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MYXOSIDIN: A NEW PEPTIDE ANTIBIOTIC

A DISSERTATION SUBMITTED TO THE SCHOOL OF GRADUATE STUDIES OF THE UNIVERSITY OF OTTAWA IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN BIOCHEMISTRY

by

David Francis Clapin

Ottawa

March, 1979

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ABSTRACT

Myxobacter 495 produces two bacteriolytic proteases. The production of another antibacterial agent became evident during a study of its interactions with a bacterium which could readily overgrow early shake-cultures of M. 495 but was strongly inhibited, without lysis, by cell-free broth from post-log cultures of M. 495. The inhibitor has been named Myxosidin. The contaminant was isolated and identified as Sphaerotilus natans.

In the first phase of this investigation it was determined that conditions for the production of Myxosidin in shake-culture are similar to those for production of the lytic proteases. A study of the growth of M. 495 in small fermentor cultures (8 litres) showed that the aeration conditions and the timing for harvesting are critical for good yields of the inhibitor. The growth study culminated in a successful large scale fermentation of a 50 litre culture. About 36 litres of broth was harvested at 72 hours. It was assayed at 1300/1716 dilution units/ml of antimicrobial activity (towards Sphaer. natans) which represented approximately 5 grams of Myxosidin or 140 mg/litre of culture broth. The conditions for large scale production of Myxosidin are compatible with production of proteases in high yield as indicated by the high yield of α-lytic protease (394 mg/litre) in the same broth.

Myxosidin in fermentor broths is a component of a large complex which is soluble in 70% acetone. The first steps of an isolation procedure developed by Dr. D.R. Whitaker exploit this property. The complex is concentrated by ultrafiltration and polysaccharides are removed by acetone precipitation. The resulting preparation is then dissociated with guanidine hydrochloride and is extracted with chloroform. The remaining components are partitioned between 3 liquid phases formed by additions of ethanol, chloroform, and ammonium sulfate. The upper phase contains Myxosidin. The final step is ion-exchange chromatography in 7 M urea or displacement chromatography with organic solvents.

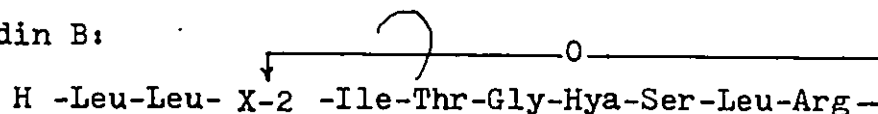
In the second phase of this investigation Myxosidin was characterized by a variety of chemical and spectroscopic methods. An acid hydrolysate of the purified inhibitor produced a mixture of eleven amino acids. An acidic amino acid, "X-1a", was identified as threo-hydroxyaspartic acid by chromatographic and electrophoretic comparisons with synthetic standards. A minor component of acid hydrolysates, "X-1b", increased in concentration with increasing durations of hydrolysis and was identified as erythro-hydroxyaspartic acid. The increase in e-Hya concentration was accompanied by a corresponding decrease in t-Hya concentration.

A residue labelled "X-2" was found to be an aromatic amino acid with a hydroxyl and a phenyl group. A minor

component of hydrolysates, identified as β-phenylserine, appeared to arise from X-2 during acid hydrolysis.

The following amino acids were present in hydrolysates of Myxosidin: Hya, Thr, Ser, Gly, X-2, Arg, Ile, Val, and Leu (molar ratio 3:3:3:3:3:3:2:1:9, respectively). This ratio is consistent with a single peptide of 30 residues or with a mixture of two kinds of decapeptides occuring in a 2:1 ratio in purified Myxosidin. Column chromatography on Sephadex G 50 indicated a molecular weight in the range of a decapeptide, $1.07 \pm 0.24 \times 10^3$ daltons. The Edman degradation reduced the Leu:Val ratio from 9:1 to 6:1 after one cycle, and to 3:1 after two cycles; the Edman reaction was blocked thereafter. Myxosidin was found to be resistant to enzyme digestion, and therefore a variety of chemical treatments were used to generate peptides for sequencing. The peptides obtained showed that Myxosidin was a mixture of two homologous decapeptides differing only in the nature of the residue at position four in their sequence, Ile in Myxosidin A and Val in Myxosidin B. The position of X-2 and Arg in the sequence was established by mass spectroscopy of permethylated N-trifluoroacetyl Myxosidin. Evidence from the above lines of investigation, supplemented by evidence from IR spectroscopy and oxidation

with chromic acid, is consistent with the following structure for Myxosidin B:



with the arrow denoting a lactone bridge between the C-terminal arginine carboxyl and the hydroxyl of the X-2 residue in position three.

The following stereo-assignments were made: Hya, Thr, Ser, Ile, Val are of the L-configuration, and L-Leu and D-Leu are present in a ratio of 2:1. The findings for X-2 and Arg are inconclusive.

The spectrum of activity of Myxosidin is similar to that of tetracycline--a broad spectrum antibiotic, inactive towards fungi and some yeasts, with minimal inhibitory concentrations in the range of 0.3 μg/ml to 2 μg/ml for a variety of bacteria.

The proposed structure of Myxosidin places it in the group of antibiotics known as the peptolides. Myxosidin is only the second antibiotic to be isolated from a myxobacterium, the other being the phenazine Myxin. Myxosidin is the first example of a peptolide which is not produced by a streptomycete, and provides further evidence of a possible evolutionary link between the myxobacteria and the streptomycetes.

ACKNOWLEDGEMENTS

The direction and instruction of Dr. D.R. Whitaker, the supervisor of this project, is gratefully acknowledged. I would like to thank Dr. K.C. Ivarson for his identification of our test organism, Dr. J. Laurence and Dr. W.D. Jamieson for their assistance with permethylations and mass spectroscopy, Dr. E.A. Peterson for donation of a collection of test microorganisms, Dr. J.R. Dillon for an extensive survey of Myxosidin against pathogenic organisms, Dr. A Jackson for collaboration in a study of acute toxicity, and Mr. R. Monahan for amino acid analyses and technical help. The isolation procedure for Myxosidin was worked out by Dr. D.R. Whitaker.

I would also like to thank the Medical Research Council of Canada for their financial assistance during my four years of graduate studies.

To the late
David Michael Clapin, M.D.

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CHAPTER I

INTRODUCTION

A. DISCOVERY OF AN INHIBITORY AGENT IN MYXOBACTER 495 FILTRATES

In 1964, Gillespie and Cook[1] reported the isolation from Ottawa soil of various myxobacteria which gave culture filtrates with lytic activity towards several species of *Staphylococcus*, *Sarcina* and *Arthrobacter*. The isolate with the highest lytic activity was designated *Myxobacter 495*. They considered it to be a species of *Sorangium*, a non-fruiting myxobacterium. The names *Sorangium Sp.*, as well as *Myxobacter 495*, are to be found in subsequent literature on the lytic enzymes of the organism. Christensen[2] has proposed a reorganization of the non-fruiting myxobacteria. According to her classification *Myxobacter 495* would be assigned to a new genus, *Lysobacter*, and would be named *Lysobacter enzymogenes*. However, this proposal has not yet been incorporated in the most recent edition of *Bergey's Manual of Determinative Microbiology*.[3] The original designation, *Myxobacter 495*, will be used throughout this thesis.

Whitaker, Cook and Gillespie[4] determined conditions for the preparation of active culture filtrate from cultures of *Myxobacter 495* grown in a fermentor. The lytic activity reported by Gillespie and Cook was shown by Whitaker to be caused by two extracellular proteases.[5] One of these, which was given the trivial name α-lytic protease, proved to be a

serine protease. The amino acid sequence of this enzyme showed that it belonged to the same group of serine proteases as the pancreatic enzymes.[6] The other lytic enzyme, given the trivial name β-lytic protease, proved to be a zinc-containing metallo-protease. Its amino acid sequence shows no homologies with any other metalloprotease which has been sequenced to date.[7]

The antibiotic discussed in this thesis was detected during an investigation of conditions for the production of the lytic enzymes of Myxobacter 495. A shake-culture was contaminated by an organism with an extremely high growth rate. It became apparent, from later observations, that the growth of the contaminant organism was strongly inhibited by an agent present in post-log phase cultures of M. 495.

The contaminant was isolated and identified later by Dr. K.C. Ivarson (Chemistry and Biology Research Institute; Canada Department of Agriculture, Ottawa) as **Sphaerotilus natans**. This organism plugs activated sludges in sewage treatment plants.[8] Recently, it has been found clogging field-drains in Southern Ontario farms.[9] It was used throughout this study as a test-organism for assays of the inhibitor by serial dilution or paper disc assay procedures.

The inhibition of Sphaerotilus natans was unaffected by a variety of pretreatments of the culture filtrate of M. 495: for example, treatment of the filtrate with

diisopropylfluorophosphonate (DFP) to inactivate serine proteases; treatment with the chelating agent 8-hydroxyquinoline; or treatment with p-chloromercuribenzoate. It was unaffected by the addition of catalase to the assay medium, or by increases in the ferric iron content of the assay medium. Inhibition was not dependent on the presence of either sucrose or glutamate in the assay medium.

The inhibitory agent was not extracted by ether from acidified culture filtrates and therefore was not myxin, a phenazine antibiotic which, as discussed in the following review of antibacterial agents from myxobacteria, is produced by a close relative of M. 495. However, the inhibition was eliminated by pretreatment of the culture filtrate with alkali. Inhibition was reduced by pretreatment with pepsin atpH 2.5. These observations suggested that the inhibitory agent might be a peptide antibiotic and merited further investigation.

B. LITERATURE REVIEW OF ANTIMICROBIAL AGENTS OF MYXOBACTERIA

The earliest reports of antibiotic activity in myxobacteria are those of Oxford and Singh[10] and Oxford.[11] Investigations of nutritional requirements of myxobacteria on solid supports led these authors to postulate that *M. virescens* produced "a true non-enzymic antibiotic substance capable of killing viable eubacteria and so rendering them susceptible to lysis by the exocellular enzymes previously elaborated by the growing myxococci."[12] When the producer

organism was cultured in submerged culture using a defined medium of casein hydrolyzate as carbon and nitrogen source, antibiotic activity of filtrates was observed against Staph. aureus during an initial ten days of incubation. The activity of filtrates disappeared if incubation was continued, although lytic activity against gelatin remained strong.

Oxford's[11] more complete report on the antibiotic activity of M. viriscens contained additional details on the antimicrobial substance (s): the better the growth of producer organism, the better production of antibiotic; solid products which were soluble in ethyl or butyl alcohol showed substantial activity;

the antibiotic was more stable than the 'gelatinase' of filtrates at 60°C. Isolation was accomplished by evaporating culture filtrate to small volume in vacuo, then drying over H_2SO_4, and extracting this solid residue with 100 parts of absolute ethanol at 55°C to 60°C for one hour. The extract was evaporated to dryness. From filtrates with an initial activity against Staph. aureus of 40 dilution units, 13 mg of a ninhydrin-positive, colourless solid was obtained which was active at a dilution of 1 part in 12,500 parts of S. aureus nutrient medium. Later authors[13,14] proved unable to confirm these findings and no further report on the structure of the substance isolated by Oxford appeared.

Solnetzewa[15] and Singh[16] demonstrated that one of the lytic factors of myxobacteria was able to diffuse through

a cellophan membrane, and was stable at 100°C for 15 minutes. In his review of the myxobacteria Dworkin[17] suggests that the factor described by these authors is likely to be a basic peptide antibiotic similar to bacitracin or gramicidin: "A peptide antibiotic would be heat-stable, dialyzable, and precipitable with organic solvents." Thus although the antibiotics demonstrated by Solnetzewa,[15] Singh,[16] and Oxford[11] all appeared to be peptide antibiotics the first two antibiotics isolated from myxobacteria proved to be phenazines. The isolation of myxosidin from M. 495 is the first confirmation of these early reports on antibiotic substances in myxobacterial filtrates.

Norén and Raper[18] disputed the claim of Oxford's group that myxobacteria grew only on previously killed host cells. In their study six myxobacteria were examined for antibiotic activity against eleven eubacter ales test organisms. All myxobacteria tested were found to have some antibacterial activity, and in each case, only against gram positive eubacteralës. Antibiotic activity was determined by streak tests on petri dishes. Inhibition zones varied greatly among the eubacteria tested. Filtrates of myxobacteria grown in submerged culture were assayed by serial dilution and the results of the streak tests were confirmed. The authors noted that antibiotic activity increased during the first few days of culture and then decreased rapidly after reaching its

maximum inhibitory effect. Inhibition of one myxobacterial species by another was noted. This may have been due to bacteriocin production rather than antibiotic substances.

