pyrrylmethoxypyrryl ketone (Rapoport and Willson, 1962).



VI

$R_1 = H$, $R_2 = OCH_3$

To test this hypothesis they synthesized several unsymmetrical methoxy-dipyrryl ketones and compared them to the precursor (Santer and Vogel, 1956). On the basis of melting point, ultraviolet and infrared spectra it was clear that the precursor isolated by Santer and Vogel was not a

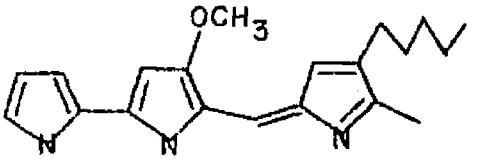
methoxydipyrryl ketone and as such the structure of prodigiosin could not be a tripyrrylmethene (Rapoport and Willson, 1962).

Interest in prodigiosin stemmed largely from the belief that the tripyrrylmethene structure was a precursor in the synthesis of porphyrins. Hoping to prove that prodigiosin was indeed a porphyrin precursor, Treibs and Hintermeir (1957) compared absorption spectra and properties of prodigiosin and a synthetic tripyrrylmethene. However, their data did not support the structure proposed by Wrede and Rothaas (1934). In 1959 Castro et al. synthesized 2, 2', 2'' tripyrrylmethene and compared it to prodigiosin on the basis of ultraviolet and infrared spectral qualities (Castro et al., 1959). The results of this work and that of Treibs and Hintermeir (1957) and Rapoport and Willson (1962) clearly demonstrated that prodigiosin was not a tripyrrylmethene.

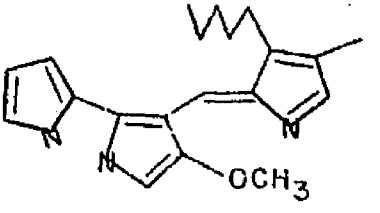
Treibs and Zimmer-Galler (1960) attempted to elucidate the structure of prodigiosin by synthesizing compounds which they felt would be logical models of prodigiosin. Although they were able to form compounds whose spectral qualities were much closer to prodigiosin than those of tripyrrylmethene, none of their products was identical to prodigiosin. A more systematic approach was taken by Wasserman et al. (1960a) who used the methylamylpyrrole isolated by Wrede and Rothaas (1933a) and the C_{10} precursor from mutant 9-3-3 to synthesize a pigment whose characteristics were indistinguishable from those of prodigiosin. From nuclear magnetic

resonance data they learned that the product isolated from mutant 9-3-3 contained a methoxy residue on carbon atom 3 and an aldehyde residue on carbon atom 2 or 4 (Wasserman et al., 1960a). These observations led them to propose two possible structures for prodigiosin:

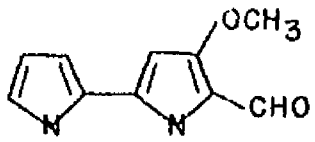
VII



or VIII



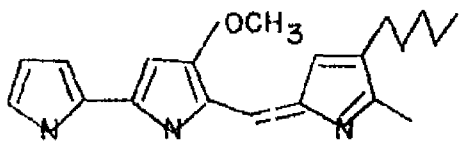
Wasserman et al. (1960b) synthesized the methyamylpyrrole (from 2-oximinooctan-3-one and ethyloxaloacetate followed by decarboxylation) which was identical to the $C_{10}H_{17}N$ degradation product of prodigiosin (Wrede and Rothaas, 1934). Cultures of Serratia marcescens 9-3-3 formed prodigiosin immediately upon exposure to the synthetic methylamylpyrrole. Thus the mutation in strain 9-3-3 prevented the organism from synthesizing the methylamylpyrrole (Wasserman et al., 1960b). Finally Rapoport and Holden (1960 and 1962) synthesized 4-methoxy-2,2'-dipyrrole-5-carboxaldehyde.



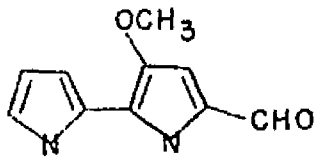
IX

This compound had the same properties as the 9-3-3 precursor (Santer and Vogel, 1956). When the synthetic dipyrrole was condensed with 2-methyl-3-amylpyrrole a red compound with ultraviolet and infrared spectra identical to prodigiosin was produced (Rapoport and Holden, 1960 and 1962).

X

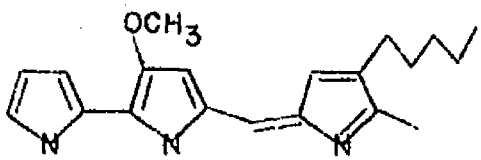


To avoid ambiguity they also synthesized 3-methoxy-2,2'-dipyrrole-5-carboxaldehyde,



XI

and condensed it with 2-methy-3-amylpyrrole to yield:

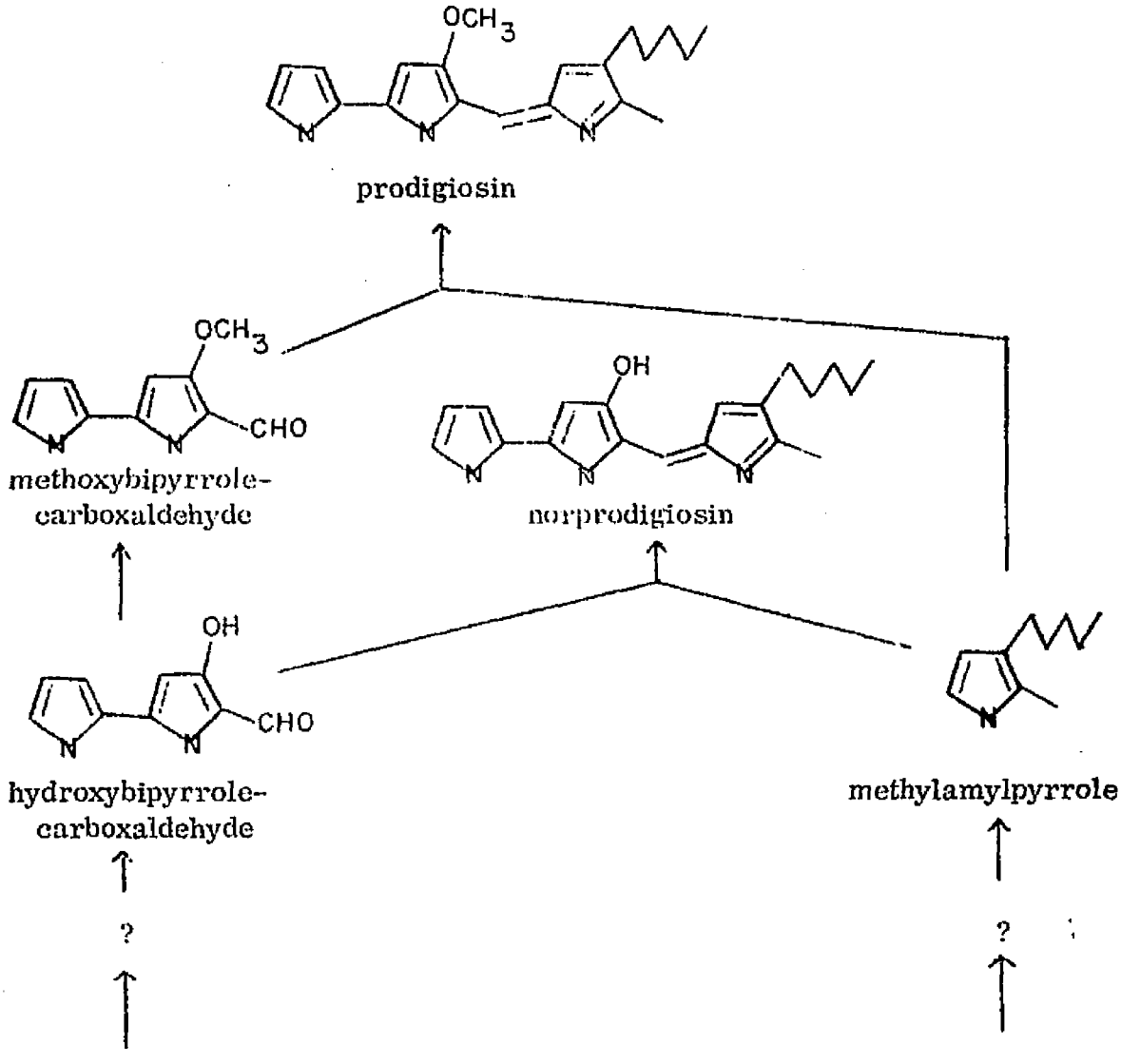


XII

This compound has an ultraviolet absorption maximum at 585 mμ as opposed to the 535 mμ maximum of both naturally occurring prodigiosin and structure X (Rapoport and Holden, 1960). Thus structure X was firmly established as the true structure of prodigiosin.

Biosynthesis

In 1958 Rizki showed that biosynthesis of prodigiosin probably took place along a bifurcated pathway (Rizki, 1958). From his earlier work with cross-feeding (Rizki, 1954a; Rizki, 1954b) he was able to classify mutants as inducers (i.e. producing a metabolite capable of diffusing to and stimulating pigment formation in another white mutant), reactors or both. Using mutants which were known to be both inducers and reactors, and a complicated system allowing the flow of metabolites to proceed in one direction only, he showed that regardless of the direction of flow only the cells receiving the metabolites were pigmented (Rizki, 1958). Such results are only possible if a bifurcated pathway is in effect and the two participants in the cross-feeding have mutations on opposite arms of the pathway. What is currently known of the pathway is shown below and taken from a review by Williams and Hearn (1967).



Although the final two or three steps in the synthesis of prodigiosin are well understood, very little is known about the initial steps of pigment formation. Before the tripyrryimethene structure (Wrede and Rothaas, 1934) was proven wrong (Rapoport and Holden, 1962), many workers felt that prodigiosin might be a natural precursor to the porphyrins. In 1950 Hubbard and Rimington presented data which they interpreted (wrongly)

as support for the tripyrrylmethene structure of prodigiosin. They also demonstrated, by the use of isotopes, that the nitrogen of prodigiosin came from glycine and that the carbon atoms came in part from the two carbons of acetate and the alpha carbon of glycine (Hubbard and Rimington, 1950). The nitrogen and alpha carbon of glycine seemed to be incorporated together. The authors suggested that the pyrrole rings of prodigiosin were synthesized in the same manner as those of the porphyrins (Shemin, 1944). Ten years later their hypothesis was tested and proven false (Marks and Bogorad, 1960). Marks and Bogorad reasoned that if the pyrrole rings of prodigiosin were formed in the same manner as those in the porphyrins, then $\triangle$-aminolevulinic acid should be incorporated into the pigment. $\triangle$-aminolevulinic acid was not incorporated into prodigiosin, however, even though it was apparently able to penetrate the cells (Marks and Bogorad, 1960). In 1941 Kost suggested proline as a possible precursor to prodigiosin. Marks and Bogorad (1960) also tested this possibility and found proline two and one-half times as effective as glycine as a prodigiosin precursor. This is in direct opposition to the results which showed glycine as a better precursor of porphyrins (Shemin and Rittenberg, 1946). More recently Qadri and Williams (1970) have shown that proline is an effective precursor of prodigiosin in stationary cultures.

Unfortunately, the value of the above experiments (Hubbard and Rittenberg, 1950; Marks and Bogorad, 1960; Qadri and Williams, 1970) is

lessened by the fact that low levels of incorporation were observed and substantial periods of time were involved in the labeling process (4-7 days for Hubbard and Rittenberg; Marks and Bogorad, and 20-24 hours for Qadri and Williams).

Pleiotropic Effects

There exists a dearth of information concerning the control of pigment formation in Serratia. It is generally accepted that pigment is not produced at temperatures above 37 C nor is it produced when the organism is grown anaerobically. Pigment is produced in chemically defined medium using glycerol as carbon source (Bunting, 1949) however, casein hydrolysate or glutamic acid were shown to inhibit pigment production (Weinberg, 1951) when added to basal medium.

Reports of pleiotropic mutations in Serratia, which involve pigment formation, might indicate a very elaborate system for the control of pigment formation.

In 1969 a report was published on the isolation of a bacteriocin in S. marcescens (Foulds and Shemin, 1969). The authors found that extracellular proteinase was destroying the bacteriocin and that bacteriocin was only produced when the cultures were grown above 37 C. A mutant was selected which could produce bacteriocin at 30 C (i.e., proteinase negative mutants). This mutant also lacked the ability to produce prodigiosin. The mutant reverted to bacteriocin temperature sensitivity and prodigiosin

production. Such mutants were also resistant to streptomycin whereas the revertants were sensitive. More direct information regarding the apparent relationship between proteinase and pigment comes from the work of McDonald on the extracellular proteinases of a red psychrophillic bacterium (Nunokawa and McDonald, 1968a). These proteinases separate into three fractions by gel filtration and chromatography on DEAE. Fraction I-1 is thought to be an aggregate of fraction III-1 while the fraction I-2 is different from these two (Nunokawa and McDonald, 1968b). A white variant of the red psychrophile was isolated and found to produce less proteinase than the red organism. The proteinase from the white organism was purified and shown to be a low molecular weight protein (component III). A larger molecular weight fraction separable into two components (I-1 and I-2) in the red organism but not in the white one is also present. Rechromatography of I-1 gives rise to component III while rechromatography of I-2 gives rise to enzymatically active I-2 and red pigment. In both red and white organisms component III was produced first, then as the culture aged the remaining fraction(s) were produced. The authors felt that component III aggregated to give component I-1 in both organisms and that component I-2 was a different proteinase somehow associated with the pigment (Nunokawa and McDonald, 1968b).

It is clear from the above two examples that pigment production and proteinase activity are associated in some manner.

Location of Prodigiosin

In Serratia marcescens it is very difficult to say with certainty where the pigment is located within the cell. For other organisms the location of the pigment in the cell membrane has been established (e.g., Sarcina lutea and Rhodospirillum rubrum: Mathews and Sinstron, 1959; Hickman and Frenkel, 1959). An initial clue to this problem was provided by Yoshida who described the isolation and characteristics of a "water-soluble" form of prodigiosin (1962). This pigment consisted of a 5,000,000 molecular weight complex made up largely of protein and carbohydrate. Prodigiosin could be extracted from this complex and it was shown to constitute 0.4% of the complex. This accounted for a molecular weight of 80,000 -- the equivalent of sixty molecules of prodigiosin per molecule of complex. The formation of this complex gradually increased after prodigiosin formation had reached a maximum (Yoshida, 1962). Williams demonstrated that this was not a "water-soluble" pigment but rather a particulate fraction (Williams and Krell, 1963; Williams and Taylor, 1959). He was able to sediment the pigment easily by centrifugation at 105,000 X G, and proposed that these pigment particles are released from disintegrating cells (Williams and Krell, 1963). Williams is of the opinion that the pigment is located in the cell "envelope" on the basis of experiments carried out in 1960 in which he followed the release of pigment and acetylhexosamine from sonicated cells treated with lysozyme (Purkayastha and Williams, 1960). Electron

microscopy and further purification by techniques known to yield cell envelopes added support to this hypothesis (Purkayastha and Williams, 1960). Recently a prodigiosin-protein complex was isolated (Cruz-Camarillo and Sanchez-Zuniga, 1968) which had all of the characteristics of the Yoshida (1962) complex. Lastly, there is a report (Hubert et al., 1969) that Serratia L-forms produced by penicillin treatment, are colorless. Since there was no evidence that the pigment had "leaked" into the medium, it appeared to the authors that the cell wall was involved in the synthesis of prodigiosin.

Prodigiosin-like pigments and analogs have been reported in Actinomadura (Nocardia) (Gerber, 1969), Actinomycetes (Dietzel, 1948), and Streptomyces (Wasserman et al., 1961).

Materials and Methods

Microorganisms

The wild-type strain used in this study was isolated from diseased cotton boll weevils (Slatten and Larson, 1967) and is maintained in the American Type Culture Collection (Serratia marcescens ATCC 25419). The mutant organisms were derived either spontaneously or through the use of N-methyl-N'-nitro-N-nitrosoguanidine (4 μg/ml) and were selected on the basis of their ability to form a white precipitate around a colony growing on 1% casein-agar plates. These were designated prd (prodigiosin) mutants. Other mutants arising spontaneously were selected by the following procedure: wild-type cells from an old slant were collected in 0.1 M phosphate buffer pH 7.5, washed, resuspended in 100 ml of brain heart infusion (Difco) and allowed to grow overnight. One-tenth milliliter of an appropriate dilution of this suspension was spread onto each of fifty plates containing 1% gelatin, 1% glycerol and medium A (components listed on page 18) with 1.5% agar (Difco). After the colonies appeared replicas were made of those plates having isolated colonies. Many colonial morphology and color mutants were observed on the original plates. After colonies had appeared on the replica plates the original plates were flooded with 3 M trichloroacetic acid (TCA) and those colonies which did not show a zone of hydrolysis were picked from the corresponding replica plate and were streaked for isolation on 1% gelatin plates. Single colonies from these

plates were picked and transferred to slants of BHI. The replica plates were again flooded with TCA and scored for clearing. Cells from mutant colonies showing no clearing were grown on complete medium and assayed for proteolytic activity. These were designated prt (proteinase) mutants.