The conclusion was reached that "if a bacterium is susceptible to an antibacterial substance produced by a myxobacterium, this fact alone does not mean that it is highly susceptible to the bacteriolytic agents of the same myxobacterium or vice versa."[19] A. aerogenes for example, was insensitive to antibiotics produced by the myxobacteria but it was rapidly lysed by all myxobacteria tested. Although an antibiotic was not apparently required to kill potential host cells the authors suggested that it might serve the function of inhibiting the growth of gram-positive cells and thereby promote the growth of gram-negative cells, a more desirable substrate type, in the vicinity of myxobacterial swarms. Subsequent reports on myxobacterial lytic activity have, however, shown that they demonstrate little or no preference for gram-negative eubacterials as substrates for lysis.

Margolith[20] reported no appreciable difference between the lysis of gram-positive and gram-negative bacteria using M. fulvus. He found that living cells were utilized more slowly than killed cells and although he could isolate no diffusible antibiotic substance he concluded that "lysis of living cells seems, therefore, to be the result of the common effort of two principles: a 'killer' and a proteolytic enzyme."[21]

In contrast to the above reports, all of which came to the conclusion that myxobacteria produced some antibacterial substance(s), Kletter and Henis[22] grew M. fulvus and M. viriscens in submerged culture and found no antibiotic activity in supernatants of any bacterial suspension at any period in the growth cycle when tested against Staphylococcus aureus or Aerobacter aerogenes. They did find that heat-killed gram-positive cells were not as susceptible to the lytic enzymes of myxobacteria as gram-negative cells and that there was no apparent difference in the utilization of living gram-positive or gram-negative bacterial substrates.

Peterson, Gillespie and Cook[23] isolated and characterized the first antibiotic substance from a myxobacter. The producer organism was an unclassified soil bacterium named Sorangium sp. (strain 3 c). The antibiotic eventually characterized was named Myxin, a phenazine with the chemical name: 1-hydroxy-6-methoxyphenazine-5, 10-dioxide. This antibiotic has been studied extensively by Lesley and Bekhi.[24,25] The producer organism was started on shake flasks in yeast-soil liquid medium and grown for large scale batch productions in a fermenter with culture volumes of 10 litres. The medium was aerated at 1 liter per min. (1/10 vvm) stirred at 300 rpm and maintained at 25°C. Maximum antibiotic activity occured at 20 to 24 hours. It was assayed against

Staph. aureus (PS80) by dilution series in a tryptone-yeast extract-dextrose broth. Paper disc assays were used to determine the spectrum of activity against bacteria, fungi, actinomycetes and yeasts.

The antibiotic could be isolated from culture filtrates by ether extraction at pH 1.0 (HCl) or by adsorption on to Amberlite C/G-50 H^+ columns. The purified antibiotic was a deep cherry red colour. Its minimal inhibiting concentration against Staph. aureus was 1.56 μg/ml. Viable cell counts showed that it was bacteriocidal towards Staph. aureus. It was active against many fungi, actinomycetes and yeasts. Although it has not yet found a clinical use, Myxin is being studied for its potential as a biological probe. It causes a rapid degradation of DNA in E. coli[24, 25] and selectively affects chloroplasts of plant leaves.[26]

Rosenberg, Vaks and Zuckerberg[27] isolated an antibiotic from Myxococcus xanthus (strain TS). The producer organism was grown in shake flasks on casitone medium with peak production of antibiotic reached after 3 to 4 days. Cells were centrifuged out and the antibiotic was extracted from supernatant with 0.8 vol. chloroform. Subsequent purification to apparent homogeneity was done by adsorption on to silicic acid, elution with 5% methanol in chloroform and preparative TLC with various organic solvent systems. Biological activity was assayed against E. coli B using both serial dilution and

paper disc techniques. The antibacterial spectrum of the antibiotic was determined by paper disc assays. It was found to be bacteriocidal, and capable of killing only actively growing cells. Preliminary experiments on the mechanism of action indicated that the antibiotic has no apparent direct effect on DNA or RNA biosynthesis. The authors concluded that the antibiotic was active against cell wall biosynthesis because of its ability to induce sphaeroplast formation under certain conditions towards several gram-positive and gram-negative bacteria tested, including some penicillin resistant strains of E. coli. The purified antibiotic was a white solid with a molecular ion at 650 and peaks indicating an aromatic compound by mass spectroscopy, and U-V spectroscopy. It was readily distinguishable from myxin on the basis of its chromatographic behaviour in different solvents and supports.(28) It is likely that this compound is a phenazine antibiotic, but to date no subsequent report on its structure has appeared.

In addition to lytic enzymes and antibiotics, two other categories of antimicrobial agents have been isolated from myxobacterial filtrates. The first of these are compounds of the class known as 'Bacteriocins'. They are, in general, bacteriophage-like particles produced by bacteria which inhibit a narrow range of bacterial strains of the same species as the producer organisms. Unlike true phage these particles are not self-reproducing or infectious.

McCurdy and MacRae[29] demonstrated the presence of a bacteriocin produced by Myxococcus xanthus strain b. The antimicrobial agent isolated by these authors was active against Cystobacter fuscus M 205, Cystobacter fuscus M 430, Melittangium lichenicolum and slightly active against Myxococcus fulvus. It was named Xanthicin. Its microscopic appearance and biochemical properties distinguished it from the bacteriocins which are phage-like particles and from O-somatic antigens produced as bacteriocins by some gram-negative bacteria. It may be of the staphylococcin type primarily composed of lipid and resembling membrane vesicles. These observations for Myxococcus xanthus were confirmed by Brown et al.[30]

Hirsch[31] reported the isolation of bacteriocin from Myxococcus fulvus strains. From filtrates of strain Mx 16 three bacteriocinic proteins of molecular weight less than 30,000 daltons were isolated and named fulvicins A, B and C. Most of the myxobacterales tested were sensitive to these fulvicins, none of the Cytophagales.

A fourth type of antimicrobial substance produced by myxobacterales was reported by Norén and Odham.[32] The organism Myxococcus xanthus secretes a variety of organic factors into the medium which have the effect of preventing germination of fungal spores and inhibiting their growth. These factors were identified as a mixture of fatty acids, both saturated and unsaturated in the C_{13} - C_{17} range. The main

one is methyl-branched with an iso-configuration. The authors also concluded that a component of antibiotic character, not a fatty acid, was present and added to the inhibitory effect of their preparations. This factor was not further identified or characterized.

The extracellular antimicrobial products of the myxobacteria are summarized in fig. 1. They are: (1) lytic enzymes, of which serine proteases, a neutral protease and a metallo-protease have been isolated from M. 495; (2) bacteriocins, xanthicin and fulvicins A, B and C from M. xanthus and M. fulvus respectively; (3) fungistatic lipids from M. xanthus and (4) antibiotics of the chemical class of phenazines, Myxin, and peptides, Myxosidins A and B, the latter isolated from M. 495. The diverse types of antimicrobial substances produced by the myxobacteria may be important for maintaining their ecological niche of utilizing other microorganisms as a food source.

(1) LYTIC ENZYMES
(i) proteases
(ii) lipases
(iii) polysaccharidases

(3) FUNGISTATIC LIPIDS ← MYXOBACTERIA → (2) BACTERIOCINS
(i) xanthicin
(ii) fulvicins

(4) ANTIBIOTICS
(i) phenazines (myxin)
(ii) peptide (myxosidin)

ANTIMICROBIAL EXTRACELLULAR PRODUCTS OF MYXOBACTERIA

FIGURE 1

CHAPTER II

GROWTH STUDY

A. OBJECTIVE

The determination of the relationships between conditions of microbial growth and the concentration of a product of interest was the goal of the growth study of *Myxobacter 495* described in this chapter.

The producer organism, *Myxobacter 495*, was studied in shake-flasks containing 800 ml of medium, in a 14-litre fermenter containing batch-cultures in 8 litres of medium, and in a 150-litre pilot plant fermenter containing batch-cultures in 50 litres of medium. In these studies, shake-cultures provided the inocula for the small fermenter, and cultures from it, in turn, provided the inocula for the large fermenter. The upper limit of 50 litres volume for cultures grown in the large fermenter was set by the capacity of a filtration unit used in the isolation of the desired product.

Different compositions of media were tried in shake-culture and several growth parameters were measured. Two different media, selected from the shake-flask study, were used to examine the effect of different aeration conditions on growth and production parameters of cultures grown in the 14-litre fermenter. Experience and techniques gained from operation of the small fermenter were applied to cultures grown in the large fermenter, the objective being to produce

the antibiotic substance (Myxosidin) on a scale sufficient to characterize its structure and antibiotic properties.

B. MATERIALS

(a) Media

Five types of media were prepared. Two of these contained 1% casamino acids (Nutritional Biochem.), an acid hydrolysate of casein, as the nitrogen (as well as a carbon) source; they differed in sucrose content-- one containing 1% sucrose, the other, 0.1%. Two media contained 1% monosodium-L-glutamate (General Biochem.) as the nitrogen source with, as above, 1% and 0.1% sucrose. These media were made up with tap water. The remaining medium was a 1% monosodium glutamate-1% sucrose medium made up with distilled water and with zinc sulfate omitted from the inorganic components.

The inorganic salts in the media (amounts in g/l) were as follows: K_2HPO_4, 0.1; KH_2PO_4, 0.1; $MgSO_4 \cdot 7H_2O$, 2; NaCl, 2; ferric citrate, 0.025; $ZnSO_4 \cdot 7H_2O$, 0.007; and $MnSO_4H_2O$, 0.003. Unless otherwise specified,all chemicals were of reagent grade. For shake-cultures, the solutions of inorganic salts and casamino acids or glutamic acid were adjusted to pH 7.00 with aqueous KOH prior to autoclaving for 20 minutes at 121°C in flasks plugged with styrofoam. Solutions of sucrose at ten-fold concentrations were autoclaved separately and, upon cooling, portions were combined

with the rest of the media to a final concentration of 1% or 0.1% sucrose.

In the case of fermenter runs in either the 14-litre or the 150-litre fermenter, media of the same composition detailed above were sterilized as three separate solutions rather than two before being combined. Solutions of the phosphate buffer (2% K_2HPO_4, 2% KH_2PO_4), adjusted to pH 7.00, and solutions of sucrose were autoclaved for 30 minutes at 121°C in glass bottles fitted with wrapped needle connectors. A solution of the remaining inorganic salts and casamino acids or monosodium glutamate was adjusted to pH 7.00 with an aqueous solution of KOH and was sterilized for 30 minutes at 121°C in the vessel of the fermenter. After cooling, the sterile phosphate and sucrose solutions were added to the rest of the medium in the fermenter through sterile needle connector ports.

(b) Fermenters

A 14-litre bench-scale (Chemap) fermenter with a glass vessel was used in initial batch-culture studies. The inside diameter of the vessel of the fermenter was 22.4 cm, its height was 27.2 cm. The working volume was 10 litres maximum. The steel top-plate of the vessel was equipped with a sterilizable pH probe and a sterilizable temperature probe. Needle connector ports located in the top-plate allowed addition or removal of solutions to the contents of

the vessel without contamination. A central shaft passed through a sterilizable pressure seal in the steel bottom-plate of the vessel and was driven by a variable-speed, electric motor. Flat-bladed, radial impellers could be attached to this shaft and fixed to any height. At the top of the same shaft two conically-shaped, radial turbines and a conical deflector, comprised a mechanical foam breaker of the design known commercially as "Fundafoam". Four plates, fixed to the bottom-plate of the vessel, functioned as baffles to promote turbulence and to prevent rotation of the medium. A supply of compressed air was set to the desired flow rate by means of a flowmeter. The air entered the vessel of the fermenter through a sterilizable, glass-wool packed, inlet filter, and was bubbled through the medium from a stainless steel, ring sparger. Air exited from the vessel via a condenser, which reduced evaporative losses, through a sterilizable, glass-wool packed, outlet filter.