Media

Cultures were routinely maintained on slants of brain heart infusion (Difco). The usual growth medium contained glycerol plus the basal salts solution used at Cold Spring Harbor for the growth of E. coli and Salmonella. It consists of the following:

(1 X strength)

K_2HPO_4	10.5	gm
KH_2PO_4	4.5	gm
$Na_3C_6H_5O_7 \cdot 2H_2O$	0.397	gm
$MgSO_4 \cdot 7H_2O$	0.107	gm
$(NH_4)_2SO_4$	1.0	gm
H_2O	1	L

The salt solution was prepared at 10 X strength and sterilized separately. Glycerol (E. H. Sargent and Co., Houston, Texas) was used as carbon source at a concentration of 1% (v/v); complete medium was made by adding 1% (w/v) gelatin (Difco) or vitamin free casein (Nutritional Biochemical Corp., Cleveland, Ohio) to the glycerol medium.

Casein used for the assay of proteinase was purchased from Nutritional Biochemical Corp., Cleveland, Ohio. Ribonucleic acid and deoxyribonucleic acid used as substrates in the assay of nuclease activity were obtained from Calbiochem, Los Angeles, Calif. The reduced carboxymethylated β-chain of insulin used in the substrate specificity experiments was obtained from Mann Research Laboratories, New York, N.Y. The 2'-7'-dichlorofluorescein used in developing TLC plates was purchased from Eastman Organic Chemicals, Rochester, N.Y. All additional organic chemicals used in this research were reagent grade and were obtained from various sources.

Methods

Proteinase assay. Proteinase activity was assayed by the casein digestion method as described by Kunitz (1955) with the following exceptions: casein was dissolved in 0.1 M phosphate buffer pH 7.5, and a separate blank was prepared for each enzyme dilution used. One unit of activity was defined as the quantity of proteinase required to solubilize sufficient casein to cause an increase in absorbance at 280 nm of 0.1 unit.

Preparation of antiserum. One rabbit was immunized with 20 mg of highly purified proteinase prepared from wild-type Serratia (Broussard, 1968). Freunds complete adjuvant was used and the emulsion (2.0 ml) injected into all four foot pads and intradermally in the scapular region and along the spine. Serum was collected sixty days after injection without

further boosting. The precipitin titer was greater than 1:100. This antiserum was used to detect cross reacting material in proteinase preparations and culture supernatant fluids obtained from the mutant organisms by the double diffusion technique of Ouchterlony.

Disc electrophoresis. The procedure for disc electrophoresis was essentially that described by Davis (1964) with the following exceptions: a) samples were dissolved in a 40% sucrose solution rather than a sample gel and layered directly over the spacer gel, b) a Beckman/Spinco Constat regulated power supply was used to supply four milliamps per tube, c) gels were stained for ten minutes with a 1% solution of Buffalo Black NBR (K & K Laboratories, Plainview, N.Y.) in 7% acetic acid. The gels were destained by shaking in several changes of 7% acetic acid.

Amino acid analysis. Amino acid analyses were performed on a Beckman 120 C amino acid analyzer. One milligram samples of protein were hydrolyzed in vacuo in 2 ml of 6 N HCl for 24, 48, or 72 hours. After drying in a vacuum desiccator and resuspension in 1 ml of 0.2 N citrate buffer, pH 2.2, samples of 0.2 ml were analyzed. The quantity of each amino acid was determined by measuring the area under the corresponding peaks by the H X W method of Spackman et al. (1958).

Purification of proteinase. Proteinase from both the wild-type and mutant organisms was purified according to the procedure outlined by Broussard (1968) for the purification of wild-type Serratia proteinase

hereafter referred to as serratolysin. The organism was grown for 36 hours in complete medium in 2 L flasks containing 1 L of broth. The cells were incubated at room temperature (25 C) on a gyrorotatory shaker and the spent medium was cleared in a Sharples centrifuge. The supernatant fluid was brought to 80% saturation with $(NH_4)_2SO_4$ and allowed to precipitate for 12 hours. The precipitate was collected in the Sharples super centrifuge (at a maximum of 20 C), redissolved in a minimal amount of 0.05 M phosphate buffer, pH 6.7, and dialyzed against three changes of the same buffer over a 12 hour period. The redissolved 80% $(NH_4)_2SO_4$ precipitate was adjusted to 10 mg/ml protein and refractionated by bringing the liquid to 30% saturation with $(NH_4)_2SO_4$ and allowing it to precipitate for one hour. The precipitate was removed by centrifugation and discarded. The supernatant fluid was brought to 50% saturation with $(NH_4)_2SO_4$ and allowed to precipitate for one hour. Again the precipitate was removed by centrifugation and this time the supernatant was discarded. The precipitate was redissolved in a minimal amount of 0.05 M phosphate buffer, pH 6.7, and dialyzed against three changes of the same buffer. The redissolved 30-50% fraction was dialyzed against three changes of 0.05 M phosphate buffer, pH 8.0, applied to the top of a 30 X 4.5 cm DEAE column which had previously been equilibrated with 0.05 M phosphate buffer, pH 8.0. Three hundred milliliters of 0.05 M phosphate buffer, pH 8.0, were washed through the column and 10 ml fractions were collected. After this a linear

gradient consisting of 1 L of 0.05 M phosphate buffer, pH 8.0, in the mixing vessel and 1 L of 0.6 M phosphate buffer pH 8.0, in the reservoir was used to elute the protein and 10 ml fractions were again collected. Protein peaks were located by measuring the 280 nm absorbance of the fractions. Fractions under these peaks were pooled, dialyzed against a single volume of deionized water and lyophilized. The dried material was stored at -70 C and redissolved in 0.1 M phosphate buffer pH 7.5 before use.

Substrate specificity. The sites of cleavage by the mutant serratolysin were determined using the reduced, carboxymethylated β -chain of insulin as substrate. Twenty-five milligrams of insulin (β -chain) were dissolved in 10 ml H_2O and adjusted to pH 8.5 with NH_4OH. Two hundred fifty micrograms of mutant serratolysin were added to the insulin and allowed to react for thirty minutes at 30 C. The digested sample was immediately lyophilized. The dried digest was dissolved in a minimal amount of 0.01 N NH_4OH and spotted along the width of a 21 X 8 inch strip of Whatman 3MM chromatography paper. The peptides in the digest were first separated by descending chromatography using a solvent of 2-butanol, 90% formic acid, and H_2O in the proportions 7:1:2. Thin strips were cut from both sides of the completed and dried chromatogram and developed by dipping in 1% ninhydrin in acetone and heating to 80 C. These strips served as convenient markers when placed back in their original position in the chromatogram. The paper containing the peptides was cut out and eluted with 0.1 NH_4OH. The peptides

were again lyophilized and dissolved in a minimal amount of 0.1 N NH_4OH. Each peptide was spotted along the width of another 21 X 8 inch strip of chromatography paper and further separated by high voltage electrophoresis. Electrophoresis was carried out at pH 3.5 in pyridine, acetic acid, water (1:10:300) using 2400 V and 140 ma supplied by a Savant power supply unit. A Savant electrophoresis tank was employed and varsol served as coolant. Again strips were cut from both sides of the electrophoretogram and stained with ninhydrin; the corresponding peptides were eluted as before. The eluted peptides were hydrolyzed with 6 N HCl for 24 hours and analyzed for amino acids. The composition of the peptides was compared to the known amino acid sequence of the reduced, carboxymethylated β -chain of insulin.

Exonuclease activity. Exonuclease activity was determined against Torula RNA (B grade) and salmon sperm DNA (A grade) according to the procedure outlined by Nestle and Roberts (1969).

Ultracentrifugation. Sedimentation velocity experiments were performed in a Beckman Model E analytical ultracentrifuge using a double sector synthetic boundary cell with quartz windows. A speed of 56,000 RPM was used employing the An D rotor and schlieren optics and photographs were taken usually at eight minute intervals beginning when 2/3 of maximum speed was obtained. All experiments were at 20 C.

The protein for these experiments was dissolved in 0.05 M phosphate buffer containing 0.1 M NaCl, pH 7.0, and then dialyzed against three changes

of the same buffer. The final dialysate was retained and used in the reference side of the cell. Photographic plates were measured on a Nikon profile projector model 6C. Sedimentation coefficients were calculated according to the following formula (Schachman, 1957):

$$S_{obs} = \frac{2.303}{60\omega^2} \quad \frac{d \log x}{dt}$$

where ω^2 = angular velocity in radians per second, x = distance (cm) from axis of rotation to protein boundary and t = time. The S_{obs} was converted to Svedbergs by dividing by 10^{-13}. S_{obs} was converted to $S_{20,w}$ according to the following:

$$S_{20,w} = S_{obs} \frac{\eta_{sol}}{\eta_{w}} \quad \frac{1 - \bar{v}\rho_{20,w}}{1 - \bar{v}\rho_{t,sol}}$$

where η = viscosity, $\bar{v}$ = partial specific volume and ρ = density. All S values were determined on varying concentrations of protein and extrapolated to zero concentration.

Sedimentation equilibrium experiments were performed according to the method of LaBar (1965). The protein concentration was 0.3 mg/ml in 0.05 M phosphate buffer containing 0.1 M NaCl pH 7.0. Equilibrium was attained after 16 hours at 26,000 RPM. After equilibrium was reached, photographs were taken at eight minute intervals on Eastman spectroscopic IIG plates. The speed was then increased to 56,000 RPM and more photographs taken. Molecular weight was computed with the following formula:

$$\frac{d \ln j_r}{dr^2} = \frac{(1 - \bar{v}\rho)\omega^2 M}{2RT}$$

where j = the number of fringes, r = radial distance, M = weight average molecular weight, R = gas constant (8.314×10^7 ergs/mole · degree) and T = absolute temperature.

Extraction of precursor. Material suspected to be a prodigiosin precursor was extracted from culture medium with equal volumes of chloroform and its presence in the solvent was determined in a Beckman DB spectrophotometer by scanning from 400 nm to 220 nm.

Cross-feeding. Complete medium A plates were inoculated with three mutants of Serratia in the shape of a triangle. Mutant 9-3-3 was used to form one leg of the triangle, WF the other leg on each plate and one of the unknown mutants formed the base. The three different organisms were not allowed to come into contact with each other, but were in reasonable proximity to one another so that metabolites could diffuse across the agar in a short period of time.

C^{14} incorporation. Uniformly labeled C^{14} glycerol was purchased from New England Nuclear Corp., Boston, Mass. and had a specific activity of 29.6 mc/mM. Cells were grown in glycerol medium for twelve hours, washed and resuspended in fresh medium containing the C^{14} glycerol. The cells were allowed to continue incubating at room temperature. The first sample was collected as soon as the label was added and additional samples

were collected at hourly intervals. The samples were extracted with chloroform, dried by evaporation in counting vials and 10 ml of liquid scintillation cocktail was added before counting in a Beckman liquid scintillation system.

The scintillation cocktail used consisted of the following: 60 g naphthaline, 4 g 2,5-diphenyloxazole (Packard Instrument Co., Inc., Downers Grove, Ill., scintillation grade), 200 mg 1,4-bis-2-(5-phenyloxazlyl)-benzene (Packard Instrument Co., Inc., Downers Grove, Ill., scintillation grade), 100 ml absolute methanol, and dioxane to make 1 L.

Thin layer chromatography. Thin layer chromatography was performed on silica gel F 254 plates purchased from Brinkman Instruments Inc., Westbury, N.Y. The solvent system used to develop the chromatograms consisted of n-hexane, ethyl ether, acetic acid in the proportions 90:10:2.

Results

Characteristics of Pigment Formation in S. marcescens ATCC 25419

The organism, S. marcescens ATCC 25419, is characteristic of those in the genus Serratia in that pigment production is repressed at high growth temperature or low oxygen tension. As shown in Table 1, growth at 37 C not only results in loss of pigment but also in severe reduction of proteolytic activity. Growth at 30 C on the other hand results in production of the deep red pigment and a concomitant high degree of proteolytic activity. When observed on solid medium containing casein, incubation at 30 C results in intensely red colonies surrounded by a wide zone of hydrolysis while incubation at 37 C results in white colonies surrounded by a zone of white precipitate similar to that surrounding the prd mutants described in materials and methods.

When glucose or sucrose is used as the carbon source for the growth of S. marcescens ATCC 25419, pigment formation is repressed even at 30 C. The results of attempts to relieve this repression by the addition of various compounds are shown in Table 2. Although serine, proline, histidine, and glycine stimulated pigment production, an acid hydrolysate of casein caused further repression (stark white area against a faint pink background). Casein caused profound stimulation of pigmentation, but casein which had been digested by purified serratolysin again resulted in further repression. Alanine, isoleucine and glutamic acid also caused additional repression.

Table 1

Comparison of extracellular proteolytic activity from cultures incubated at different temperatures[1].

Temperature	Color	Proteolytic Activity u/ml
30	Red	83
37	White	4

[1] S. marcescens ATCC 25419 was grown at 30 C in BHI. Culture supernates were assayed after 36 hours growth according to the procedure outlined in Materials and Methods.

Table 2

Reversal of glucose-mediated pigment repression.

Compound Applied[1]	Pigmentation[2]
Casein	+++
Casein hydrolysate	--
Casein digest	--
Alanine	--
Aspartic acid	N
Arginine	N
Cystein	N
Glutamic acid	--
Glycine	+
Histidine	+
Isoleucine	--
Leucine	N
Lysine	N
Methionine	N
Phenylalanine	N
Proline	++
Serine	+++
Threonine	N
Tryptophan	N
Tyrosine	N
Valine	N

[1] Plates of medium A with 1% (w/v) glucose were spread with a suspension of S. marcescens ATCC 25419 and spotted with either vitamin free casein, acid casein hydrolysate, all common amino acids, or a preparation of vitamin free casein that had been digested by purified wild-type serratolysin. The plates were then incubated for 24 hours at room temperature.

[2] +++ Red, ++ Dark pink, + Pink, N No effect (faint pink), -- White.

Characteristically Serratia can produce pigment when grown on a medium consisting only of basal salts and glycerol (Bunting, 1949). Although ATCC 25419 produces pigment on minimal medium, proteinase production depends upon the presence of exogenous protein in the growth medium as shown in Table 3. The high proteinase activity observed with growth on acid hydrolyzed casein is assumed to be induction by residual peptides found in the commercial preparation of the hydrolysate since a synthetic mixture of amino acids results in total repression of proteinase activity.

Further examination of cells grown in the presence and absence of a protein source indicates that enzyme synthesis and not release is affected (Table 4). Culture supernatants of cells grown on minimal medium as well as sonicates of the cells themselves showed no proteolytic activity. Supernatants but not sonicates of cells grown on medium containing protein showed normal proteolytic activity. When supernatants and sonicates of induced and non-induced cultures were tested for cross-reacting material using antiserratolysin antiserum (Figure 1), the supernatant from induced cultures contained a protein that forms a band of identity with purified serratolysin; sonicates of induced cultures had a slow-diffusing band of cross-reacting material which was identical to the slow-diffusing band of cross-reacting material found in sonicates of uninduced cells and fraction II of prd A2 proteinase (see Figure 12): supernatants of non-induced cultures

Table 3

Control of proteinase production[1].

Medium	Proteinase Activity u/ml[2]
1% glycerol	0.5
1% glycerol + 1% casein	45.5
1% glycerol + 1% casein hydrolysate (acid)	26.0
1% glycerol + 18 amino acids (total 1%)	0

[1] S. marcescens ATCC 25419 was grown for 36 hours at room temperature. Culture supernates were assayed for proteolytic activity.

[2] Average of two determinations.

Table 4

Location of proteolytic activity in cells and culture fluids of Serratia marcescens.

Medium	Fraction[1]	Activity u/ml
1% glycerol	Supernatant	0
	Sonicate	0
1% glycerol + 1% casein	Supernatant	45
	Sonicate	0

[1] Cell supernatants were obtained by clearing spent medium by centrifugation at 25,000 X G for 20 minutes. Cell sonicates were prepared by a three minute exposure to a 20 Kc Branson sonifier at full power. The resulting suspension was cleared of cell debris by centrifugation at 25,000 X G for 10 minutes.

Figure 1. Representation of Ouchterlony plate showing cross reactivity of various preparations to antiserratolysin antiserum.

Center well: antiserratolysin antiserum.

Well No. 1: sonicate of induced culture of S. marcescens ATCC 25419.

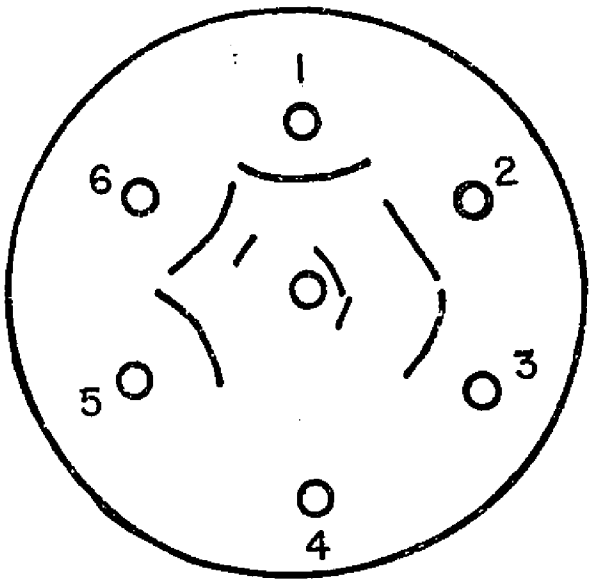
Well No. 2: purified serratolysin.

Well No. 3: supernatant of induced S. marcescens ATCC 25419.

Well No. 4: supernatant of non-induced S. marcescens ATCC 25419.

Well No. 5: sonicate of non-induced S. marcescens ATCC 25419.

Well No. 6: purified prd A2 proteinase.


1
2
3
4
5
6

did not react with antiserratolysin antiserum.

Characterization of prd and prt Mutants

Table 5 summarizes the pigmentation and proteolytic activity of the mutants and the mutagen utilized in deriving them from S. marcescens ATCC 25419. All seven prd mutants and all eight prt mutants have drastically reduced proteolytic activity (10% or less of wild-type activity) with concomitant loss of pigment. It is also clear from Table 5 that S. marcescens ATCC 25419 produced a highly active proteinase in comparison to other wild-type strains of S. marcescens (nima and urinae). Five revertants of the prd mutants were isolated and these regained both pigment and full proteinase activity. The rate of appearance of revertants for those mutants which did revert was grossly different for each mutant.

Because of the obvious relationship between proteinase production and pigmentation, attempts were made to effect reversion of the phenotype of the prd mutants by supplying missing metabolites. Addition of purified serratolysin to lawns of the seven prd mutants growing on plates of casein complete medium resulted in hydrolysis of the casein leaving a zone of clearing, but did not stimulate pigment production. Addition of serratolysin-digested casein to the growth medium of prd mutants also failed to stimulate pigment production. Although Marks and Bogorad (1960) have shown that Δ-aminolevulinic acid is not incorporated into prodigiosin, no reports have appeared regarding pyrrolidone carboxylic acid as a possible precursor.

Table 5

Characteristics of organisms studied.

Organism[1]	Mutagen	Color	Proteolytic Activity u/ml
ATCC 25419	--	R[4]	121
prd A1	S[2]	W[5]	10
prd A2	S	W	12
prd A3	NG[3]	W	10
prd A4	NG	W	9
prd A5	NG	W	11
prd A6	NG	W	12
prd A7	NG	W	15
prt 11B	S	W	1.2
prt 17B	S	W	2.6
prt 19B	S	W	5.0
prt 20D	S	W	1.2
prt 20E	S	W	1.8
prt 28A	S	W	0
prt 32A	S	W	2.0
prt 35A	S	W	0.8
prd A1 R1	S	R	105
prd A3 R3	S	R	93.5
prd A4 R4	S	R	79
prd A6 R6	S	R	86.5
prd A7 R7	S	R	94
nima	--	R	5.2
urinae	--	R	9

[1] All mutant strains were derived from S. marcescens ATCC 25419. R1, R3, R4, R5, R6, and R7 are spontaneous revertants.

[2] Spontaneous.

[3] N-methyl-N'-nitro-N-nitrosoguanidine.

[4] Red.

[5] White.

Pyrrolidone carboxylic acid did not stimulate the prd mutants to form pigment; thus if it is a precursor of prodigiosin, it would have to be assumed that the biosynthetic block occurs after the synthesis of pyrrolidone carboxylic acid.

Cross-feeding

Using the mutants 9-3-3 and WF attempts were made to locate the site of the prd mutations in relation to the partially elucidated biosynthetic pathway of prodigiosin (Williams and Hearn, 1967). Mutant 9-3-3 is a white mutant known to accumulate the bipyrrole precursor (Santer and Vogel, 1956) and WF is a white mutant which produces the other half of prodigiosin -- the volatile methylamylpyrrole. In the presence of condensing enzyme these two precursors form prodigiosin. All seven prd mutants were tested for their ability to cross-feed with 9-3-3 and WF. No cross-feeding was observed between any of the prd mutants and these two mutants. If, however, 9-3-3 and WF were spread on the same plate along with any of the prd mutants, pigment was observed in that area on the prd streak where diffusion of the bipyrrole of 9-3-3 would overlap with the volatile monopyrrole. This indicates that the prd mutants possess the condensing enzyme but they do not synthesize any of the known precursors.

Precursor Studies

A culture of mutant prd A3 when extracted with an equal volume of

chloroform yielded a substance with a characteristic absorption spectrum (Figure 2). The absorption maxima at 315 nm and 255 nm seemed to occur in a constant ratio for several separate extractions. All of the prd mutants yielded a similar product upon extraction with chloroform. S. marcescens ATCC 25419 when extracted with chloroform gives the pattern shown in Figure 3 which is characteristic for prodigiosin. Note the large absorption maximum at 540 nm and the total lack of 315 nm absorbing material. Clearly the chloroform-soluble compounds extracted from the prd mutants and the wild-type organism are different. Mutant 9-3-3 also yields a chloroform soluble product, the bipyrrole, and its spectrum is shown in Figure 4 for comparison. The bipyrrole exhibits a symmetrical peak with an absorption maximum at 363 nm which is also markedly different from the spectrum of the product of the prd mutants. Since the growth conditions and the medium for all three of the above were the same there is obviously no product in the medium which could cause the observed effect. Clearly then prd mutants of S. marcescens ATCC 25419 accumulated a product which is not found in the pigmented varieties. This product was excreted into culture medium and was not found in the cells per se as shown in Figure 5 a and b.

The chloroform extract of prd A3 was taken to dryness and the melting point was determined on a Fisher-Johns melting block. The material, which was tan in color, melted at 217-218 C. The appearance of the product in culture supernates as a function of time was studied and the results are

Figure 2. Spectrum of prd A3 chloroform-soluble product.

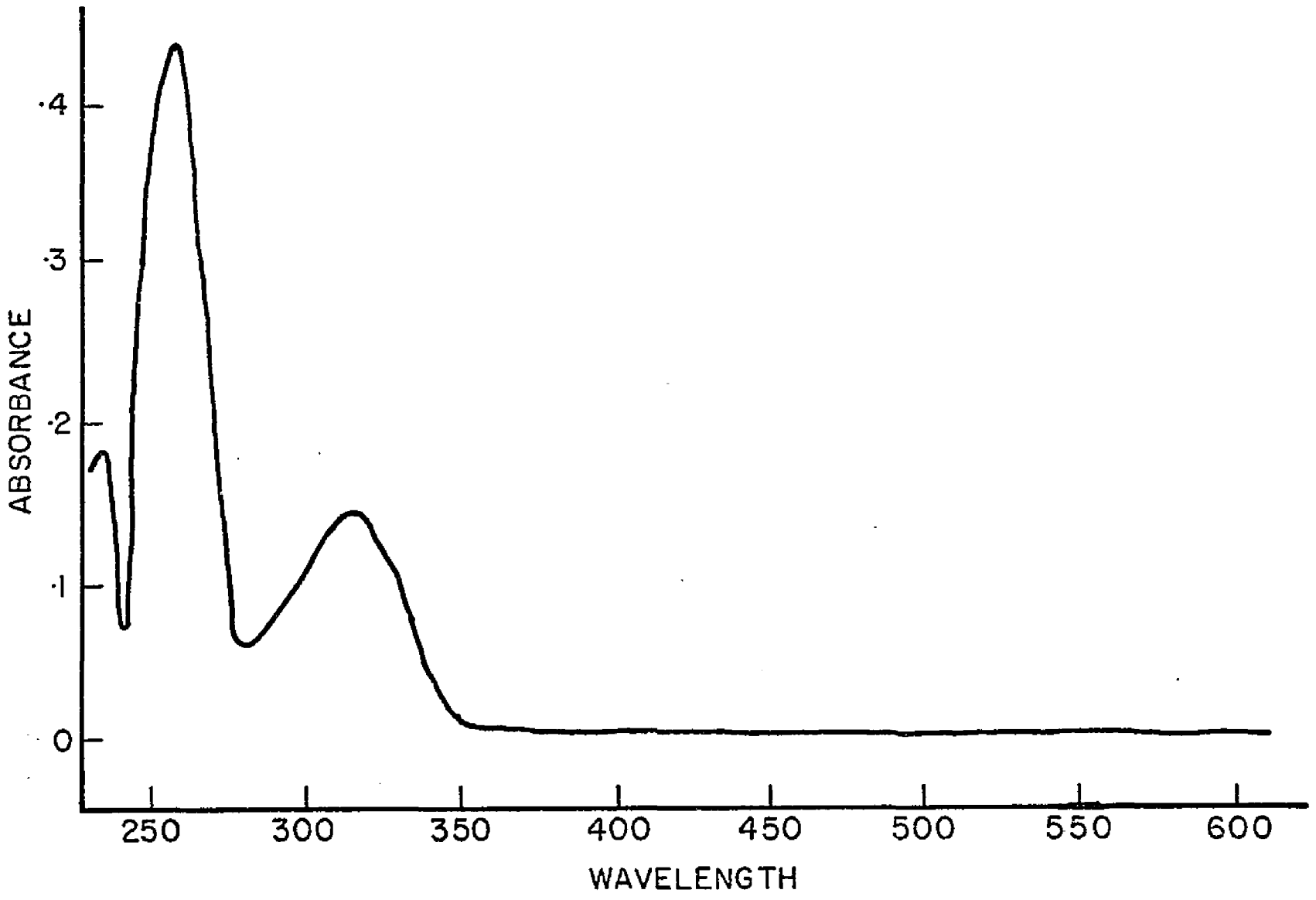

ABSORBANCE
·4
·3
·2
·1
0
250
300
350
400
450
500
550
600
WAVELENGTH

Figure 3. Spectrum of chloroform extract of S. marcescens ATCC 25419.

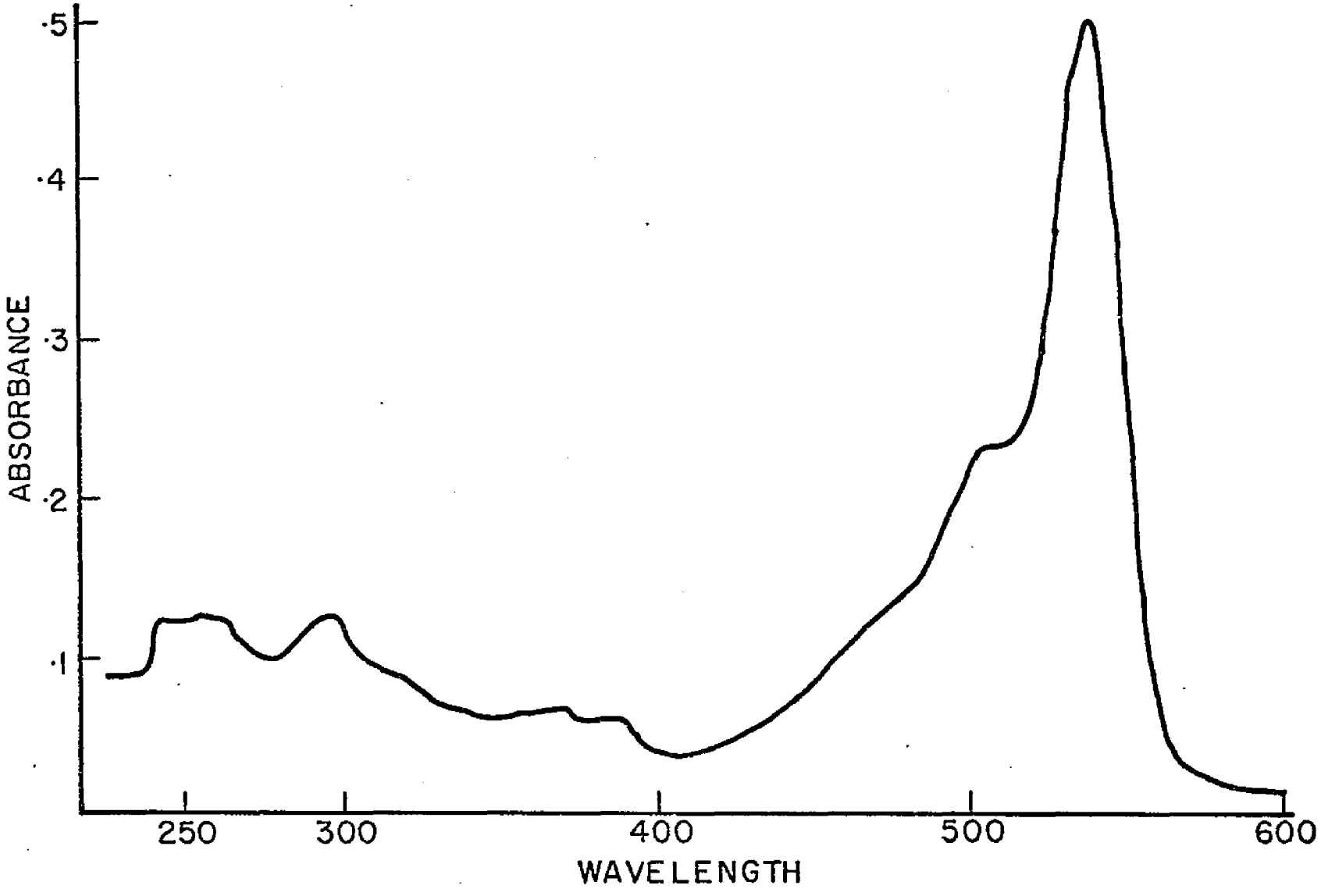

·5
·4
·3
·2
·1
ABSORBANCE
250
300
400
500
600
WAVELENGTH

Figure 4. Spectrum of chloroform extract of S. marcescens mutant 9-3-3.

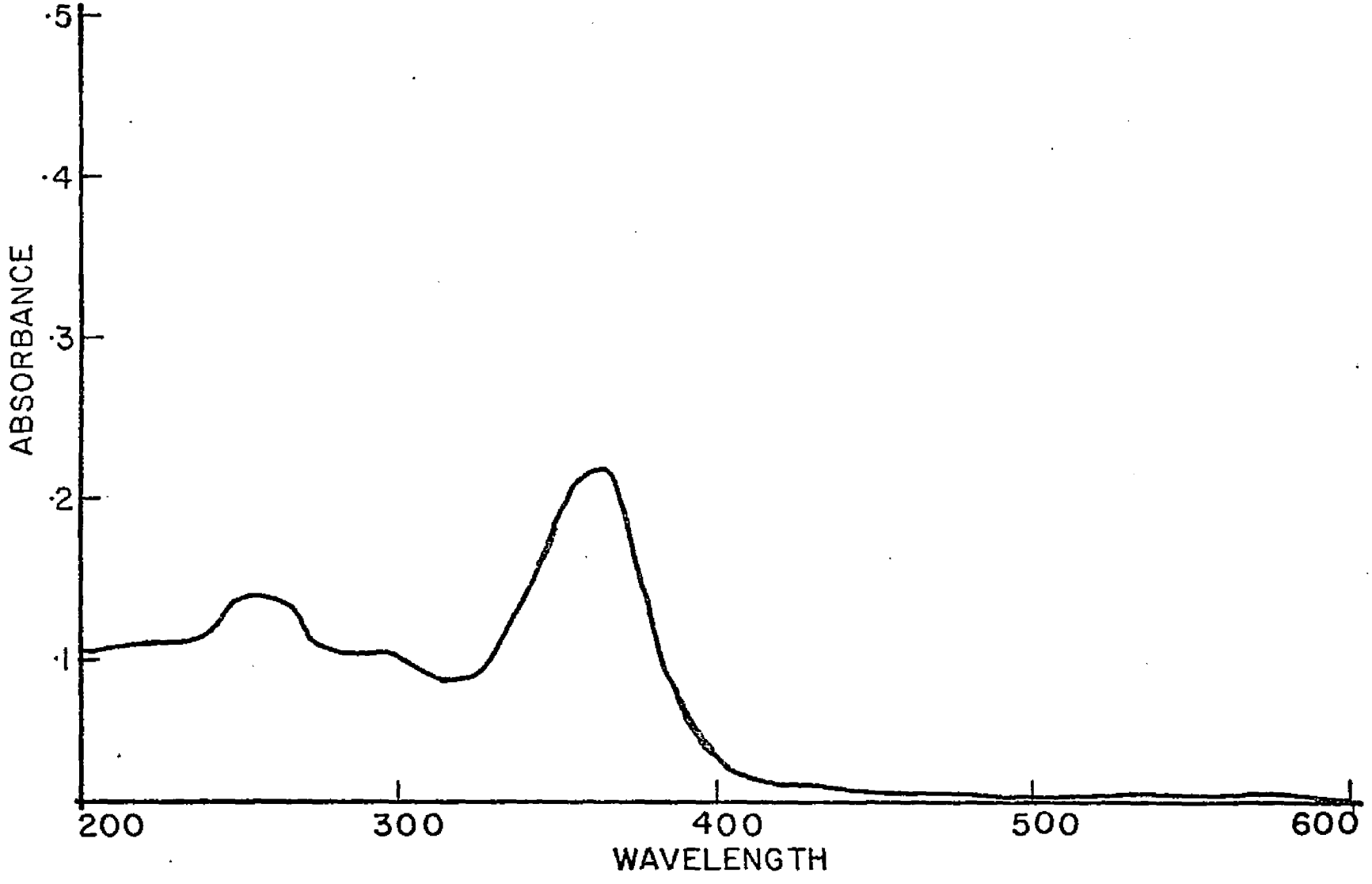
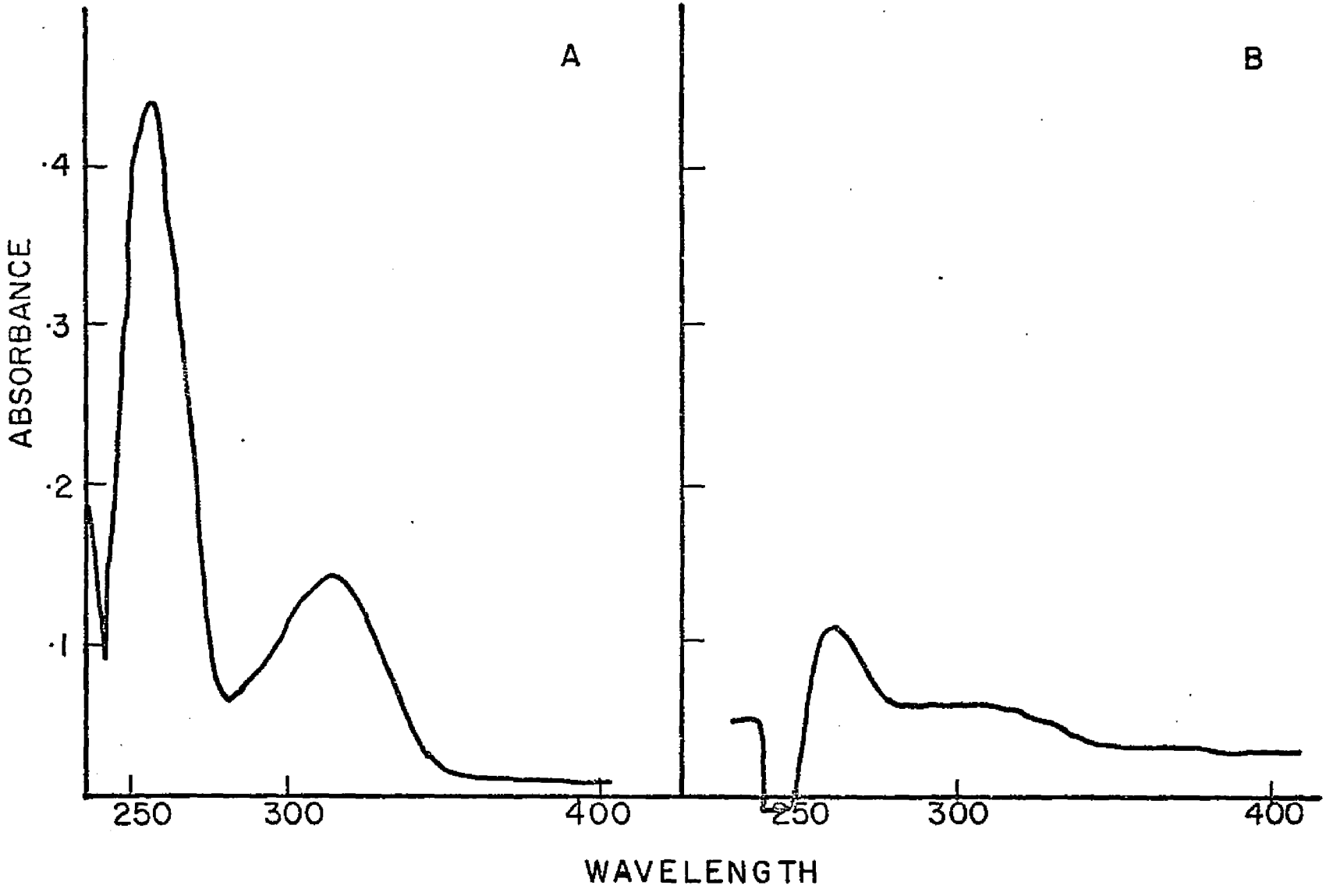