A pilot plant fermenter (Chemap), with a vessel of 150 litres volume was used for large scale production runs. Its vessel was of stainless steel construction with a maximum working volume of 100 litres. Ports passing through the sides of the vessel held a sterilizable pH probe and a sterilizable temperature probe. Needle connector ports located in the top plate allowed addition of solutions to

the contents of the vessel without contamination. A glass inspection window and light fixture in the top plate allowed the contents of the fermenter vessel to be viewed during a run. Samples could be withdrawn through a sterilizable valve system connected to the bottom plate of the vessel. A central shaft passed through a steam-sealed gland of the type used on large fermenters. This design allowed steam to circulate between the packing layers during sterilization of the fermenter vessel. Three flat-bladed, radial impellers were set at fixed positions on the shaft. The stirring speed of the shaft was variable and was set by a pulley and clutch drive-belt device driven by a large electric motor. A mechanical foam breaker of the "Fundafoam" type (see above) was fixed to the top plate of the fermenter vessel and was driven by a separate, direct drive electric motor at a single speed of 1800 rpm. Four baffle plates were attached to the walls of the vessel. Air-line pressure was maintained at 25 psi by means of an air pressure regulator. Air flow into the vessel was measured by a flowmeter. Both the air inlet and the air outlet filters were of the ceramic candle type. Air was supplied to the contents of the vessel through a ring sparger located at the bottom and encircling the lowest impeller. A condenser was fitted to the air outlet line to reduce evaporative losses. The fermenter vessel was wrapped in a jacket supplied with water and steam lines. An electro-pneumatic temperature control mechanism maintained

jacket temperature at the desired preset value. Two independently sterilizable reservoirs were connected by a valve system to the fermenter vessel. These were used to add solutions of the phosphate and sucrose components of the medium to the sterile solution of inorganic salts and glutamate in the vessel.

C. METHODS

(a) Growth of Myxobacter 495 in Shake-Cultures

Cultures of Myxobacter 495, in 40 ml of each medium, were inoculated with samples of a previous culture which had been frozen in a dry ice-acetone bath and stored at -60°C. All cultures were grown at 25°C on a rotary shaker describing a circle, 1" in diameter, at about 150 rpm. The starting cultures were subcultured four times, the third and fourth subcultures were in duplicate. Approximately 38 ml of each of the final subcultures was used to inoculate a 2800 ml Fernbach flask containing 800 ml of medium. A small amount of each inoculum was set aside for turbidity measurements and microscopic examination.

(b) Growth of Myxobacter 495 in Fermenters

Batch fermentation cultures with 8 litres of either the 1% casamino-1% sucrose medium or 8 litres of 1% glutamate-1% sucrose were grown at a variety of aeration rates. Cultures were inoculated with samples of a previous 24 hour culture in 5 ml of casamino medium. These 5 ml cultures were propagated in 50 ml cultures of casamino or glutamate media for at least two subcultures. The inocula for the fermenter

were 24 hour 400 ml cultures in the appropriate medium. These were added through a sterile needle connector and a small sample was kept for pH and turbidity measurement. The temperature of the vessel was regulated at 26°C. After inoculation the outlet air filter was replaced with a length of sterilized rubber tubing in order to ensure atmospheric pressure in the vessel.

Cultures of Myxobacter 495 tend to foam violently when air is sparged through them in a fermenter, particularly during the first 24 hours of growth. Foam production is a function of the aeration rate and of the stirring speed. High stirring speeds reduce the size of the air bubbles rising through the medium from the sparger and give a dense, stable foam which is difficult for the mechanical foam breaker to separate. The excessively foamy nature of these cultures required (a) a high turbine speed to control foam and (b) a low stirring speed to reduce foam production. These conditions could not be realized in the 14-litre fermenter since, as indicated above, the foam-breaker turbine and the stirring blades were on the same shaft. Attempts to lower the rate of foam production by reducing the length of the stirrer blades and changing their position on the shaft were unsuccessful. However, it was found that good growth could be obtained by relying only on the sparged air for agitation. The stirrer was therefore removed from the shaft allowing the foam breaker to be operated at the

high speeds (1200 - 1800 rpm) which were necessary for effective foam control.

The inocula for 50 litre batch-cultures were 48 hour mid-log phase, 8 litre cultures prepared as described above. Five litres of inoculum was transferred to 50 litres of 1% glutamate-1% sucrose medium in a 150-litre fermenter via a sterile transfer tube. There were some variations in technique from one production run to amother but essentially the procedure was as follows: initial aeration was accomplished by stirring the culture at 200 rpm while air passed over the top of the medium (vortex aeration)--this avoided loss of inoculum into a large foam layer and led to shorter lag phases; temperature was regulated at 26°C; at 24 hours when cultures had an optical density of about 1, air was sparged from a pipe at the bottom of the vessel at 17 l/min which corresponded to ½ vvm on the 8 litre scale (see scale-up discussion in Chapter II section D (c) "Growth on 50 litre Scale"). The vessel immediately filled with foam and the foam breaker was switched on at 1800 rpm; as a safety measure, the outlet filter was removed and replaced with a long section of tubing leading to a drain--this allowed the air pressure inside the vessel to remain at atmospheric pressure and eliminated the risk of a pressure build-up should excessive foam overflow the foam breaker and plug the outlet air filter. The culture was harvested at 72 hours by connecting the vessel outlet

drain directly to the input of a CEPA continuous-centrifuge and pumping it through the centrifuge under a head of air pressure created in the fermenter by closing the air outlet valves.

(c) Sampling Procedure

Immediately after inoculation of the Fernbach flasks or the fermenters, a zero-time sample was withdrawn. Further samples were taken during each run. Smears were made, stained with safranin and examined for evidence of contamination. A portion of each sample was diluted with 1% NaCl for measurement of turbidity at 660 nanometers. The remainder was centrifuged for 30 minutes at 11,000 rpm (Servall) and a portion of the supernatant was removed for pH measurement. Another portion was treated with an equal volume of 5% aqueous trichloracetic acid (TCA) to remove protein. Samples were stored at -20°C pending determination of enzyme and antibiotic activity.

(d) Analytical Procedures

(i) Sucrose Assay

Sucrose was determined by the phenol-sulfuric acid method.[33] TCA-treated samples were diluted 1 to 101 (1% sucrose media) or 1 to 11 (0.1% sucrose media) with distilled water. Fresh reagent grade sulfuric acid (Fisher) and phenol (BDH) were used. Absorbances were measured at 490 nm. Glutamic acid or casamino acids at the levels present in these analyses do not interfere with the colorimetric reaction.[34]

(ii) Amino Acid Analyses

Analyses were done with a Beckman 121 amino acid analyzer (the resins and buffer systems used are described in Chapter IV). The TCA-treated samples were diluted as required with the appropriate citrate buffer (pH 3.25 for acidic and neutral amino acid analyses, pH 5.25 for basic amino acids and ammonia). Some samples were centrifuged or filtered to remove any residual protein precipitate.

(iii) α-lytic Activity

Samples of culture supernatant were assayed with a pH-stat (Ole Dick) for esterase activity towards N-benzyl-L-alanine methyl ester.[35] The titrant was 0.05 M KOH. The reaction mixture was maintained at pH 8.0. The amount of α-lytic protease present in the supernatant was calculated using a value for the second order velocity constant of $k = 723\ M^{-1}\ sec^{-1}$.[36]

(iv) Turbidity Measurements

Turbidities of samples of cell-suspensions, diluted with 1% NaCl, were measured at 660 nanometers, using 1 cm glass cells in a Beckman DB spectrophotometer.

(v) Protease Activity Towards Casein

Samples were assayed for proteolytic activity towards casein.[37] The activity was also measured after treatment of the samples with diisopropylfluorophosphonate (DFP). Thirty microlitres of a 10% solution of DFP in isopropanol

was added to each 3.0 ml sample of supernatant solution. The DFP-treated solutions were left at room temperature for 3 hours and assayed for proteolytic activity towards casein.

(vi) Myxosidin Assay by Serial Dilution

The assay procedure for determination of antibiotic activity of Myxosidin is described in more detail in Chapter V. Briefly, activities of culture filtrates were determined by a serial dilution procedure. A series of flasks containing graded amounts of supernatants in 5.0 ml of 1% glutamate-1% sucrose medium was inoculated with a 24 hour culture of Sphaerotilus natans and incubated for 18 hours at 26°C on a rotary shaker describing a circle 1" in diameter at 130 rpm. Distilled water was used for sample dilution and the blank contained medium and inoculum alone. The flasks were scored as "clear" (complete inhibition of growth), "turbid" (heavy growth), or "marginal" (partial inhibition of growth). If, for example, 100 μl of a sample solution in 5.0 ml of medium resulted in a turbid culture (dilution 1:51) after 18 hours of incubation, while 125 μl in 5.0 ml of medium resulted in a clear culture (dilution 1:41), the activity would be expressed as 41/51 dilution units/ml. In practice, often two or more sets of assays were required. The first, with a broad range of serial dilutions, determined the approximate level of antibiotic activity. The second, and

subsequent assays, using a narrower range of dilutions, determined the level of antibiotic activity more exactly.

(vii) pH Measurements

Aliquots of culture supernatants were set aside for pH measurement using a Radiometer pH meter. Readings differed very little before and after removing cells by centrifugation.

D. EXPERIMENTAL RESULTS

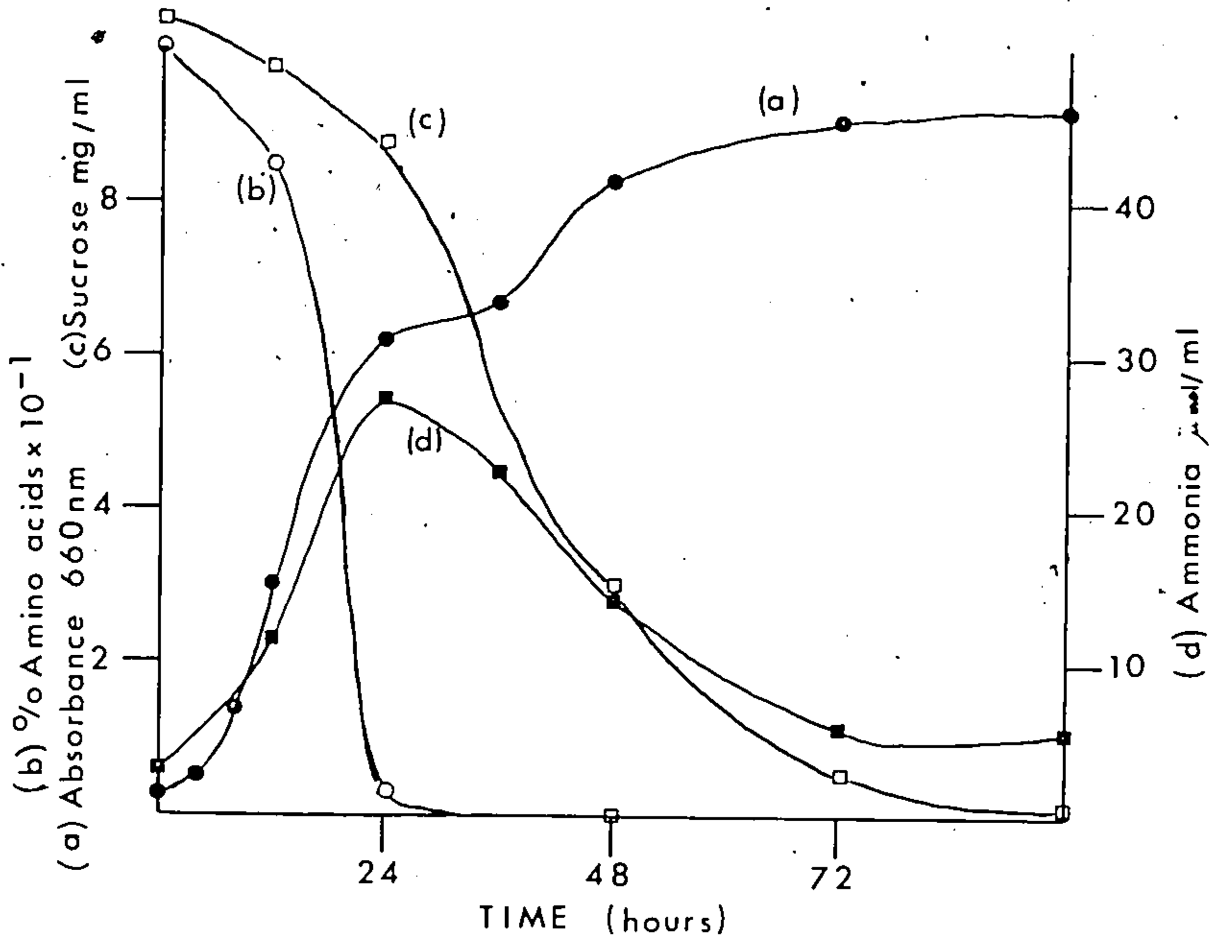
(a) Growth in Shake Cultures

The producer organism, Myxobacter 495 was grown in 800 ml shake flask cultures. Two different nitrogen sources were used, hydrolyzed milk proteins and monosodium L-glutamic acid. The carbon source of all media was commercially available sucrose used at two concentrations: 1% and 0.1%. All media were adjusted to pH 7.0. A special medium was prepared using distilled water and inorganic salts omitting zinc sulfate. Media depleted in zinc gave culture filtrates with reduced levels of β-lytic protease activity. This enzyme is a zinc-containing metallo-protease abundant in culture filtrates of Myxobacter 495.