·5
·4
·3
·2
·1
ABSORBANCE
200
300
400
500
600
WAVELENGTH

Figure 5. a) Spectrum of chloroform extract of prd A3 spent culture fluid.
b) Spectrum of chloroform extract of prd A3 cells.


A
B
ABSORBANCE
·4
·3
·2
·1
250
300
400
250
300
400
WAVELENGTH

shown in Figure 6. The absorbance at 315 nm was plotted against time and the product was shown to accumulate at 11-12 hours. Similar experiments with 9-3-3 show the appearance of the bipyrrole at 16-17 hours. Prodigiosin usually does not appear in colonies growing on plates until approximately 24 hours.

Precursor Identification

The infrared spectrum of the dried chloroform-soluble product was determined in a Beckman infrared spectrop[illegible] and the results appear in Figure 7. The sample was prepared in a K[illegible]let and the peaks observed at 3.1, 4.2, 6.2 and 7.1 microns were also found in a KBr blank pellet. The spectrum was consistant with that of a lactum. However, more information is required to determine precisely the structure of the compound.

Degradation of the precursor by acid hydrolysis (6 N HCl in vacuo for 24 hours) resulted in the release of ninhydrin reactive material. Paper chromatography of hydrolyzed and unhydrolyzed chloroform-soluble material in n-butanol, ethanol, water (7:1:2) resulted in the chromatogram shown in Figure 8. The chromatogram was first checked for ultraviolet fluorescence then ninhydrin reactivity. The unhydrolyzed product gave a single band of fluorescence at the solvent front; this band was also faintly ninhydrin positive. The hydrolyzed material gave three bands; bands A and B fluoresced blue and yellow respectively while the band which remained at the origin did not fluoresce but gave a strong reaction with ninhydrin. Thus it is clear that

Figure 6. Appearance of prd A3 chloroform-soluble product as a function of time. Samples of a culture growing at room temperature were extracted by shaking with equal volumes of chloroform and scanned from 400 nm to 220 nm.

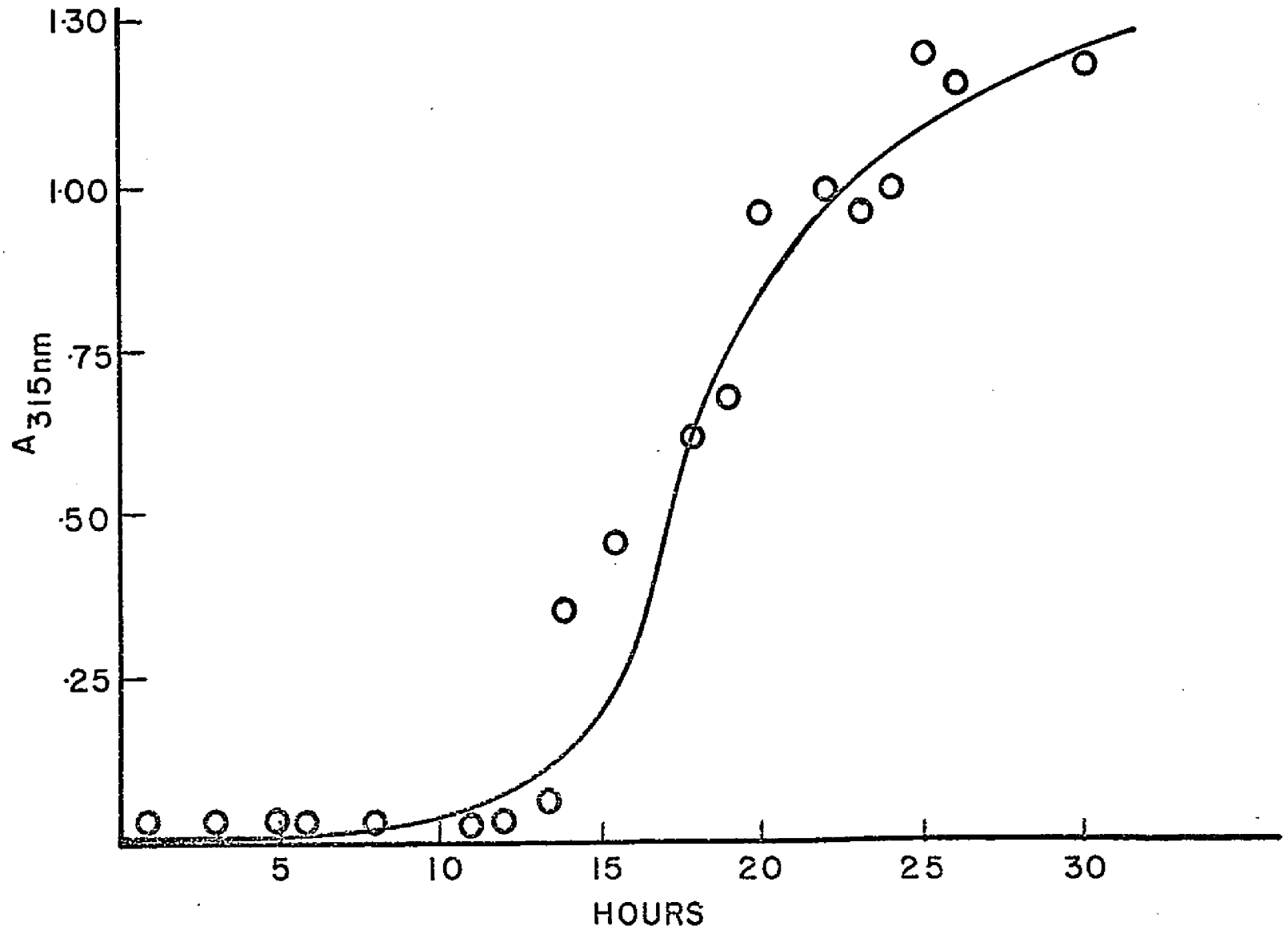

1·30
1·00
·75
·50
·25
A_{315nm}
5
10
15
20
25
30
HOURS

Figure 7. Infrared spectrum of prd A3 chloroform-soluble product.

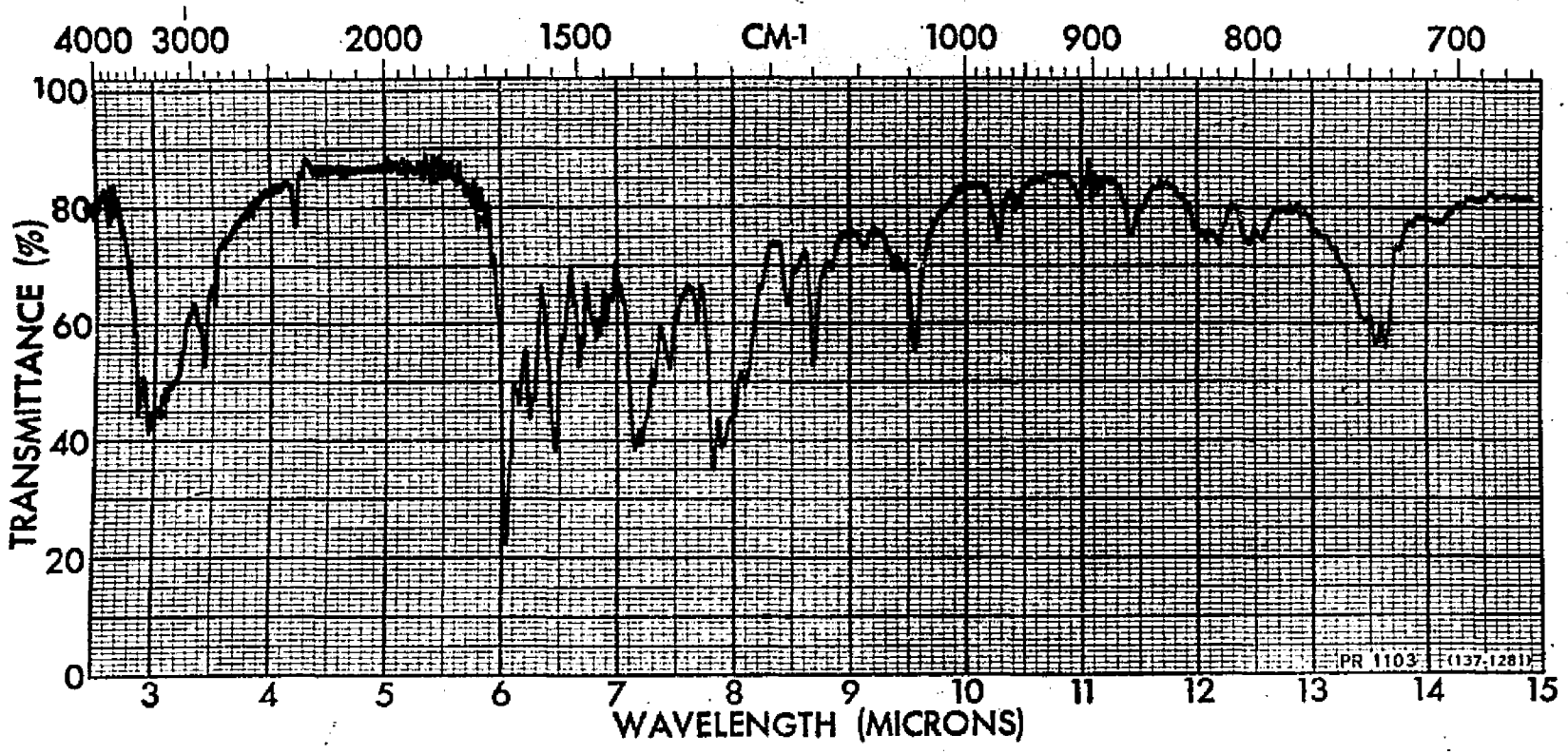
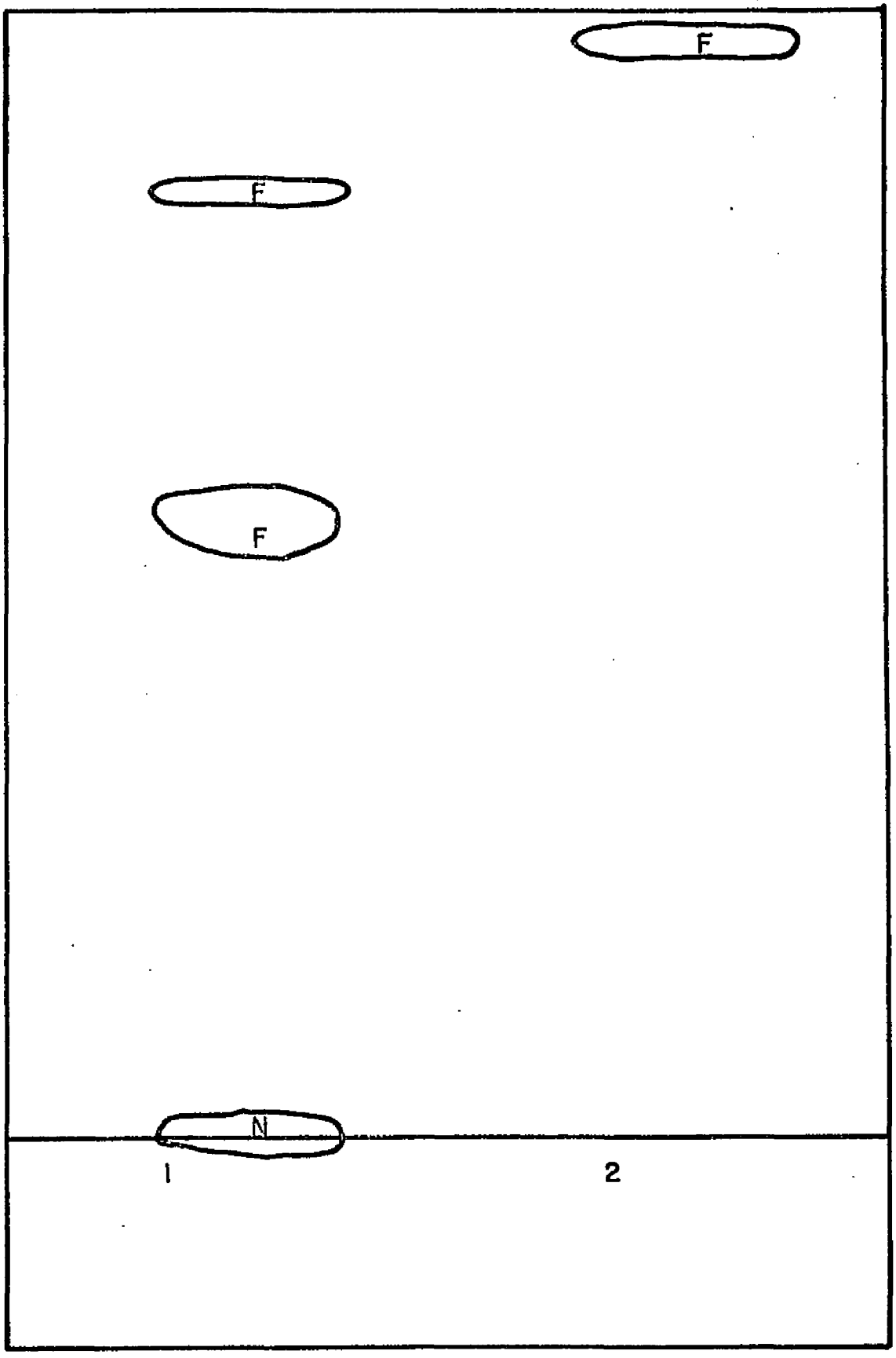

4000 3000 2000 1500 CM-1 1000 900 800 700
100
80
60
40
20
0
TRANSMITTANCE (%)
3 4 5 6 7 8 9 10 11 12 13 14 15
WAVELENGTH (MICRONS)
PR 1103 (137-1281)

Figure 8. Paper chromatogram of prd A3 chloroform-soluble product. Sample 1: acid hydrolyzed product. Sample 2: unhydrolyzed product. The chromatogram was developed in a solvent system consisting of n-butanol, ethanol, and water (7:1:2). Spots marked N were ninhydrin positive. Spots marked F fluoresced in UV light.


F
F
F
N
1
2

acid hydrolysis of the chloroform-soluble product releases ninhydrin reactive material and alters the product. Table 6 shows the results of amino acid analysis of the hydrolyzed material. The product released by acid hydrolysis is the amino acid threonine. If the precursor is degraded by saponification in 1 N NaOH, the spectrum is changed to that shown in Figure 9 and the product becomes water soluble. The maximum at 315 nm disappears and a new maximum at 280 nm appears. Other maxima and minima for saponified material are at 251 nm and 265 nm respectively.