Changes in growth parameters are illustrated in figures 2 to 11. These graphs show the growth of the cell population measured as increase in turbidity at 660 nm; consumption of casamino acids or glutamic acid; consumption of sucrose; pH changes; production of extracellular lytic enzymes measured in a specific assay for α-lytic protease;

FIGURE 2

Growth parameters for 800 ml shake-flask cultures on 1% casamino-1% sucrose medium: (a) —●—●— turbidity at 660 nm; (b) —○—○— % amino acid concen., zero-time set to 100%; (c) —□—□— sucrose concentration of medium, mg/ml; (d) —■—■— ammonia concentration of medium, μmol/ml. The lower graph illustrates pH changes in the same cultures. Experimental points are the arithmetic mean of measurements made on duplicate cultures.



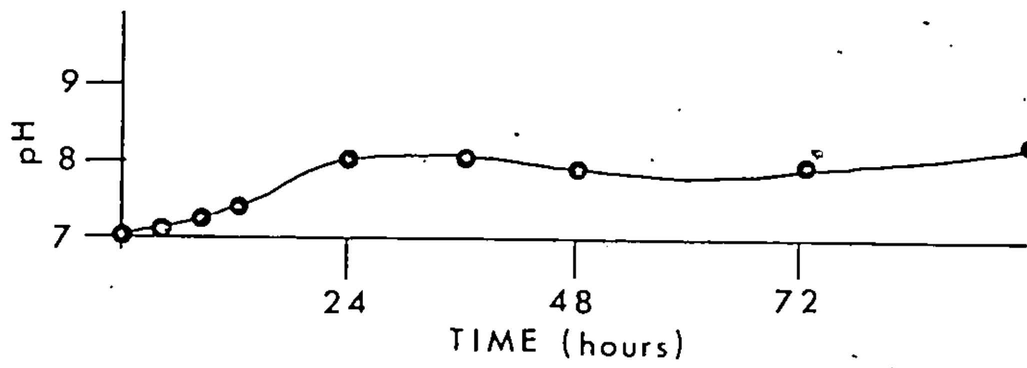


FIGURE 2

FIGURE 3

Enzyme activity of culture filtrates from 800 ml shake-flask cultures grown on 1% casamino-1% sucrose: (a) —●—●— α-lytic protease activity expressed as μg protein per ml culture filtrate; (b) —▲—▲— casein lytic activity in micromoles of tyrosine released/ 10 min/ml culture filtrate; (c) —■—■— casein lytic assay following DFP treatment of culture filtrates. The DFP treatment had the effect of inactivating serine proteases.

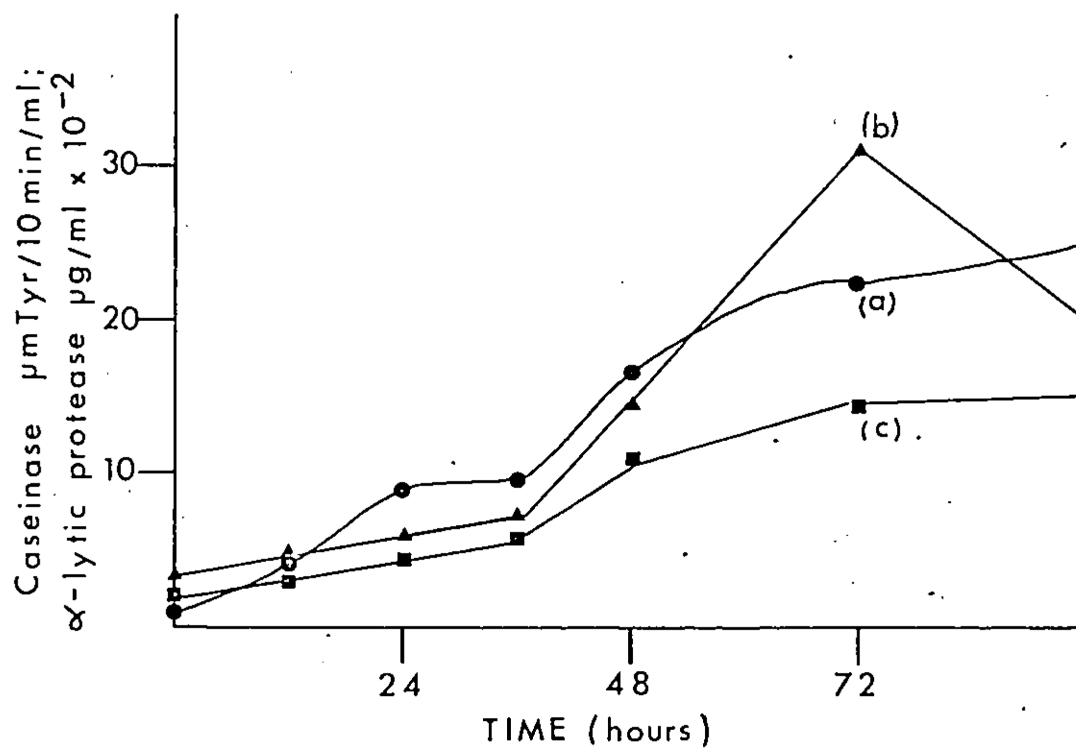
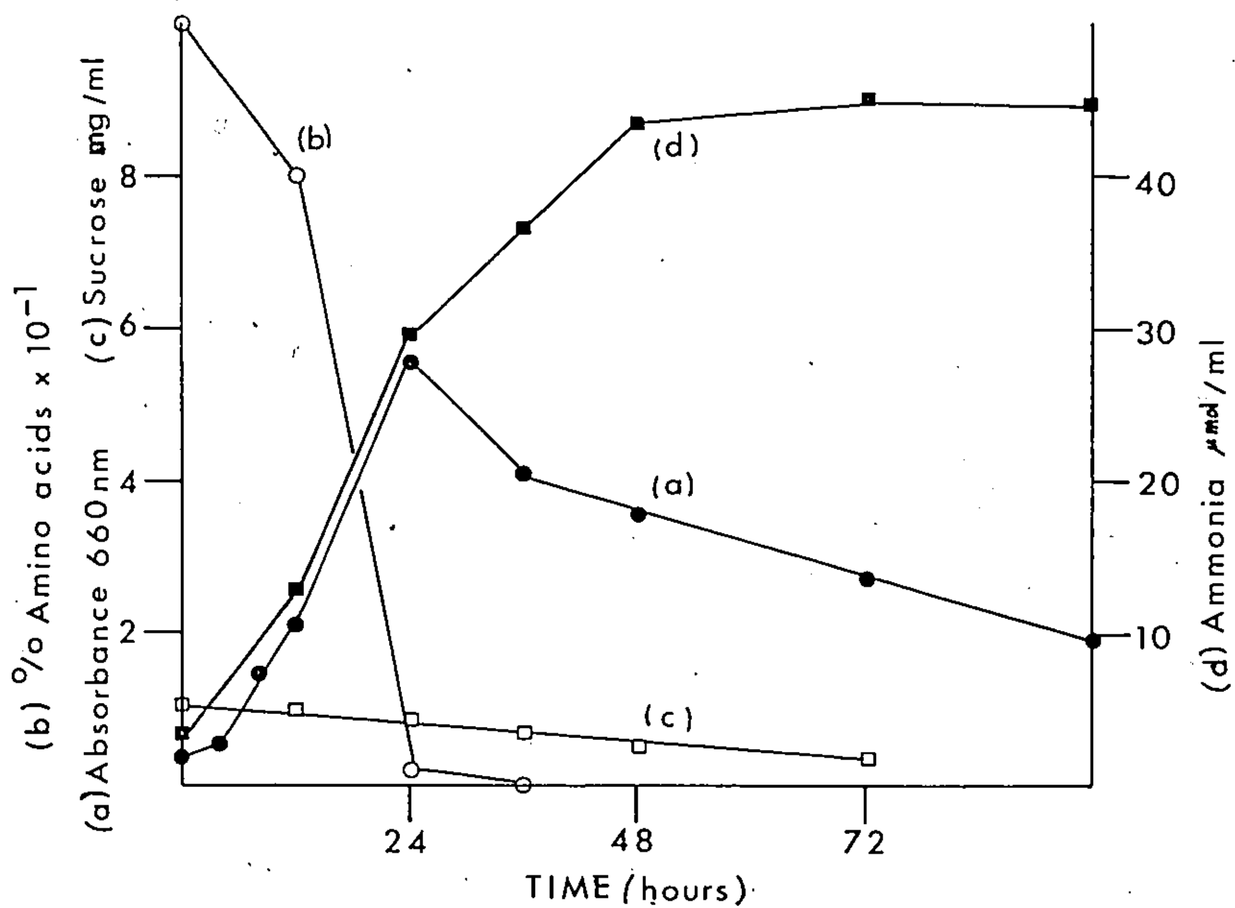


FIGURE 3

FIGURE 4

Growth parameters for 800 ml shake-flask cultures on 1% casamino-0.1% sucrose medium: the symbols and units are as in Fig. 2.



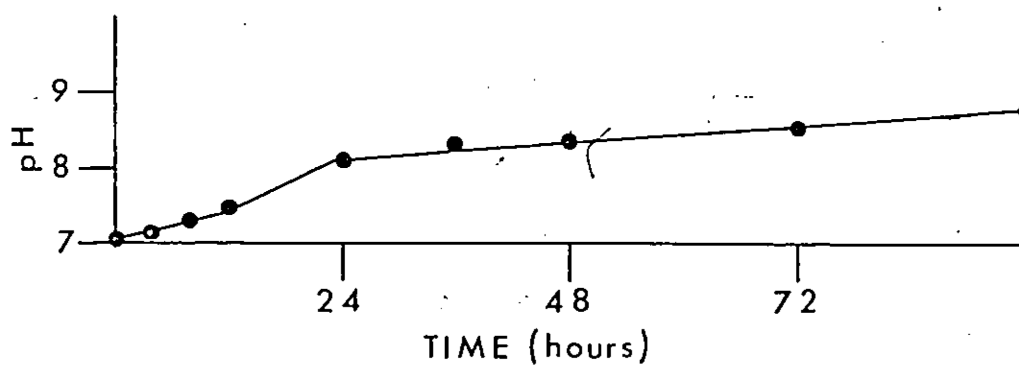


FIGURE 4

FIGURE 5

Enzyme activity of culture filtrates from 800 ml shake-flask cultures grown on 1% casamino-0.1% sucrose: the symbols and units are as in Fig. 3.

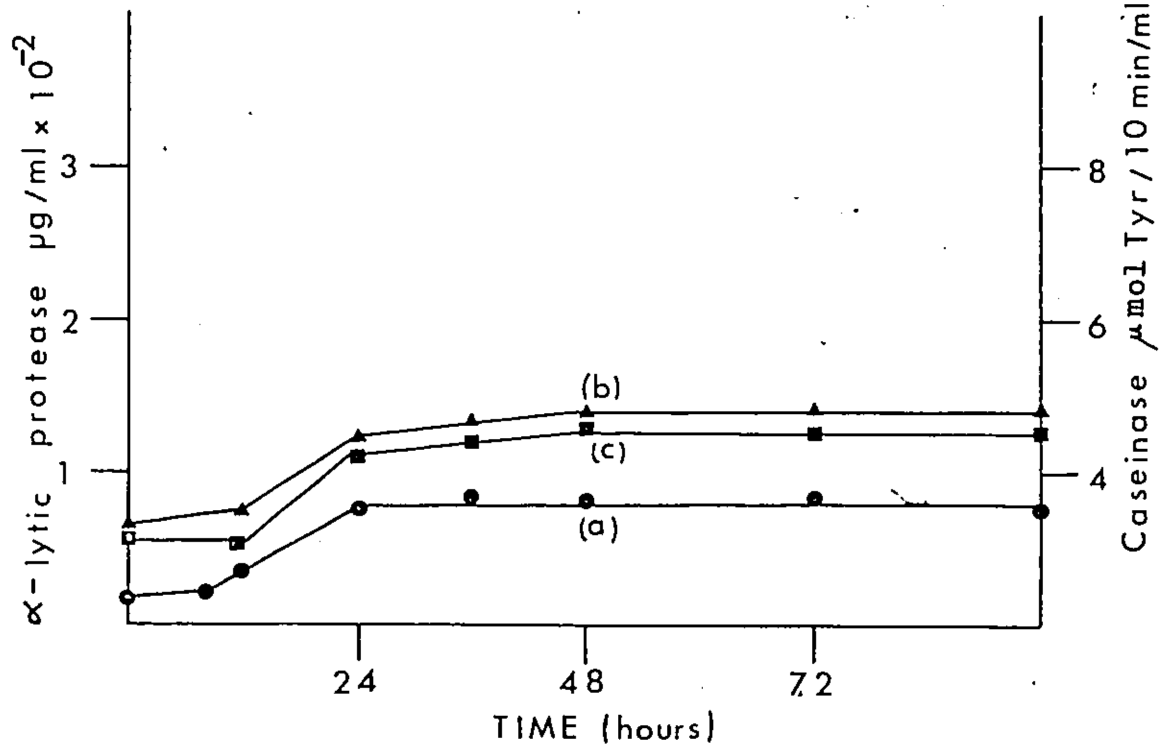
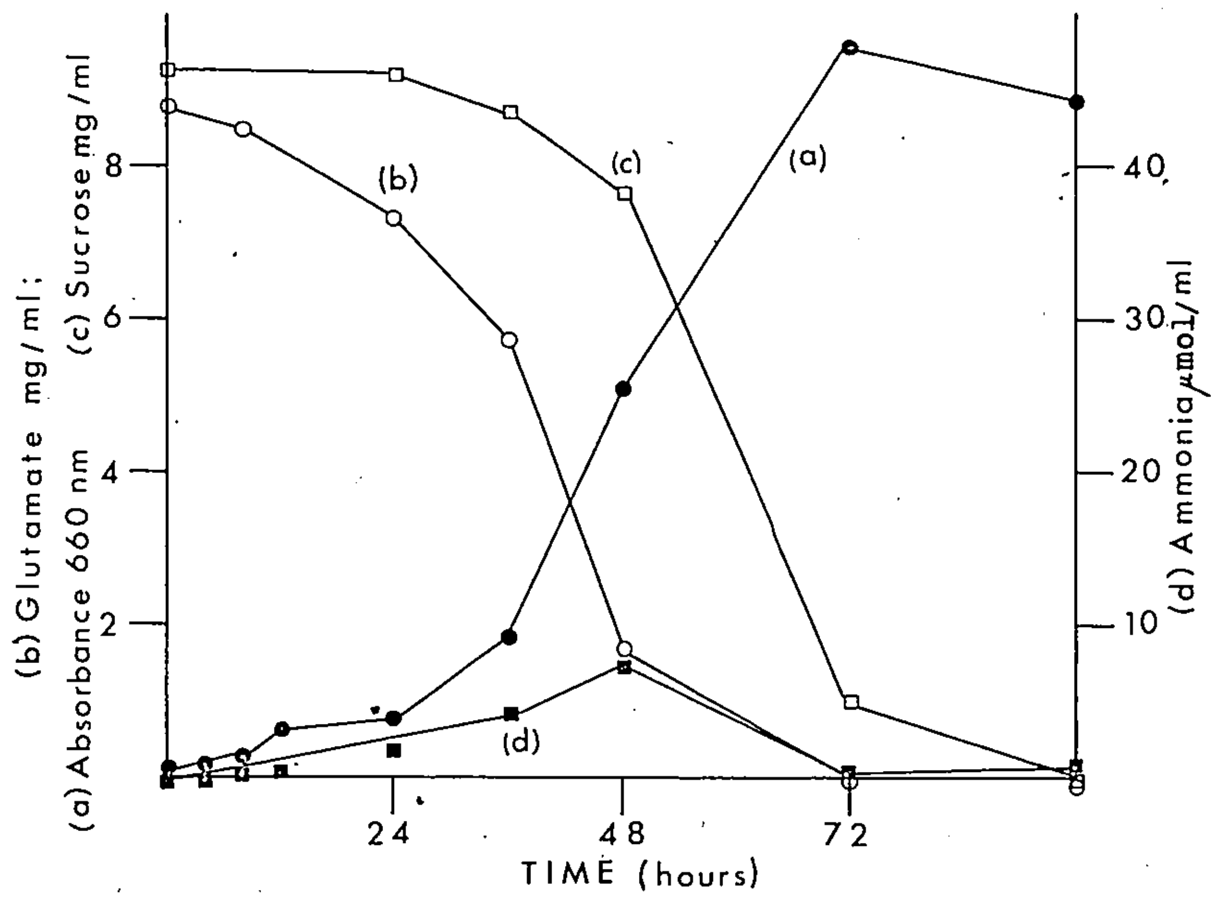


FIGURE 5

FIGURE 6

Growth parameters for 800 ml shake-flask cultures on 1% glutamate-1% sucrose medium: the symbols and units are as in Fig. 2.



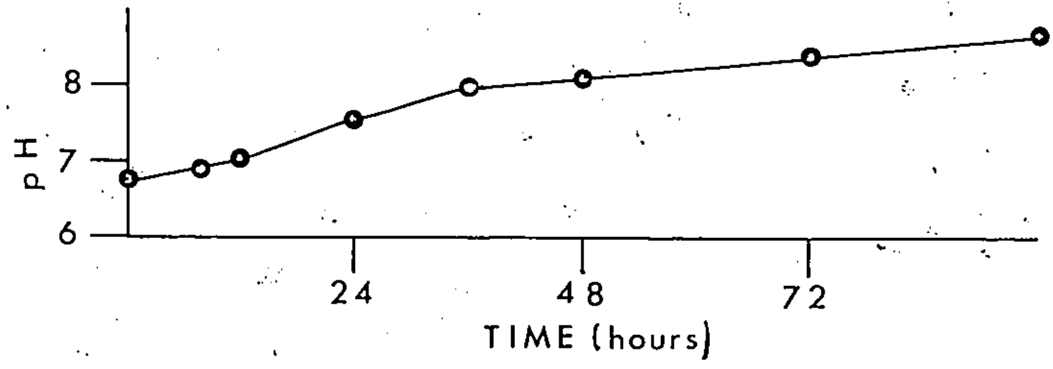


FIGURE 6

FIGURE 7

Enzyme activity of culture filtrates from 800 ml shake-flask cultures grown on 1% glutamate-1% sucrose medium: the symbols and units are as in Fig. 3.

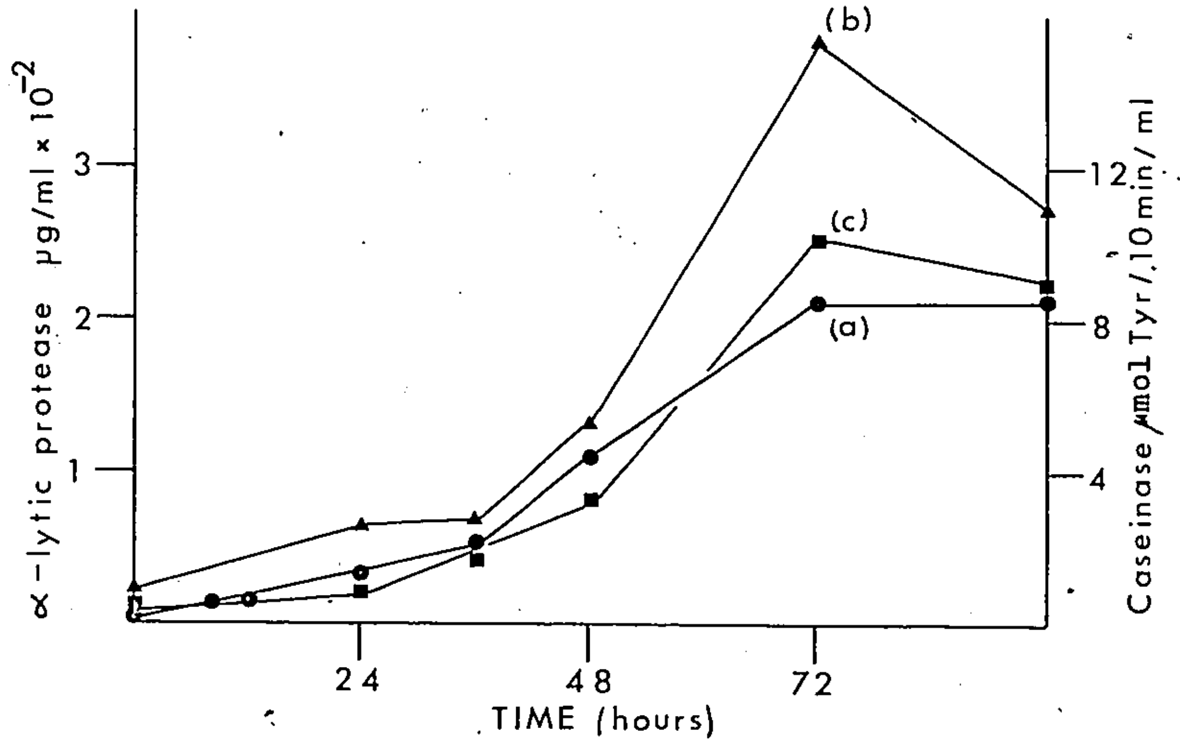
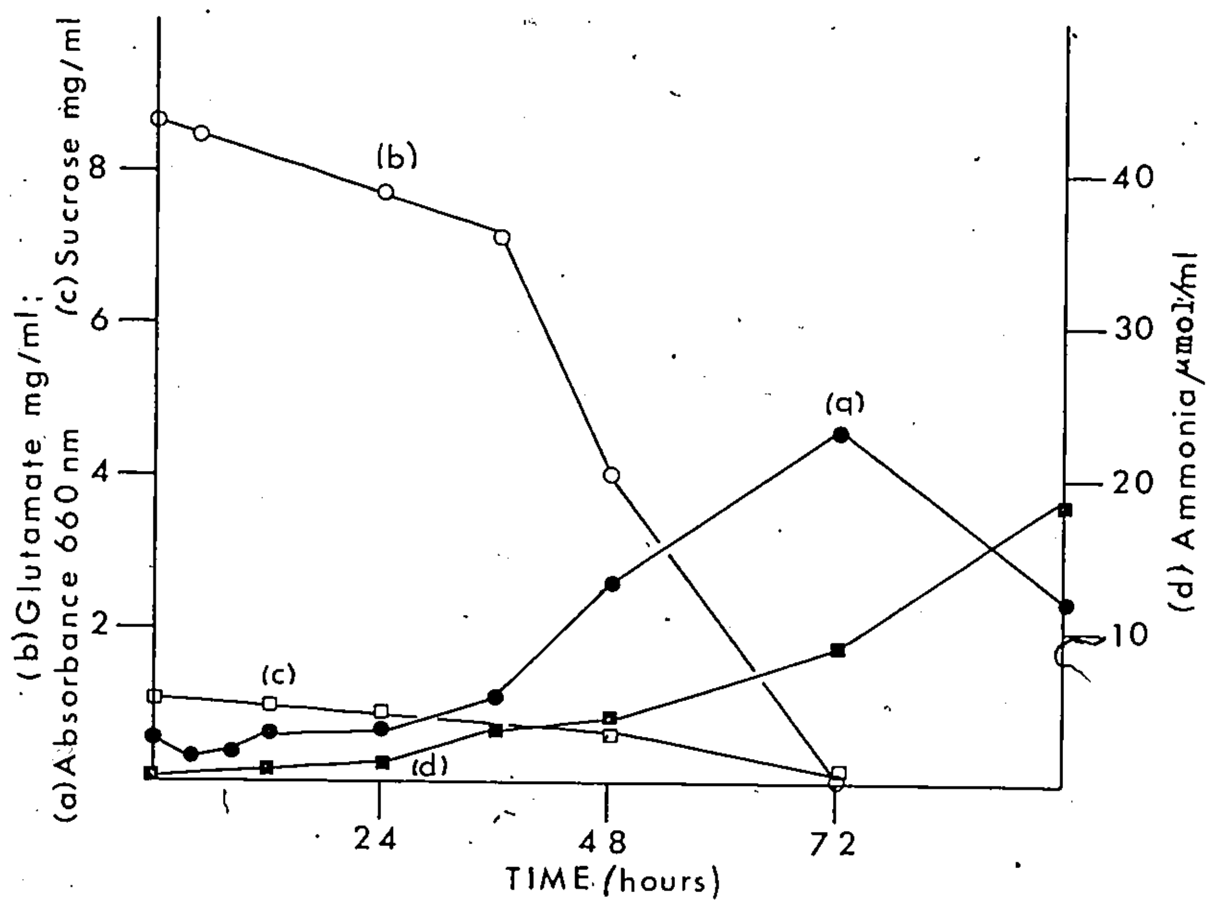


FIGURE 7

FIGURE 8

Growth parameters for 800 ml shake-flask cultures on 1% glutamate-0.1% sucrose medium: the symbols and units are as in Fig. 2.