Since saponified material was rendered water soluble it was decided to attempt separation of the saponification product by TLC. Saponified material was acidified with concentrated HCl, extracted with n-hexane and the extract was chromatographed in a solvent of hexane, diethyl ether, acetic acid (90:10:2). The chromatogram was developed with 2',7'-dichlorofluorescein and the results appear in Figure 10. The unhydrolyzed material gave only one spot which absorbed uv light and fluoresced after spraying with fluorescein while the hydrolyzed material gave three fluorescent spots with fluorescein. None of the spots co-chromatographed with the standards which were 2-tridecanone, undecanol, undecanoate and undecanoyl acetate. Attempts to alter the spectrum of the suspected precursor by treatment with serratolysin were unsuccessful. Using uniformly labeled C^{14} glycerol as substrate only random incorporation (approximately 200 CPM) of label was observed in the prd A3 chloroform soluble product.

Table 6

Amino acid analysis of prd A3 chloroform-soluble material[1].

Amino Acid	uMoles
Aspartic acid	0.017
Threonine	0.491
Serine	0.045
Glutamic acid	0.025
Proline	0.052
Glycine	0.098
Alanine	0.029
Half cystine	0.006
Valine	0.014
Methionine	0.009
Isoleucine	0.010
Leucine	0.010
Tyrosine	0.009
Phenylalanine	0.009

[1] The dried precursor was hydrolyzed in 6 N HCl for 24 hours in vacuo. The hydrolyzed product was analyzed with 10 µl of Beckman standard calibration mixture.

Figure 9. Spectrum of prd A3 chloroform-soluble product treated with 1 N NaOH.

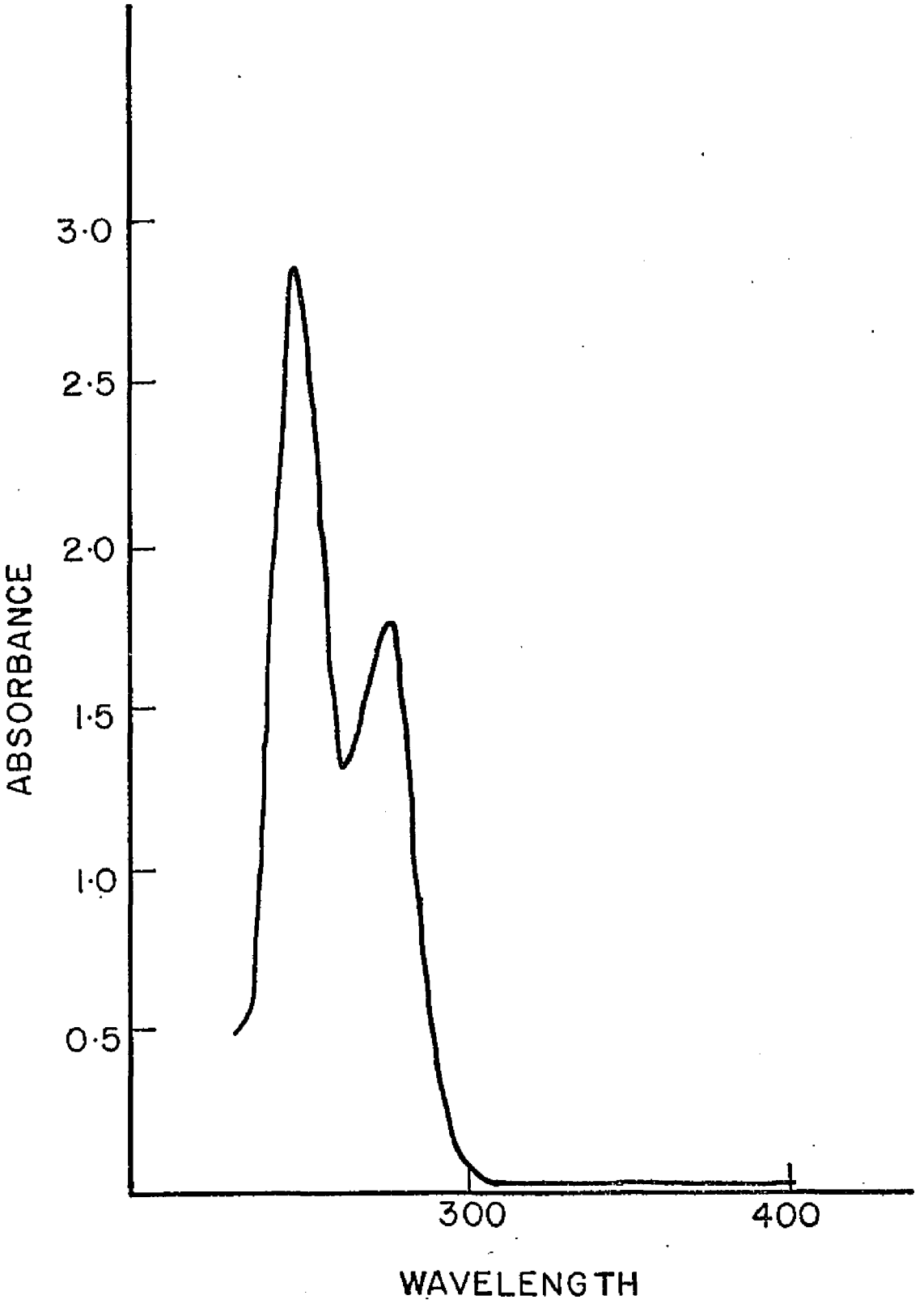
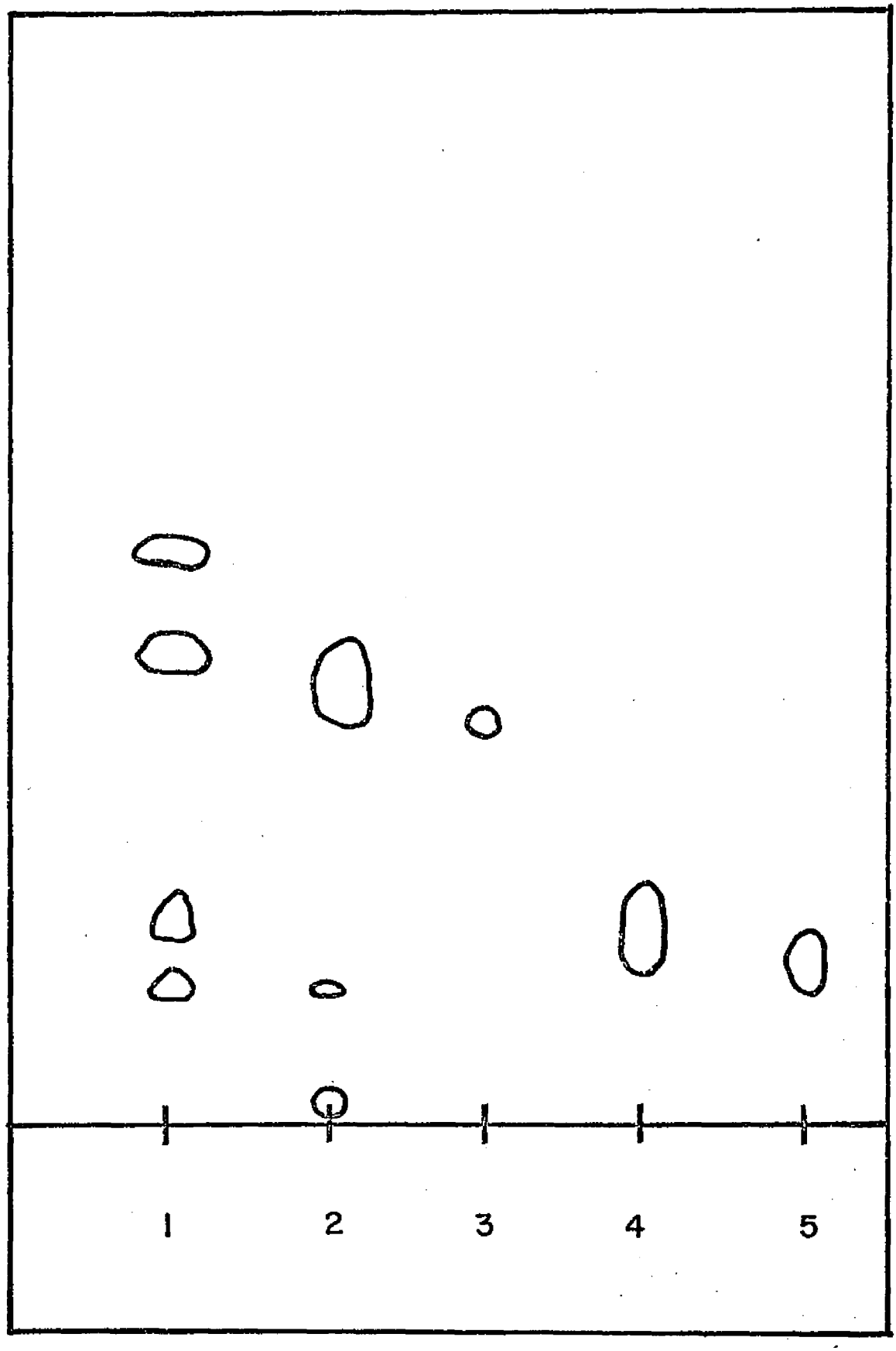

3·0
2·5
2·0
1·5
1·0
0·5
ABSORBANCE
300
400
WAVELENGTH

Figure 10. Thin layer chromatogram of prd A3 chloroform-soluble product. Sample 1: standard consisting of undecanol, undecanoic acid, 2-tridecanone and undecanyl acetate (from bottom to top). Sample 2: hydrolyzed product. Sample 3: unhydrolyzed product. Sample 4: octanoic acid. Sample 5: octanol. The chromatogram was developed in a solvent consisting of n-hexane, diethyl ether, and acetic acid (90:10:2). The compounds were detected by spraying with 0.2% 2',7'-dichlorofluorescein in ethanol. The plates were dried and examined for fluorescence in UV light.


1
2
3
4
5

Proteinase Studies

The results of the purification of the proteinase from mutant prd A2 are shown in Table 7. The purification scheme used was the same as that for the purification of serratolysin (Broussard, 1968) and the yields achieved at each step closely paralleled those for wild-type serratolysin. The specific activity however, was only one-tenth that of wild-type serratolysin at each step of the purification. A representative elution profile from DEAE cellulose chromatography is shown in Figure 11. Only fraction I had appreciable proteolytic activity and unlike serratolysin possessed little if any charge under the conditions of DEAE chromatography. Both fractions I and II were tested for CRM using antiserum produced to wild-type serratolysin by the Ouchterlony technique and the results in Figure 12 show that both fractions cross react with serratolysin antiserum. Fraction I is similar but not identical to serratolysin whereas fraction II although sharing some of the same antigenic determinants is clearly a modified protein. Based on the position and shape of the precipitin band for fraction II one can surmise that it is a larger molecule than IgG. Disc gel electrophoresis of the two peaks from DEAE chromatography show a single band for fraction II and three bands for fraction I (Figure 11). The single band of fraction II seems to be the same as the most negatively charged band in fraction I. Results similar to these were recorded for mutants prd A3 and prd A5. When discs such as those shown in Figure 11 are placed on casein agar plates and the protein

Table 7

Purification of prd A2 proteinase.

Fraction	Protein mg/ml	Specific Activity	Fold Purification	CRM[2]
Crude	15.20	0.13	--	+
Dissolved 80% $(NH_4)_2SO_4$ Precipitate	32.00	0.47	3.65	+
Dissolved 30-50% $(NH_4)_2SO_4$ Precipitate	25.50	5.88	45.3	+
DEAE Fr I	5.60	10.54	81.2	+
DEAE Fr II	3.15	0.56	--	±
2nd DEAE Ia[1]	--	0	--	--
2nd DEAE Ib	0.98	23.5	181	+

[1] In most instances the purification was terminated after the first DEAE column.
[2] Cross-reacting material to anti-serratolysin antiserum.

Figure 11. Elution profile of prd A2 proteinase. (●) absorbtion at 280 nm. (0) concentration of ions as parts per million NaCl. (□) activity units per milliliter. Insert: representation of disc electrophoresis gels for the first and second protein peaks.

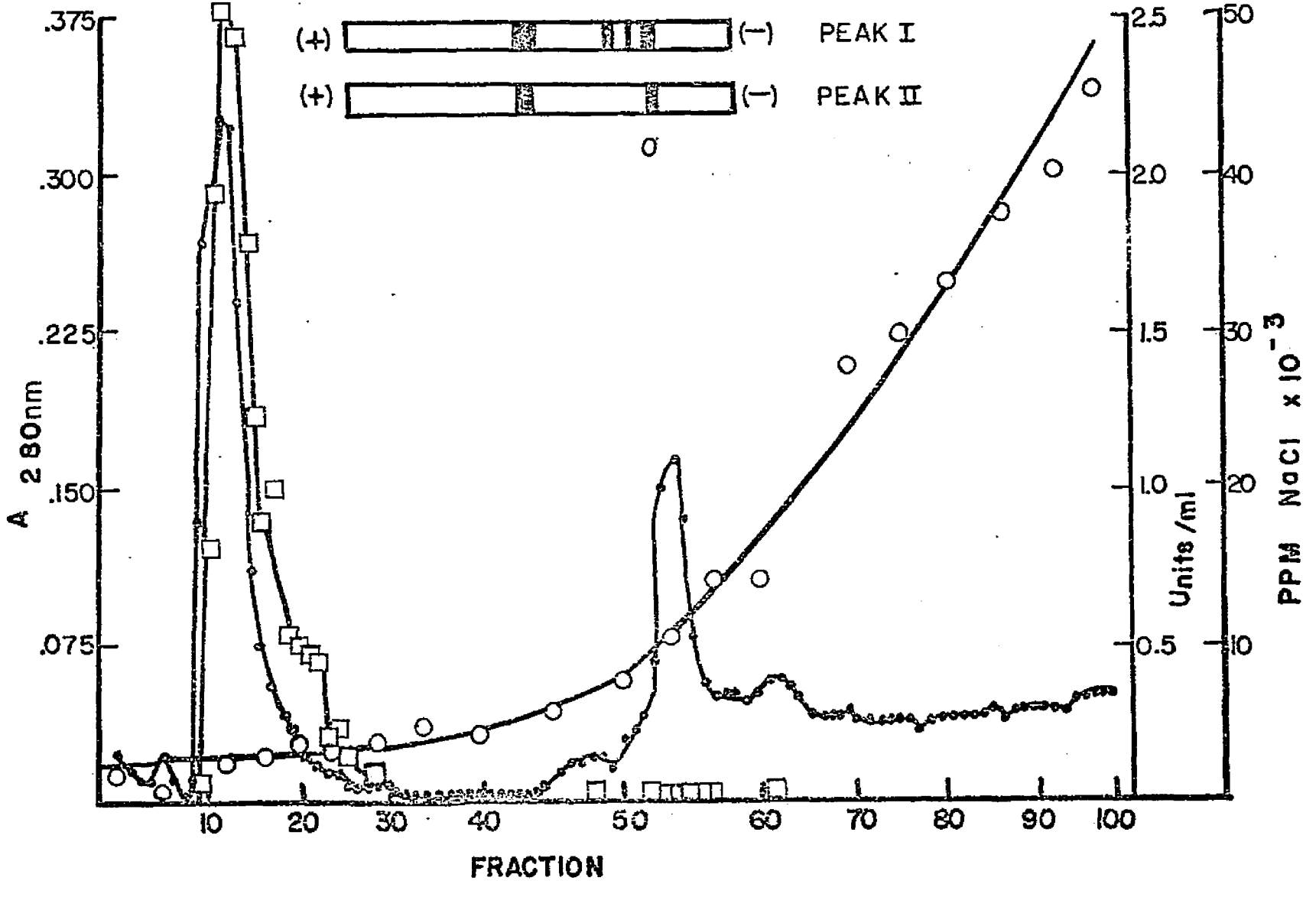
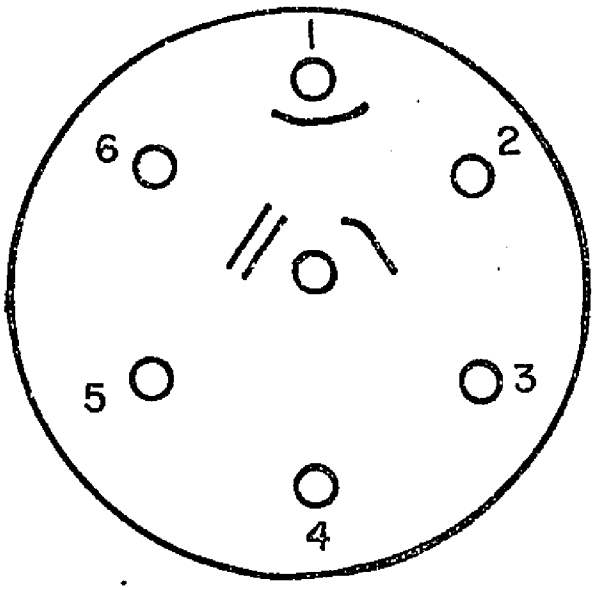

(+)
(−)
PEAK I
(+)
(−)
PEAK II
0
.375
.300
.225
.150
.075
A 280nm
2.5
2.0
1.5
1.0
0.5
Units /ml
50
40
30
20
10
PPM NaCl x 10⁻³
10
20
30
40
50
60
70
80
90
100
FRACTION

Figure 12. Representation of Ouchterlony plate showing cross reacting material in preparations of prd A2 proteinase.
Center well: antiserratolysin antiserum.
Well No. 6: purified serratolysin.
Well No. 1: prd A2 proteinase fraction II.
Well No. 2: prd A2 proteinase fraction I.


1
2
3
4
5
6

allowed to diffuse out of the gels, fraction I material shows a zone of hydrolysis in the region of the protein bands near the cathode only; fraction II material shows no zone of hydrolysis. The mutant enzyme and serratolysin both hydrolyzed the β -chain of insulin. Both enzymes depleted the substrate by 30 minutes as shown in Figure 13.

Amino Acid Analysis

The amino acid composition for both fractions of prd A2 and both fractions of prd A5 as well as for serratolysin appear in Table 8. Only slight differences are noticeable between fractions I and II, and the two mutants have very nearly the same composition. The composition of serratolysin is decidedly different from that of either mutant.

The composition of the mutant proteinase will vary depending on the substrate used for growth of the organism. Table 9 depicts both fractions I and II from prd A2 grown on gelatin and BHI. The gelatin-grown cells yield a proteinase of much higher proline and glycine content than the BHI-grown cells; one can also find evidence of hydroxyproline in the gelatin-grown cells. This effect was not observed with serratolysin which gave a uniform amino acid composition irrespective of the growth substrate.

Ultracentrifugation Analysis

Figure 14 is photograph of the pattern observed with schlieren optics when fraction I of prd A2 proteinase was centrifuged at 56,000 RPM. Only

Figure 13. Rate of hydrolysis of β -chain of insulin. (0) serratolysin. (●) prd A2 proteinase. Note that with both enzymes release of ninhydrin reacting material levels off after 30 minutes.

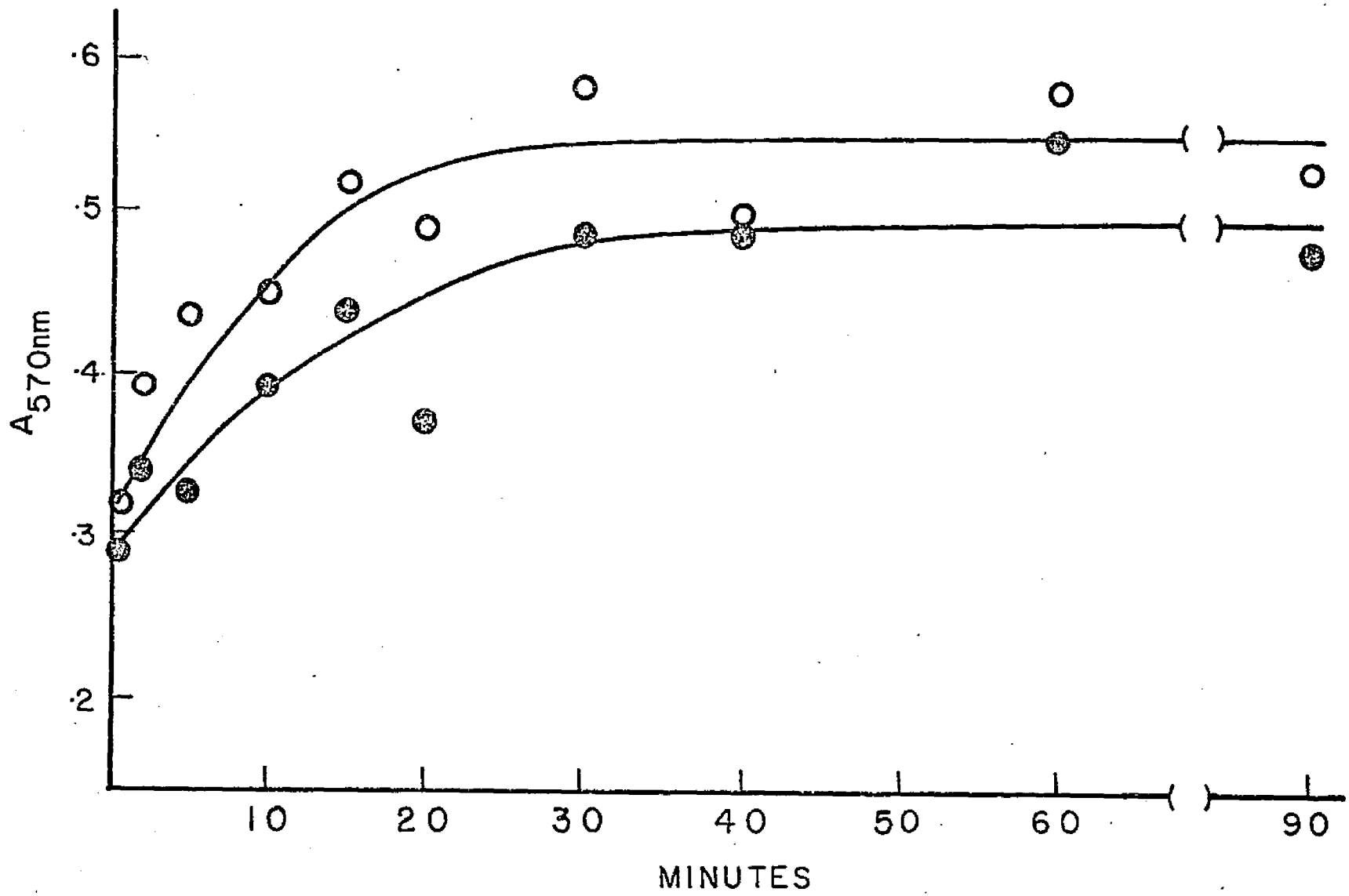

·6
·5
·4
·3
·2
A_{570nm}
10
20
30
40
50
60
90
MINUTES

Table 8

Amino acid composition of proteinases from mutants prd A2 and prd A5, and S. marcescens ATCC 25419.

	Percent Composition				
	prd A2I	prd A2II	prd A5I	prd A5II	WT
Lysine	2.91	2.16	2.17	2.24	3.39
Histidine	1.01	0.63	1.04	1.18	2.33
Arginine	4.75	3.99	3.52	3.13	1.76
Aspartic acid	7.89	6.23	4.51	7.97	15.66
Threonine[1]	3.04	1.76	1.04	1.66	7.39
Serine[1]	5.11	4.45	4.42	5.13	7.77
Glutamic acid	6.97	11.15	8.53	10.24	9.21
Proline	14.41	19.10	17.90	12.63	2.33
Glycine	29.24	32.08	35.39	32.23	13.30
Alanine	11.25	7.08	9.36	8.77	10.72
Valine	3.38	1.74	2.99	3.46	5.55
Methionine	0.30	1.60	1.54	1.53	-----
Isoleucine	2.54	1.83	2.20	2.31	4.20
Leucine	3.36	3.14	2.00	2.81	5.32
Tyrosine[1]	1.48	1.21	1.76	2.35	3.68
Phenylalanine	1.59	1.73	1.50	1.83	5.54
Tryptophan[2]	0.10	ND[3]	0.13	0.52	1.84

[1] extrapolated to zero hydrolysis.
[2] determined spectrophotometrically (Goodman and Morton, 1946).
[3] not determined.

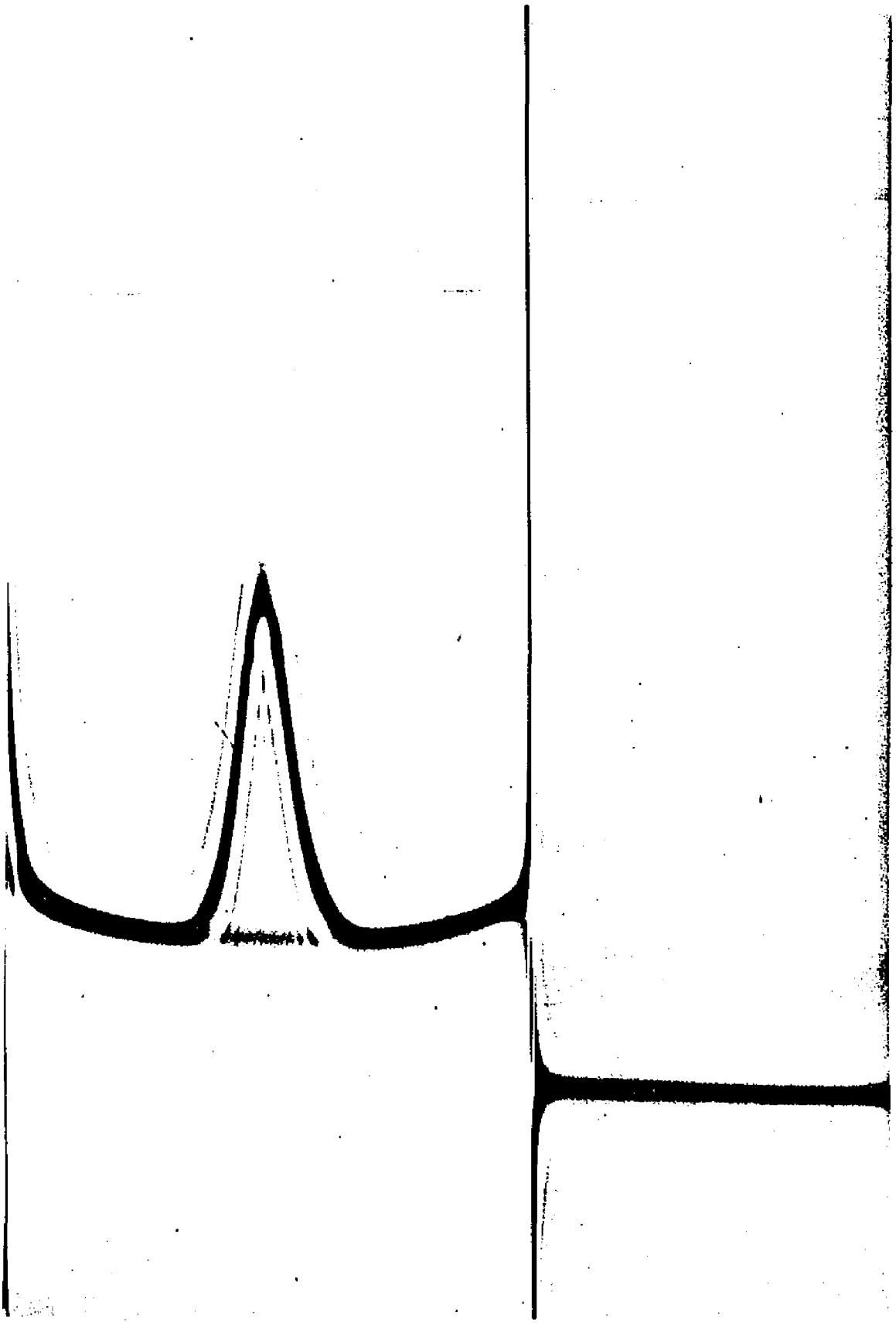
Table 9

Effect of growth substrate on amino acid composition of mutant proteinases.

	Percent Composition			
	BHI-I[1]	BHI-II[2]	GEL-I[3]	GEL-II[4]
Lysine	3.64	3.64	2.91	2.16
Histidine	1.38	2.34	1.01	0.63
Arginine	2.33	1.27	4.75	3.99
Aspartic acid	8.13	13.16	7.89	6.23
Threonine[5]	4.43	7.13	3.04	1.76
Serine[5]	4.08	8.00	5.11	4.45
Glutamic acid	6.27	9.58	6.92	11.15
Proline	10.58	5.90	14.41	19.10
Glycine	18.58	14.74	29.94	32.08
Alanine	17.80	9.44	11.25	7.08
Valine	6.44	7.61	3.38	1.74
Methionine	0.52	0.23	0.30	1.60
Isoleucine	3.49	3.79	2.54	1.83
Leucine	5.67	4.20	3.36	3.14
Tyrosine[5]	2.88	2.77	1.48	1.21
Phenylalanine	3.12	4.21	1.59	1.73
Tryptophan[6]	1.20	ND[7]	0.10	ND

[1] amino acids in prd A2 proteinase fraction I isolated from cells grown on brain heart infusion.
[2] amino acids in fraction II from BHI grown cells.
[3] amino acids in fraction I from gelatin grown cells.
[4] amino acids in fraction II from gelatin grown cells.
[5] extrapolated to zero hydrolysis.
[6] determined spectrophotometrically (Goodman and Morton, 1946).
[7] not determined.

Figure 14. Photograph of schlieren pattern obtained when prd A2 fraction I proteinase is sedimented at 56, 000 X G.



a single boundary is evident in these photographs. Figure 15 is a plot of $\log_{10}x$ vs. time for a representative sedimentation velocity experiment. Values for S_{obs} were calculated at several concentrations and extrapolated to zero concentration. The S^0_{obs} for prd A2 fraction I proteinase is 1.65. Fraction II has the same S^0_{obs}. In contrast the $S^0_{20,w}$ for serratolysin is 3.98.

Figure 16 is a plot of $\log_n j$ vs. r^2 for prd A2 fraction I proteinase. The curved line for this plot indicates an associating enzyme, and maximal and minimal molecular weights must be estimated from lines drawn tangent to the curve (see Figure 16). The molecular weights determined in this manner are 32,400 maximum and 8,400 minimum.

Extracellular Nuclease

Serratia produces an extracellular nuclease and both S. marcescens ATCC 25419 and prd A2 were tested to determine if production of this exonuclease had been affected in any way by the prd mutation. As shown in Table 10 the prd mutant produces a far less active exonuclease. The total protein content in the medium was approximately the same for both the mutant and wild-type organisms. Purified serratolysin does not possess nucleolytic activity.

Inhibitor Studies

Serratolysin and the proteinase from prd A2 can both be inhibited by

Figure 15. Plot of $\log_{10}$ distance of protein boundary from center of rotation versus time.

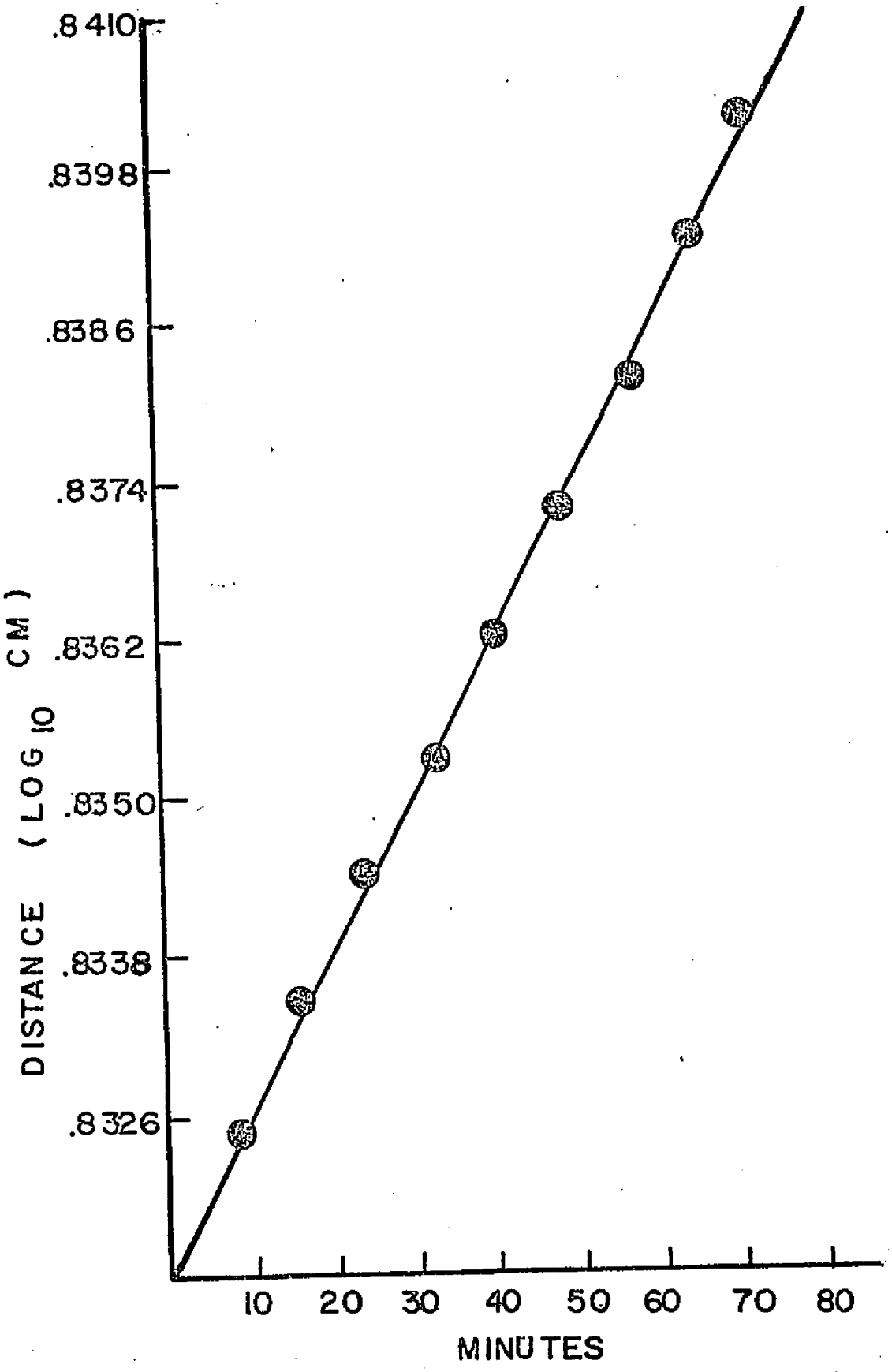

.8410
.8398
.8386
.8374
.8362
.8350
.8338
.8326
DISTANCE (LOG 10 CM)
10
20
30
40
50
60
70
80
MINUTES

Figure 16. Plot of $\log_e$ net fringe displacement versus r^2 for prd A2 fraction I proteinase. The curved plot indicates aggregation and the lines drawn tangent to the curved line are used to calculate minimum and maximum molecular weights.