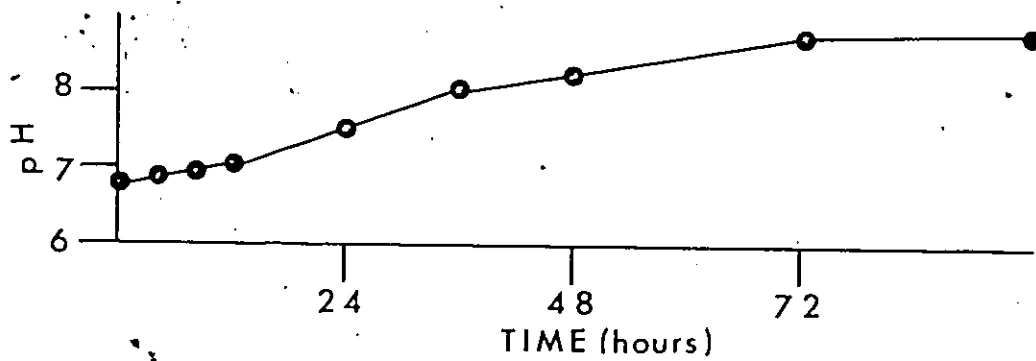


FIGURE 8

FIGURE 9

Enzyme activity of culture filtrates from 800 ml shake-flask cultures grown on 1% glutamate-0.1% sucrose medium: the symbols and units are as in Fig. 3.

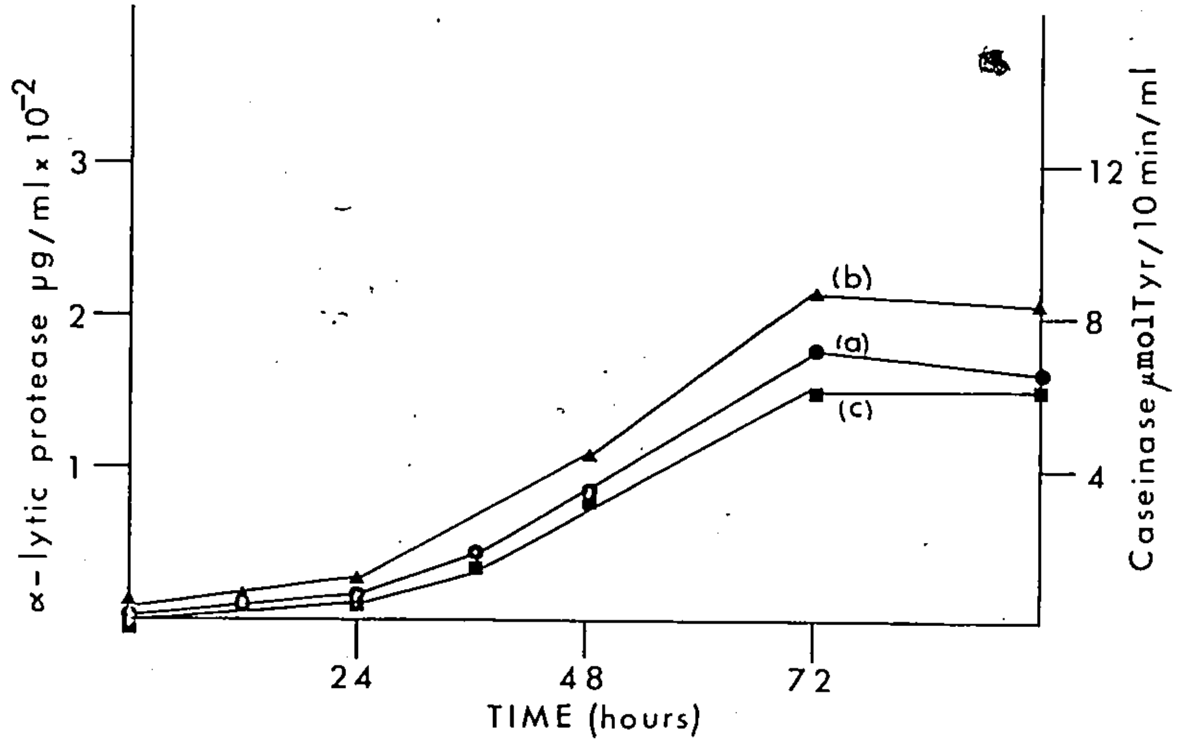
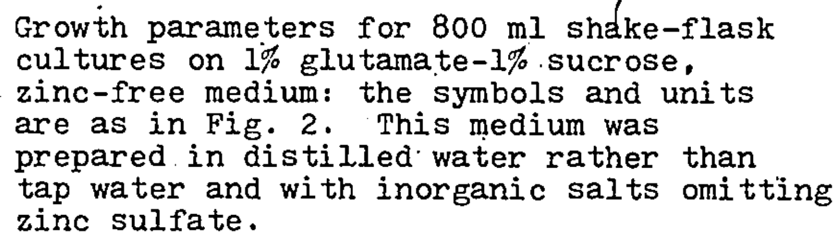


FIGURE 9

FIGURE 10



Growth parameters for 800 ml shake-flask cultures on 1% glutamate-1% sucrose, zinc-free medium: the symbols and units are as in Fig. 2. This medium was prepared in distilled water rather than tap water and with inorganic salts omitting zinc sulfate.

(c)

(a)

(b)

(d)

(b) Glutamate mg/ml; (c) Sucrose mg/ml

(a) Absorbance 660 nm

(d) Ammonia μmol/ml

8 6 4 2

40 30 20 10

24 48 72

TIME hours

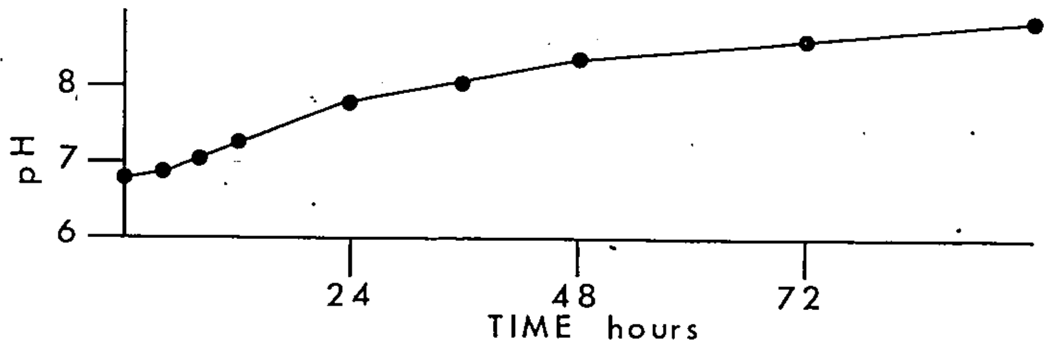


FIGURE 10

FIGURE 11

Enzyme activity of culture filtrates from 800 ml shake-flask cultures grown on 1% glutamate-1% sucrose, zinc-free medium: the symbols and units are as in Fig. 3. The activity of β-lytic protease, a zinc-containing, extracellular metallo-protease of *Myxobacter 495* should be depressed or absent in these culture filtrates.

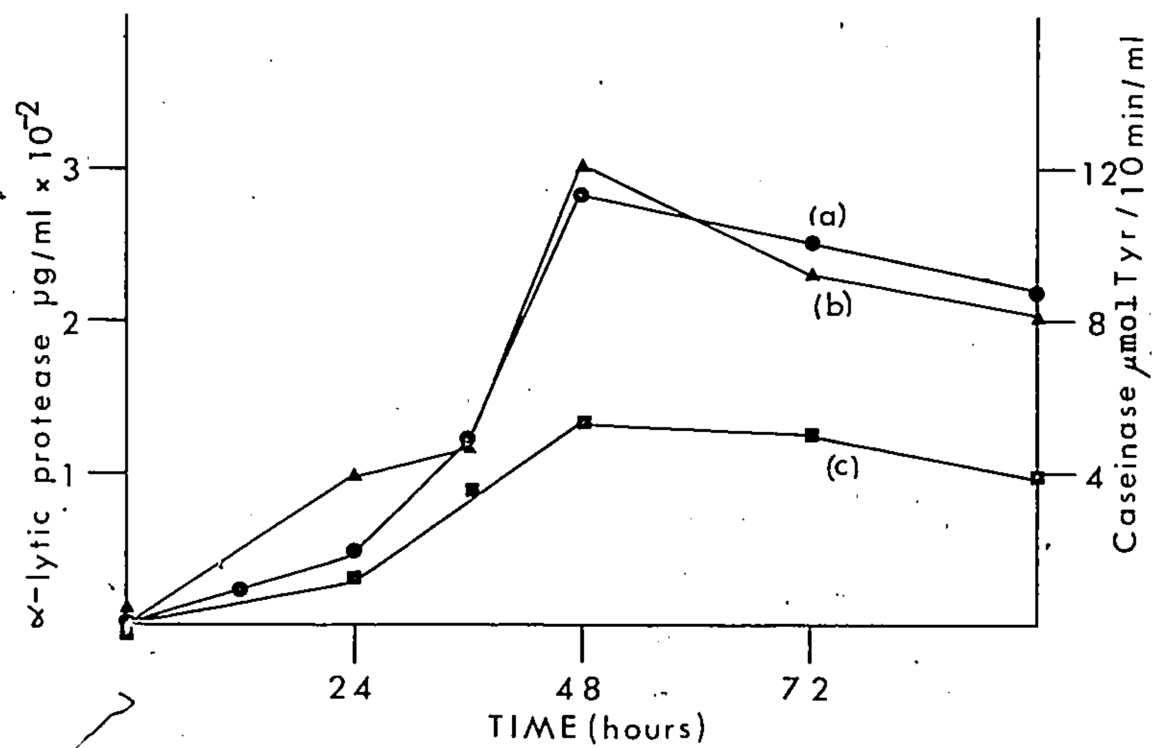


FIGURE 11

levels of protease activity towards casein before and after treatment with DFP; and changes in ammonia concentration of the media. Antibiotic activity as assayed by serial dilution against the test organism Sphaerotilus natans is recorded for the same cultures in Table 1.

Cultures grown on 1% casamino-1% sucrose medium (fig.2) demonstrated the phenomenon known as diauxie.[38] The growth curve, as observed by turbidity measurements at 660 nm, showed two periods of log-phase growth separated by a brief stationary phase. Free amino acids in solution were depleted during the first log-phase growth period. Sucrose was exhausted during the second log-phase growth period. It was observed in all media tested that Myxobacter 495 used amino acids as substrates in preference to sucrose.

Table 1 shows the levels of antibiotic activity of culture filtrates from shake-flask cultures. The test organism was Sphaerotilus natans. The units are dilution units per millilitre of culture filtrate. The zero-time samples were taken immediately after inoculation and all had some activity due to the presence of the antibiotic in the inoculum. For the culture shown in fig.2, maximum antibiotic activity was reached at 24 hours. The activity remained fairly constant from 24 to 96 hours. In fig. 3, enzyme activities for the same culture are illustrated. Peak enzyme activity was reached at 72 hours.

TABLE 1

COMPARISON OF ANTIBIOTIC PRODUCTION IN 800 ml SHAKE FLASK CULTURES OF MYXOBACTER 495

MEDIUM	TIME (HOURS)				
	0	24	48	72	96
1% CAS-1% SUC	21/31	125/155	80/90	125/155	125/155
1% CAS-0.1% SUC	21/31	155/205	205/305	205/305	205/305
1% GLU-1% SUC	16	41/61	155/205	155/205	105/125
1% GLU-0.1% SUC	16	16	21/31	16/21	61/80
1% GLU-1% SUC (No Zinc)	16	16	125/155	105/125	80/90

Figures are given in units of activity per ml of culture filtrate; the test organism was *Sphaerotilus natans*; zero-time samples were taken after inoculation and contained activity added with the inocula; the two numbers given for each sample are the lower and upper limits of activity respectively except for samples with very low activity for which only the upper limit is shown.

The pattern of amino acid consumption for a culture grown on 1% casamino-1% sucrose is shown in Table 2. Free amino acids in solution were determined by amino acid analyses and the results are given in micromoles/ml of culture filtrate. The total number of micromoles of each amino acid present at zero-time was arbitrarily assigned a value of 100%. The total number of micromoles of amino acids remaining at 12, 24 and 48 hours was expressed as a percentage of the zero-time total and plotted on graphs of growth parameters of 1% casamino cultures as % amino acid consumption.

Cultures grown on 1% casamino-0.1% sucrose media (fig. 4) grew well initially but began to autolyze at about 24 hours, coinciding with the depletion of free amino acids in the medium. The autolysis was evidenced by the rapid decline in the growth curve, as represented by turbidity measurement at 660 nm, and by the dark brown appearance of the culture in the shake flask. The ammonia levels of these cultures reached very high values during the period of autolysis. When samples of the cultures were examined by phase-contrast microscopy damaged cells and aggregations of cell debris could be seen. Production of extracellular lytic enzymes (fig. 5) was much lower than that for cultures grown on 1% casamino-1% sucrose medium. Levels of antibiotic activity obtained for this culture were somewhat higher than the other casamino culture (Table 1). However, it was

TABLE 2

AMINO ACID UPTAKE FROM MEDIUM:
800 ml SHAKE CULTURE, 1% CAS-1% SUC

AMINO ACID	TIME(HOURS)			
	0	12	24	48
ASPARTIC ACID	3.08	3.39	0.208	0
THREONINE	1.88	1.82	0	0
SERINE	3.26	2.97	0	0
GLUTAMIC ACID	8.69	8.75	0.956	0
PROLINE	5.81	5.08	0	0
GLYCINE	1.84	1.41	0	0
ALANINE	2.44	1.58	0	0
VALINE	2.13	2.48	0	0
METHIONINE	1.67	1.67	0.508	0.528
ISOLEUCINE	1.46	1.66	0	0
LEUCINE	3.69	2.68	0	0
TYROSINE	0.992	0.928	0	0
PHENYLALANINE	0.716	0.456	0	0
LYSINE	2.94	3.19	0	0
HISTIDINE	0.740	0.720	0	0
ARGININE	1.04	0.832	0	0
TOTAL (μmoles/ml)	42.38	39.62	1.67	0.528
AMINO ACIDS %	100	93.5	3.94	1.25

Amounts are given as μmoles/ml of culture filtrate; 42.38 μmoles/ml of the amino acids listed was present at zero-time; totals of amino acids present at 12, 24, and 48 hours are expressed as a percentage of the zero-time amount; zero in the above table indicates less than 0.01 μmole/ml. The pattern of amino acid consumption was quite similar in all cultures grown on 1% casamino media.

observed during exploratory studies of methods for the isolation of the inhibitor that by-products of autolysis led to difficulties during initial filtration or column treatments of culture filtrates. Filtrates from cultures which had undergone autolysis tended to be very viscous to the point of being a syrup if the autolysis were allowed to go to completion.

Cultures grown on 1% glutamate-1% sucrose (fig. 6) had a longer lag-phase than the casamino cultures. Peak levels of antibiotic activity were observed at 72 hours (Table 1) and of lytic enzyme activity at 72 hours (fig.7). In glutamate media with 0.1% sucrose (fig. 8) poor growth and low levels of antibiotic activity were observed. However, production of lytic enzymes (fig. 9) was comparable to that on glutamate medium with 1% sucrose.

Cultures grown on 1% glutamate-1% sucrose medium which was zinc-free (fig. 10) showed excellent growth as observed by turbidity measurements. It was anticipated that the activity of metallo-proteases which required zinc would be reduced in a culture grown in zinc-free medium. The zinc-containing enzyme, β-lytic protease, is a major component of the extracellular proteolytic activity found in culture filtrates of <u>Myxobacter</u> <u>495</u>.[5] DFP-treated lytic activity towards casein (fig. 11) was reduced compared to the activity measured for the glutamate medium with zinc (fig. 7).

The medium which was designated "zinc-free" may have contained some zinc introduced into the medium in the distilled water or one of the solid components, especially casamino acids, and this would account for some of the proteolytic activity observed in the DFP-treated samples from the culture of fig. 11.

Antibiotic activity (Table 1) for the zinc-free medium was comparable to peak activity obtained for the other cultures grown on media containing 1% sucrose. There appeared to be a significant decline in activity as incubation was continued past 48 hours.

(b) Growth in Small Fermenter

Two types of medium from the shake-flask study, 1% casamino-1% sucrose and 1% glutamate-1% sucrose, were used to study the effect of several different aeration conditions on the growth of Myxobacter 495 and production of antibiotic. All cultures were grown on 8 litres of medium at 26°C. Aeration rates were from 4 l/min or ½ vvm (volumes of air per volume of medium per minute) to 16 l/min or 2 vvm, for glutamate cultures, and ½ vvm to 1½ vvm for casamino cultures. As discussed previously, the stirring blades were removed from the shaft and agitation was accomplished by the action of bubbles of sparged air rising from the bottom of the fermenter vessel.

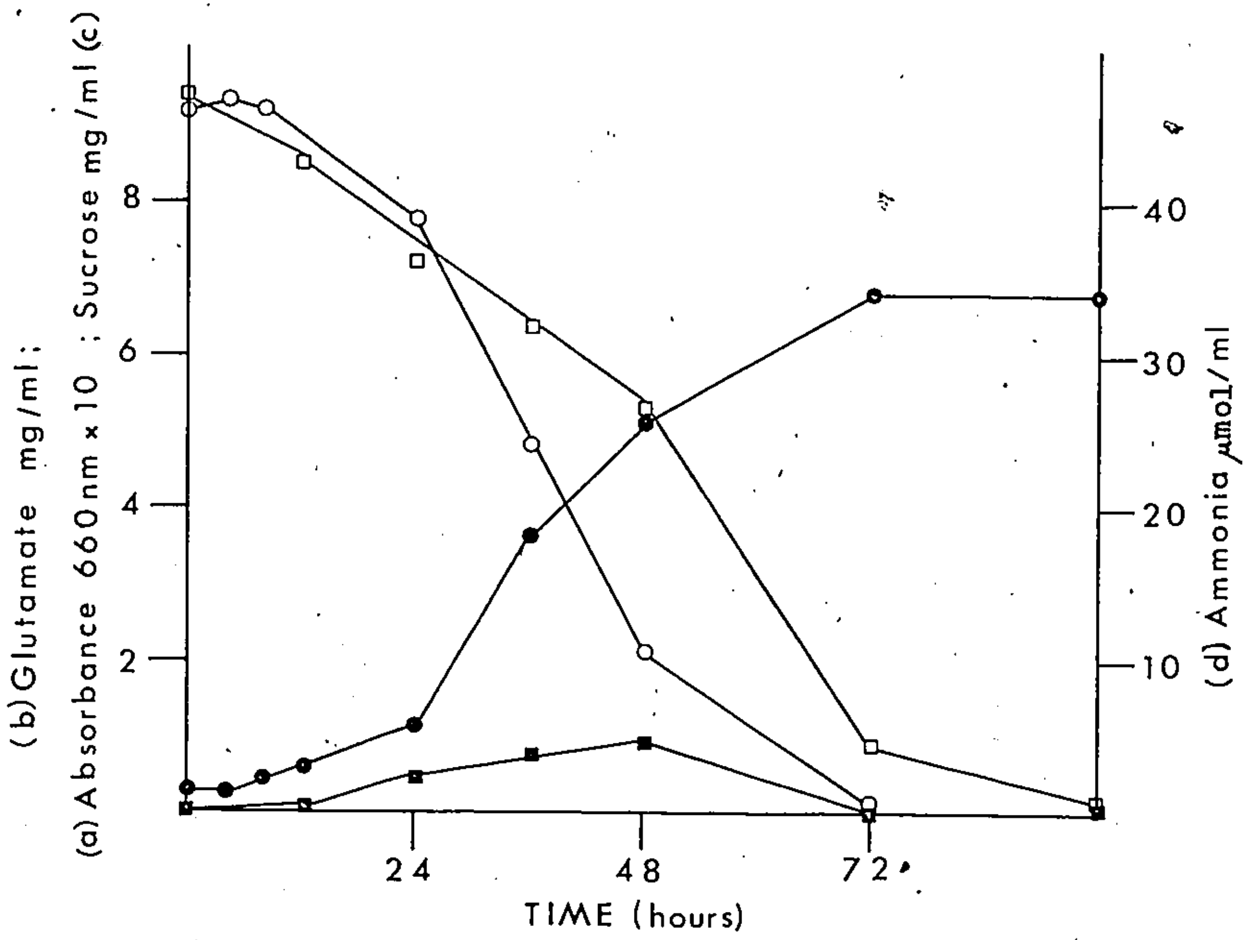
Figure 12 illustrates the growth parameters measured for a culture grown on 8 litres of 1% glutamate-1% sucrose medium aerated at 4 l/min or ½ vvm. Antibiotic activity for the glutamate fermenter cultures is shown in Table 3. This culture reached its maximum turbidity at 72 hours. Peak antibiotic activity was observed at 72 hours.

Figures 13, 14, and 15 are cultures grown on 8 litres of glutamate medium aerated at 1 vvm, 1½ vvm, and 2 vvm respectively. In these cultures the growth curve, turbidity at 660 nm, peaked at 72 hours. The maximum turbidity reading increased with increasing aeration rate. Antibiotic activity (Table 3) tended to peak at 72 hours and was highest at ½ vvm.

Figures 16 and 17 show the results of 8 litre fermentations on 1% casamino-1% sucrose medium aerated at ½ vvm and 1 vvm respectively. As for shake-flask cultures, the free amino acids were consumed in preference to sucrose. The peak turbidity reading for the culture grown at ½ vvm (fig. 16) was reached at 96 hours. The culture grown at 1 vvm reached its peak turbidity at 48 hours (fig. 17). A rapid decline in turbidity and an increase in ammonia concentration indicated that the culture aerated at 1 vvm had begun autolysis after 48 hours. When higher aeration rates were attempted using the casamino medium, many problems in foam control arose. A culture aerated at 1½ vvm (not illustrated) had poor growth. Several cultures aerated at 2 vvm did not grow at all.

FIGURE 12

Growth parameters for 8 litre 1% glutamate-1% sucrose culture aerated at 4 l/min (½vvm) in 14-litre fermenter: (a) —●—●— turbidity measured at 660 nm; (b) —○—○— glutamate concentration, mg/ml; (c) —□—□— sucrose concentration mg/ml; (d) —■—■— ammonia concentration µmol/ml. The lower graph illustrates pH changes in the same culture.