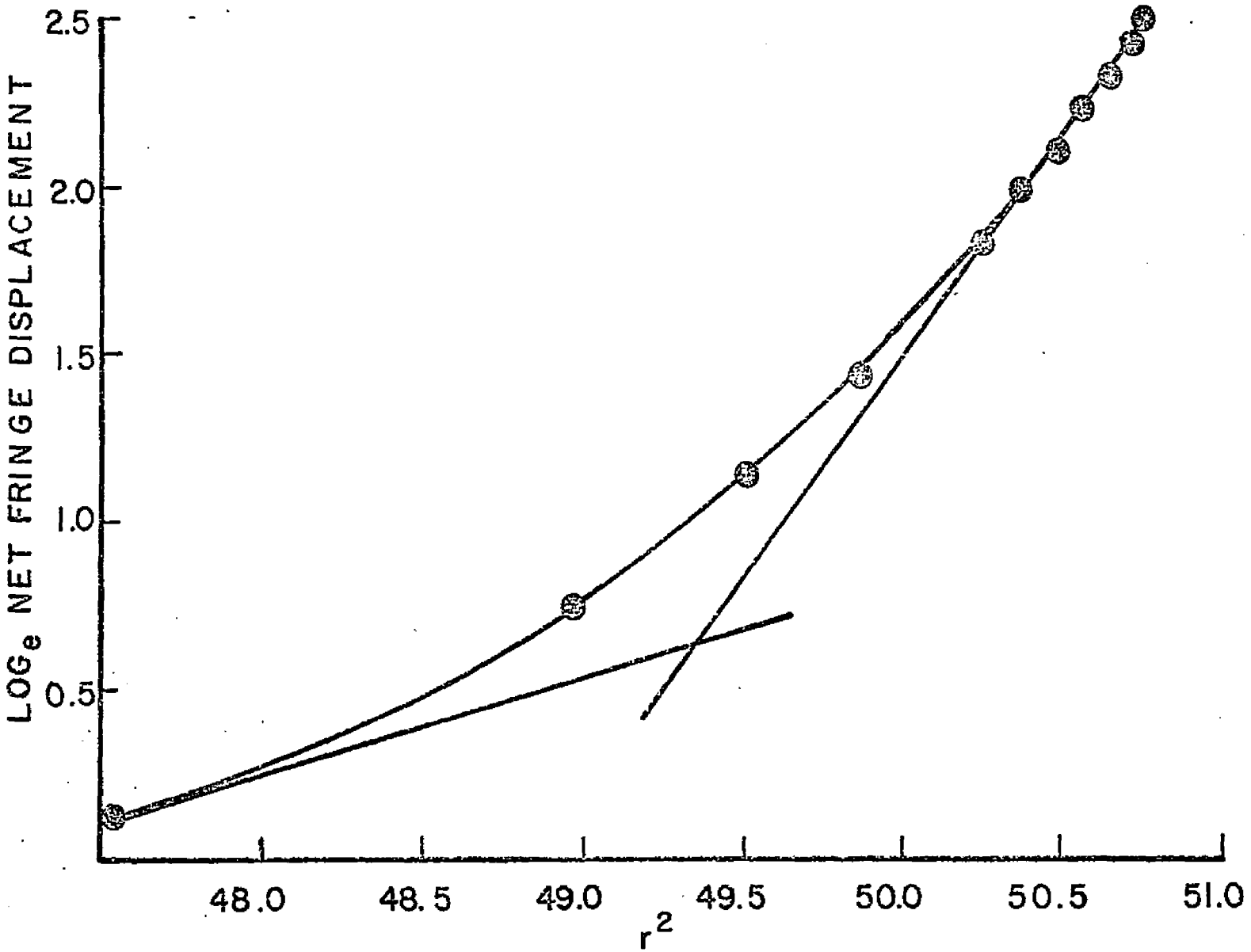

2.5
2.0
1.5
1.0
0.5
LOG$_e$ NET FRINGE DISPLACEMENT
48.0
48.5
49.0
49.5
50.0
50.5
51.0
r^2

Table 10

Comparison of exonuclease activity in culture supernatant of wild-type and mutant organisms.

Organism[1]	u/ml	u/mg
S. marcescens ATCC 25419	65	23.16
prd A2	10.5	3.17

[1] cells were grown on BHI at room temperature for 24 hours and the supernatant fluid assayed as described in Materials and Methods.

a sonicate of S. marcescens ATCC 25419. Table 11 shows the results of incubation of enzyme and sonicate for 10 minutes at room temperature. The proteinase was completely inhibited even when the incubation period was reduced to the shortest period of time possible (5-10 seconds). If the proteinase is allowed to remain in the presence of the sonicate for a prolonged period, full activity will return usually in about seven hours (Figure 17). The inhibitory substance is heat labile. Sonicates of prd A2 possess an inhibitor of similar characteristics.

Substrate Specificity

Figures 18 and 19 are tracings of peptide fingerprints obtained when reduced, carboxymethylated β -chain of insulin is digested with serratolysin and prd A3 fraction I proteinase respectively. There are almost no identical spots on the two fingerprints and it is clear that the points of attack of serratolysin and prd A2 fraction I proteinase are different. Table 12 shows the composition of peptides formed by the action of prd A2 fraction I proteinase on β -chain of insulin and Figure 20 shows the points of attack of the mutant and wild-type enzyme on the β -chain of insulin. The peptide present in the largest amount is the N-terminal tetrapeptide. This peptide is sharply separated from any other peptide. The C-terminal tripeptide is also clearly separated. The remainder of the peptides appear to be contaminated with other peptides but it is still possible to determine the peptides present in highest concentrations.

Table 11

Inhibition of proteinase by S. marcescens intracellular fluid.

S. marcescens[1]	prd A2[2]	sonicate[3]	time[4]	activity
0.5 ml	--	--	0 min	6 u/ml
0.5 ml	--	1.0 ml	10 min	0 u/ml
0.5 ml	--	--	10 min	6 u/ml
--	1.0 ml	--	0 min	8 u/ml
--	1.0 ml	1.0 ml	10 min	0 u/ml
--	1.0 ml	--	10 min	8 u/ml
--	--	1.0 ml	10 min	0 u/ml

[1] serratolysin in 0.10 M phosphate buffer pH 7.5.

[2] proteinase purified from prd A2 in 0.1 M phosphate buffer pH 7.5.

[3] undialyzed sonicate of S. marcescens ATCC 25419.

[4] incubation was at 30 C.

Figure 17. Proteinase recovery from inhibition by cell sonicate. (0) serratolysin plus sonicate. (●) serratolysin alone.

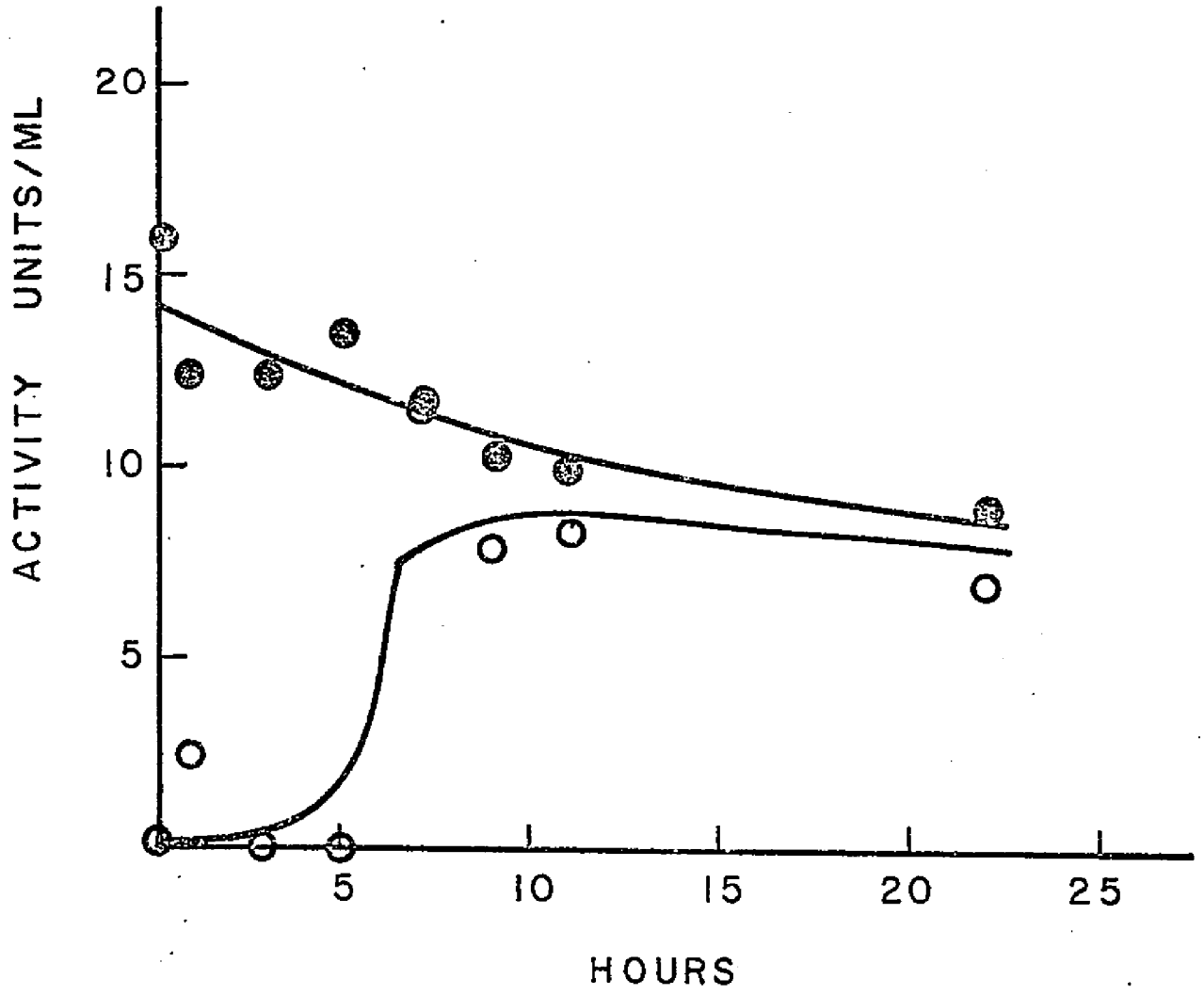

ACTIVITY UNITS/ML
20
15
10
5
5
10
15
20
25
HOURS

Figure 18. Fingerprint of β -chain of insulin digested by serratolysin. (+) origin. (MR) methyl red--chromatography marker. (QS) quinine sulfate--electrophoresis marker. Chromatography was carried out in the vertical direction in 2-butanol, formic acid 90%, and water (7:1:2). Electrophoresis was carried out in a horizontal direction at pH 3.5 and 2,400 volts.

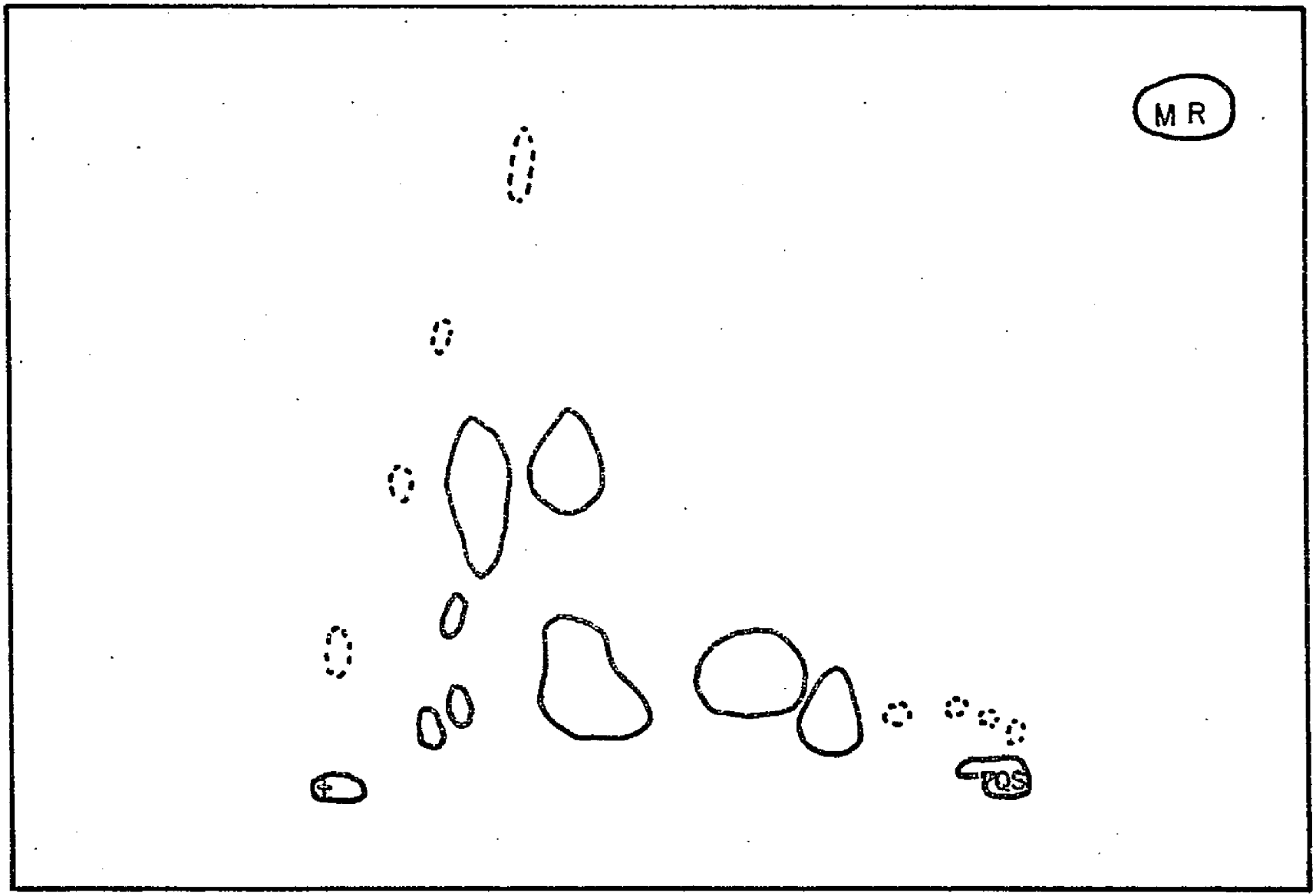

MR
PQS

Figure 19. Fingerprint of β -chain of insulin digested with prd A2 fraction I proteinase. The conditions were the same as in figure 18.

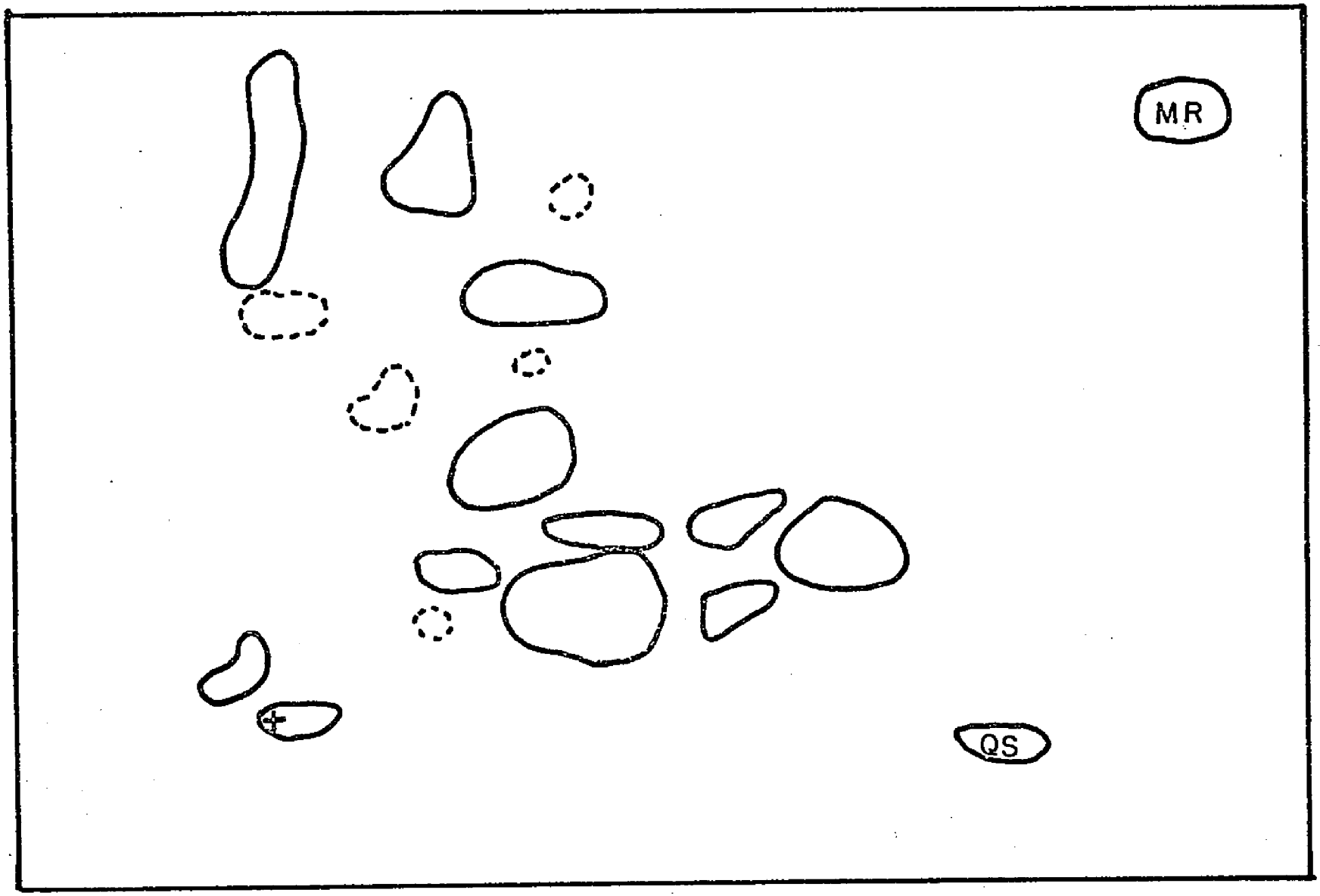

MR
QS

Table 12

Substrate[1] specificity of prd A2 fraction I proteinase.

Peptide Composition	Sequence	Peptide Bonds Cleaved
Asn, Gln, Val, Phe	Phe-Val-Asn-Gln	Gln-His
Lys, Pro, Ala	Pro-Lys-Ala	Thr-Pro
Lys, Thr, Pro, Gly, Ala, Phe	Gly-Phe-Phe-Tyr-Thr-Pro-Lys-Ala[2]	Arg-Gly
His, Ser, Glu, Gly, Val, Leu[3]	Gly-Ser-His-Leu-Val-Glu[4]	Cys-Gly Glu-Ala
Glu, Val, Ala, Leu	Leu-Val-Glu-Ala[5]	His-Leu Ala-Leu
Cys, Glu, Gly, Ala, Val, Leu, Tyr	(Glu)-Ala-Leu-Tyr-Leu-Val-Cys-(Gly)	Val-Glu Gly-Glu or Glu-Ala Cys-Gly

[1] reduced carboxymethylated β-chain of insulin.

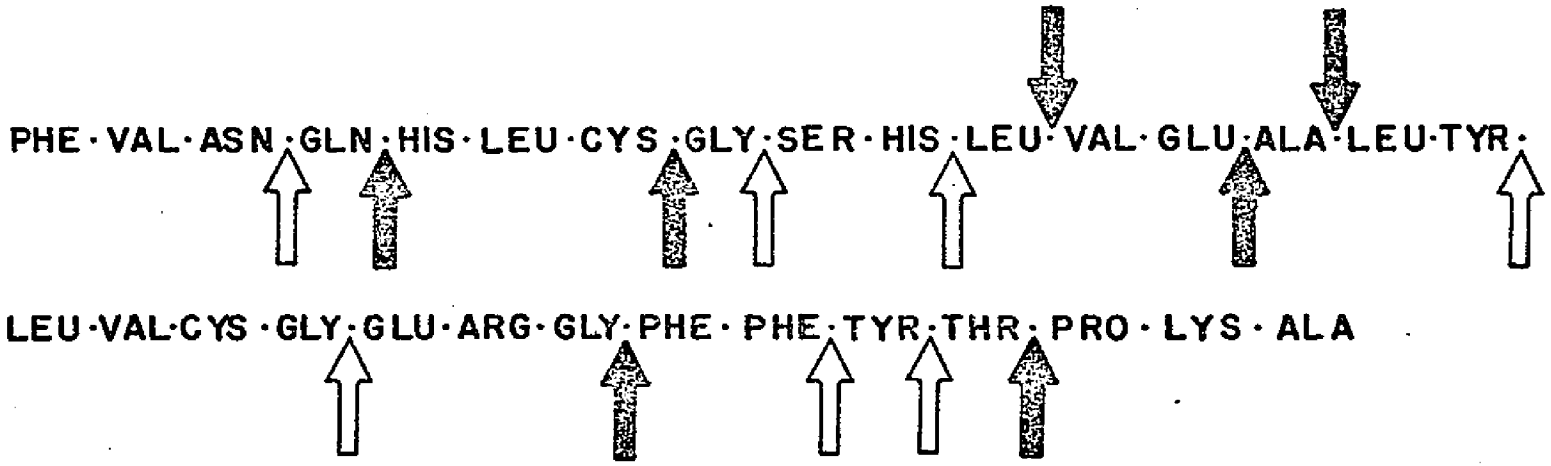
[2] contaminated with Thr-Pro-Lys-Ala.

[3] contaminated with Lys, Arg, Cys, Asp, Thr, Pro, Ala.

[4] possible contaminant: Gly-Glu-Arg-Gly-Phe-Phe-Tyr-Thr-Pro-Lys-Ala.

[5] contaminated with Gly-Ser-His-Leu-Val-Glu-Ala-Leu.

Figure 20. Map of β -chain of insulin showing major points of attack by prd A2 fraction I proteinase (closed arrows) and serratolysin (open arrows).


PHE · VAL · ASN · GLN · HIS · LEU · CYS · GLY · SER · HIS · LEU · VAL · GLU · ALA · LEU · TYR ·
LEU · VAL · CYS · GLY · GLU · ARG · GLY · PHE · PHE · TYR · THR · PRO · LYS · ALA

Discussion

Several possibilities are immediately evident to explain the relationship of pigment to the extracellular proteinase of S. marcescens ATCC 25419. If the genetic information for the proteinase and at least one of the prodigiosin biosynthetic enzymes were both contained on an episome, it would be possible that incorporation of the episome into the chromosomal material might result in repression of the episomal elements in much the same fashion as repression of the bacteriophage lambda when it is in the lysogenic state. This of course would result in concomitant loss of pigment and proteinase. One appealing facet of this possibility is that it could explain the occurrence of such a hyperactive proteinase in this strain of Serratia marcescens. Although Serratia sp. are generally recognized as possessing extracellular proteinases, S. marcescens ATCC 25419 shows proteolytic activity far in excess of other strains. The ability to produce this hyperactive proteinase might have been transferred on an episome from some other species or possibly some other genus. Falkow et al. (1961) reported on episomic transfer from Salmonella typhosa to S. marcescens; it is not difficult to postulate the incorporation of such an episome into the chromosome and subsequent excision carrying some chromosomal element (a prodigiosin gene) into the episomic state. Assuming such an episome existed it would be possible that the genes on the episome are repressed by high temperature or low oxygen tension and in this manner

account for the loss of pigment and proteinase in the wild-type organism under such circumstances. Although this could be possible in the wild-type organism, clearly the pleiotropic mutant is not simply a control mutant. If it were a control mutant (i. e., non-derepressed) one could only expect to observe differences in the amount of and not the nature of the proteinase. The mutant proteinase is different from the wild-type proteinase in several respects such as molecular weight, charge and amino acid composition.

Loss of the hypothetical episome would also result in the observed pleiotropism, however this cannot be the case since pure cultures of the prd mutants spontaneously give rise to full revertants.

The inability to find pigmented organisms lacking proteinase argues strongly for the dependence of pigment production on proteinase. It is relatively easy to isolate white mutants which contain normal levels of proteolytic activity. Since pigmentation is not restored in the prd mutants exposed to the proteinase, it would appear that the proteinase serves some function in pigment production other than supplying an essential metabolite or precursor through its action on proteins. Presumably such a function would be carried out while the proteinase remained inside the organism.

Another possible explanation of the pleiotropic mutation would be the so called polar effect in which a nonsense codon arising in one gene of a polycistronic messenger not only terminates synthesis of the protein coded in the particular gene in question, but also reduces the total amount of

protein synthesized from all genes translated after the nonsense codon. In this particular case one could propose the occurrence of such a nonsense codon toward the end of the proteinase gene enabling the proteinase to be synthesized almost entirely cutting off only a small portion of the C' terminus. This of course would result in the production of an altered proteinase, one which would have decreased activity yet still possess cross-reacting capacity as does the proteinase of the prd mutants. If such a nonsense codon did exist, then any genes following it would not be read efficiently. Thus if one of the prodigiosin genes happened to be on the same polycistronic messenger as the proteinase gene, its production would be reduced or stopped and in this manner a nonsense mutation could be responsible for the pleiotropism. There are two serious shortcomings to this hypothesis: 1) it assumes a polycistronic mRNA consisting of mRNA for the proteinase gene and mRNA for one of the prodigiosin genes. There is no reason to assume that these two genes would be included in a single operon. 2) If there were such an operon, one could logically expect to isolate missense mutations which would result in altered proteinase yet have no effect on pigment formation. However all of the many proteinase mutants isolated were colorless. Furthermore no revertants were observed which regained pigment only.

Although it is known that prodigiosin is synthesized from a branched pathway, nothing is known of the origin of the pyrrole nucleus in S. marcescens.

Williams and Hearn (1967) are of the opinion that at some early point in the biosynthetic pathway there is a common precursor or a branch point for the pathway. Their opinion is based on the existance of certain mutants which are incapable of forming pigment unless supplied with the products from both arms of the pathway. The results of cross-feeding experiments using mutants 9-3-3 and WF indicate that the prd mutants may have a block before this branch point. Since 9-3-3 and WF each accumulate the final products of their respective branches only a mutant which accumulates a product prior to the branch point of the pathway would require both products to produce the pigment. The prd mutants are such mutants (i.e., they do not accumulate the products of either branch of the pathway). Certainly then the prd mutants are blocked at or before a branch point for it would be impossible that two mutations took place, one in each arm, both of which revert at the same frequency and the same time.

It was desirable to study the pleiotropic mutation from two vantage points; first, a study of the suspected precursor shown to accumulate in prd mutants and second, a comparison of the wild-type and mutant forms of serratolysin.

That the compound isolated from prd mutants is in fact a precursor of prodigiosin has not yet been firmly established. However, the time at which the product appears (shortly before the bipyrrole accumulates in 9-3-3) is consistant with it being a precursor. It is also significant that no similar

product was found in pigmented organisms or those mutants which accumulate late intermediates. The ultimate proof that the product is in fact a precursor of prodigiosin will have to come from tracer experiments in which the compound is labeled, fed to wild-type Serratia, and shown to be specifically incorporated into prodigiosin. The radio specific activity of the compound extracted from glycerol-U-C^{14} grown cells was too low to warrent its use as a source of label for prodigiosin. Presumably glycerol is too far removed, biosynthetically, from the compound to achieve anything other than random incorporation. If the compound can be identified, then labeled compounds which would logically be expected to be incorporated directly into it would be fed to the mutants thus increasing the possibility of labeling the compound at sufficiently high radiospecific activity. A good candidate for incorporation may be threonine since hydrolysis of the suspected precursor compound yields threonine.

Although the chloroform-soluble product obtainable from prd mutants has not been fully identified, it is certain from its reaction with dichlorofluorescein and its behavior in chromatography that the compound is lipid-like and reasonably nonpolar. The fact that it contains theonine suggests that it may be related to serratamolide (Wasserman et al., 1961). Serratamolide is a depsipeptide consisting of two molecules of serine and two molecules of 3-hydroxydecanoic acid joined to form a 14 membered ring. There are significant differences between serratamolide and the product

isolated from the prd mutants. Serratamolide melts at 159-160 C, has no absorption in the UV range above 200 nm, and demonstrates strong absorption at 5.79, 6.05 and 6.46 μ as well as a peak at 3.01. The chloroform soluble product of the prd mutants other hand melts at 217 C, has absorption maxima at 255 nm and 315 nm, and IR maxima at 6.05, 6.45, 7.85, 8.70, 9.55 μ. Saponification of serratamolide with 1 N NaOH at room temperature results in the release of serratamic acid (seryl-3-hydrodecanoic acid), whereas similar treatment of the prd chloroform soluble product results in the release of ninhydrin positive material. Hydrolysis with acid results in release of threonine. Serratamolide is extracted from the organism not from the culture medium as is the suspected precursor of the prd mutants. It is possible that the chloroform soluble product of the prd mutants is in some way related to serratamolide but the differing characteristics mentioned would necessitate several structural differences between the two molecules.

Nonetheless the existence of a peptide bond in serratamolide and the evidence that a peptide bond may exist in the chloroform soluble material (e.g., unhydrolyzed material reacts poorly if at all with ninhydrin while hydrolyzed material gives a strong ninhydrin reaction) seemed to be significant in light of the dependence of pigment formation on proteinase. One could postulate that proteinase of a given specificity is required to attack this peptide bond and that this might be a significant step in the production of prodigiosin. This possibility seems unlikely however since treatment of

the chloroform soluble product with serratolysin had no effect on the spectrum of the product. It would be reasonable although not necessary to assume that cleavage of this peptide bond would alter the spectrum of the product.

The second aspect of this research involved a comparsion of the wild-type and mutant serratolysin. Both are extracellular enzymes and can be purified by the same scheme. It is important to note that during the course of the purification the specific activity of the mutant enzyme remains roughly one-tenth that of the wild-type. This indicates that the observed decrease in proteolytic activity of the mutants is not due simply to a decreased production of the wild-type serratolysin, but rather that an altered proteinase is produced. Although the mutant proteinase must be different than the wild-type serratolysin, both fractions I and II clearly share some antigenic determinants with the wild-type enzyme. Based on the results of the Ouchterlony plates fraction I appears to be quite similar to wild-type serratolysin; fraction II on the other hand seems to be a larger molecule of similar antigenic character to serratolysin.

Despite the apparent immunological similarity of mutant and wild-type serratolysin, there are many significant differences between them. The mutant proteinase elutes from DEAE at a lower molarity than does the wild-type. The difference in mobility of the two enzymes on disc electrophoresis at pH 9.3 may be a reflection of differences in charge and/or molecular size. The greatest discrepancies are in sedimentation rate, molecular weight and amino acid composition. Both fractions I and II from prd A2 have considerably

lower S values than the wild-type. The molecular weight and S value of fraction I are a function of concentration which would indicate aggregation of the mutant enzyme. However, even in the aggregated state the mutant enzyme is a much smaller protein based on the low S value of 1.65 and molecular weight of 32,400. The molecular weight of serratolysin is 51,600 by comparison. It may be possible to invoke this aggregation as a means of explaining the apparent discrepency between the low S value for fraction II and its extremely slow diffusion through agar (Ouchterlony plates). Based on the diffusion of fraction II relative to that of IgG one would expect a very large molecule; the S value however, indicates a very small molecule. If one assumes extensive aggregation of the protein at higher concentrations then it is possible, although not very likely, that the apparent size of the molecule would be large on the Ouchterlony plates and small in the ultracentrifuge cell. Two other possible explanations of the slow diffusion through agar are that the protein has some special affinity for agar or that it is a random coil molecule.

Regardless of the reasons behind the slow diffusion through agar, the fact that association takes place indicates that the mutant enzyme is composed of subunits. This of course would make it possible that one of the subunits (if there is in fact more than one type) is also active in prodigiosin synthesis and functions inside the organism in that capacity yet once associated with the remaining subunits (outside the cell perhaps) forms part of the

proteinase and functions as a proteinase. Thus a mutation affecting only this peptide would: 1) block pigment synthesis, 2) reduce but not destroy proteolytic activity, 3) maintain immunological cross reactivity, and 4) show an aggregation phenomenon where none is obvious in wild-type serratolysin.

A more disturbing discrepancy is the amino acid composition of the two forms of serratolysin. Based on the unusually high proline and glycine content of prd A2 proteinase, it appeared that the amino acid composition may, to some extent, be a function of the content of the growth medium. Mutant proteinase from cells grown on BHI has a significantly different composition from proteinase isolated from cells grown on gelatin. It appears that the mutant serratolysin attaches to its substrate but does not fully cleave it, so that a portion of the substrate remains in association with the enzyme. Perhaps this hypothetical portion of substrate which remains attached to the enzyme partially blocks the active site thus reducing the activity of the proteinase. This phenomenon of proteins with vastly different amino acid compositions giving immunological reactions of near identity was also reported for the external and internal varieties of yeast invertase (Gascon et al., 1968).

The sites of cleavage of the two enzymes on the β-chain of insulin are different even though they both degrade substrate. All of the points of cleavage for the mutant proteinase have not been determined as yet but of those that have been determined several are different from the wild-type.

This difference in specificity probably does not account for the loss in pigment in the mutant (in terms of providing some peptide product) since the products of wild-type serratolysin digestion of casein do not stimulate pigmentation in the mutants. Although there is a clear relationship between proteinase and prodigiosin synthesis in S. marcescens, the relationship remains obscure. Oddly enough the pigment is produced in the wild-type organism under conditions which do not stimulate proteinase production (i.e., in the absence of exogenous protein); however, a protein showing immunological cross reactivity to antiserratolysin antiserum has been shown in sonicates of such non-induced cells. This cross-reacting material is a slow diffusing product which in Ouchterlony plates appears to be very similar to the slow diffusing prd A2 fraction II proteinase. The supernatant fluid of these non-induced cultures had no proteolytic activity nor cross-reacting material. It is not clear whether or not the slow diffusing cross-reacting material from non-induced wild-type cultures is identical to the slow diffusing cross-reacting material of the prd mutants. However it is apparent that active proteinase itself is not directly involved in pigmentation since non-induced cultures of S. marcescens ATCC 25419 are red and addition of active proteinase to cultures of mutant organisms has no effect on pigmentation. It seems most probable that the relationship of pigmentation and proteinase involves the slow diffusing cross-reacting material mentioned earlier and that in the mutant this material is slightly altered so that it loses its activity in pigment

production yet manages to retain its antigenicity. This could be achieved as mentioned earlier by the alteration of a subunit which also has activity in prodigiosin production.

It will be interesting to eventually purify and characterize the cross-reacting material found in non-induced cells and to compare this to fraction II of the mutant proteinase. Perhaps if this product could be purified it would be capable of stimulating pigment formation in the prd mutants.

Since it appears that the pigment is associated with the cell envelope (Purkayastha and Williams, 1960), the possibility exists that in the mutant cells some damage has been done to the envelope which simultaneously causes loss of the pigment synthesizing site and damage to some proteinase activating mechanism. This theory is interesting in light of the differences between the wild-type and mutant organisms regarding the extracellular nuclease of Serratia (the mutant organism shows much less nucleolytic activity). Perhaps the mutation is one which damages the membrane affecting not only the site of pigment synthesis but also the release of all extracellular enzymes.

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