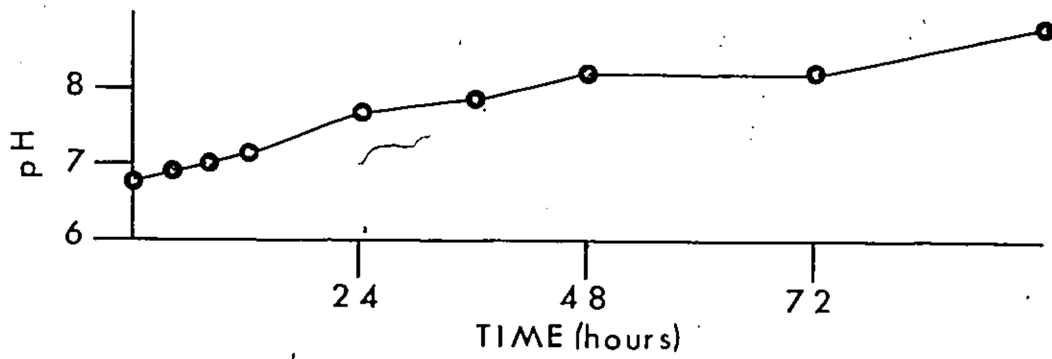
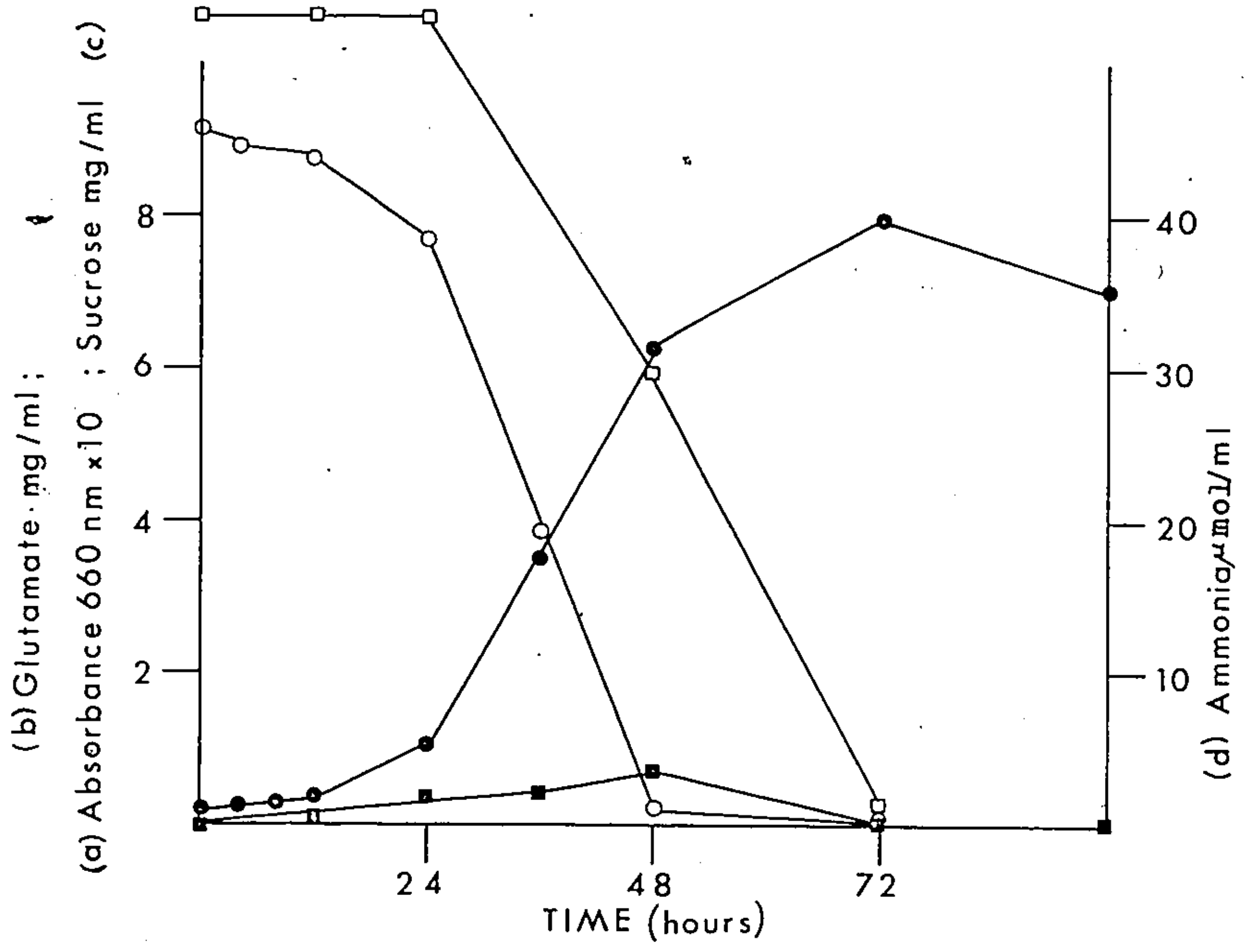


FIGURE 12

FIGURE 13

Growth parameters for 8 litre 1% glutamate-1% sucrose culture aerated at 8 l/min (lvvm) in 14-litre fermenter: the symbols and units are as in fig. 12.



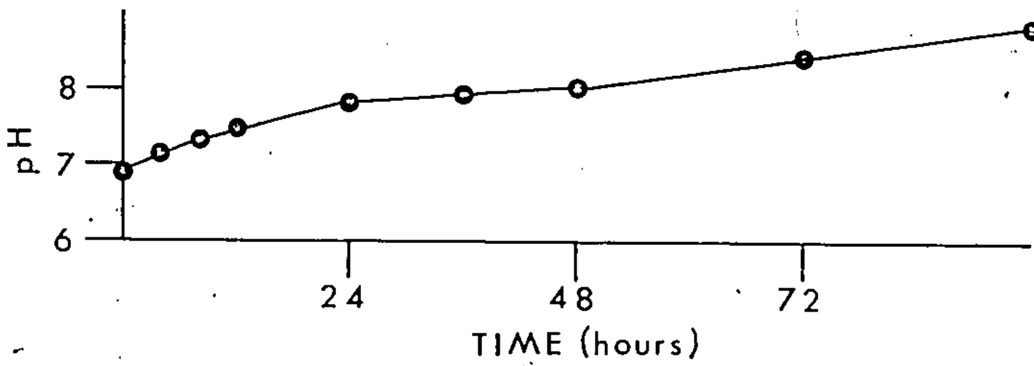


FIGURE 13

FIGURE 14

Growth parameters for 8 litre 1% glutamate-1% sucrose culture aerated at 12 l/min ($1\frac{1}{2}$vvm) in 14-litre fermenter: the symbols and units are as in fig. 12.

(b) Glutamate mg / ml ;

(a) Absorbance 660 nm × 10 ; Sucrose mg / ml (c)

8
6
4
2

40
30
20
10

(d) Ammonia μ mol / ml

24
48
72
TIME (hours)

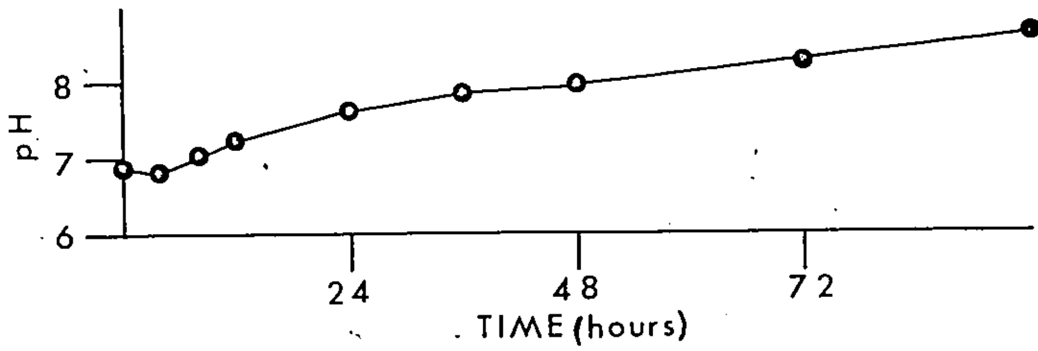


FIGURE 14

FIGURE 15

Growth parameters for 8 litre 1% glutamate-1% sucrose culture aerated at 16 l/min (2 vvm) in 14-litre fermenter: the symbols and units are as in fig. 12.

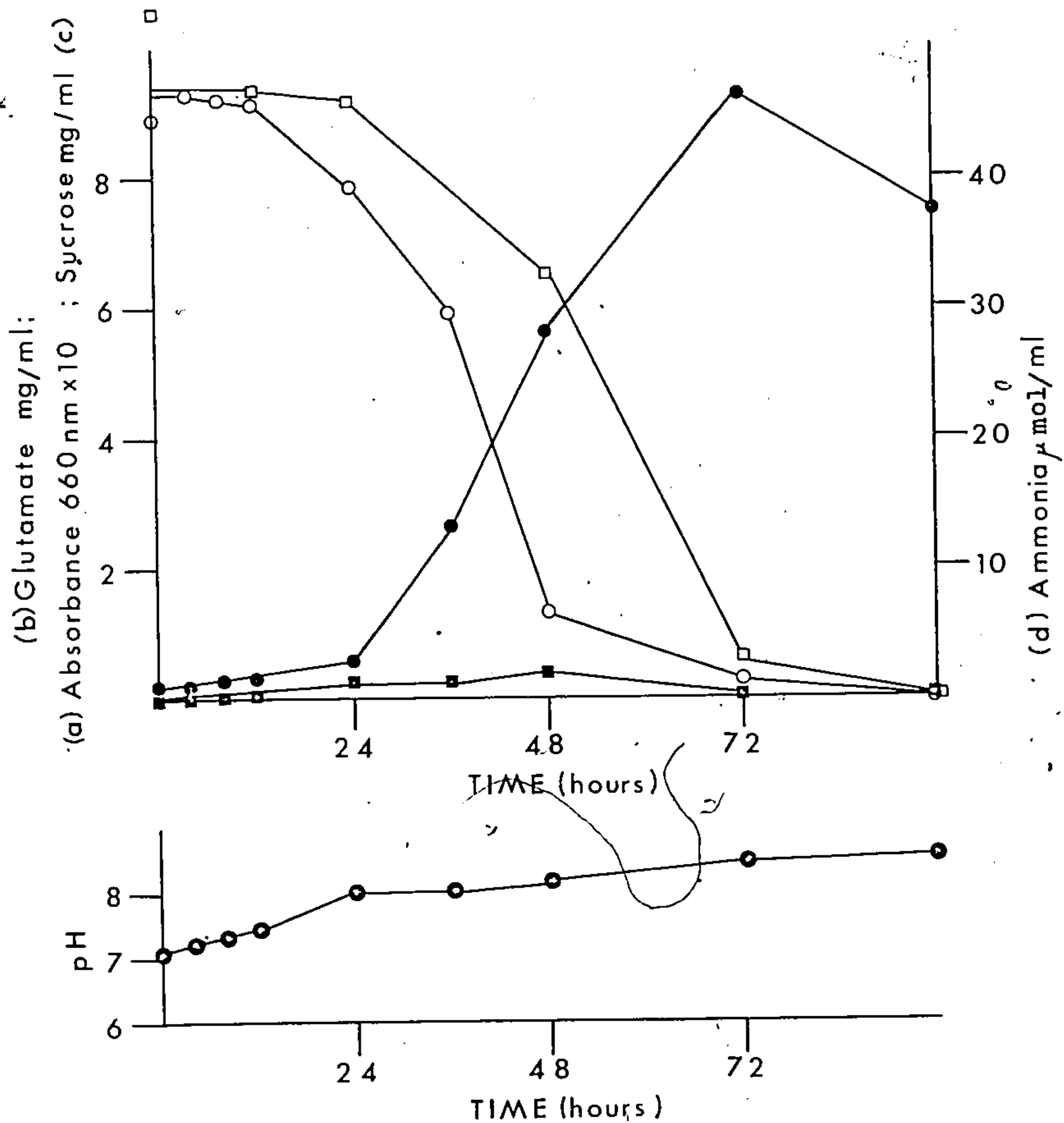


FIGURE 15

FIGURE 16

Growth parameters for 8 litre 1% casamino-1% sucrose culture aerated at 4 l/min ($\frac{1}{2}$ vvm) in a 14 litre fermenter: (a) —●—●— turbidity measured at 660 nm; (b) —○—○— % amino acids in medium, zero-time set to 100%; (c) —□—□— sucrose concentration mg/ml; (d) —■—■— ammonia concentration μmol/ml. The lower graph illustrates pH changes in the same culture.

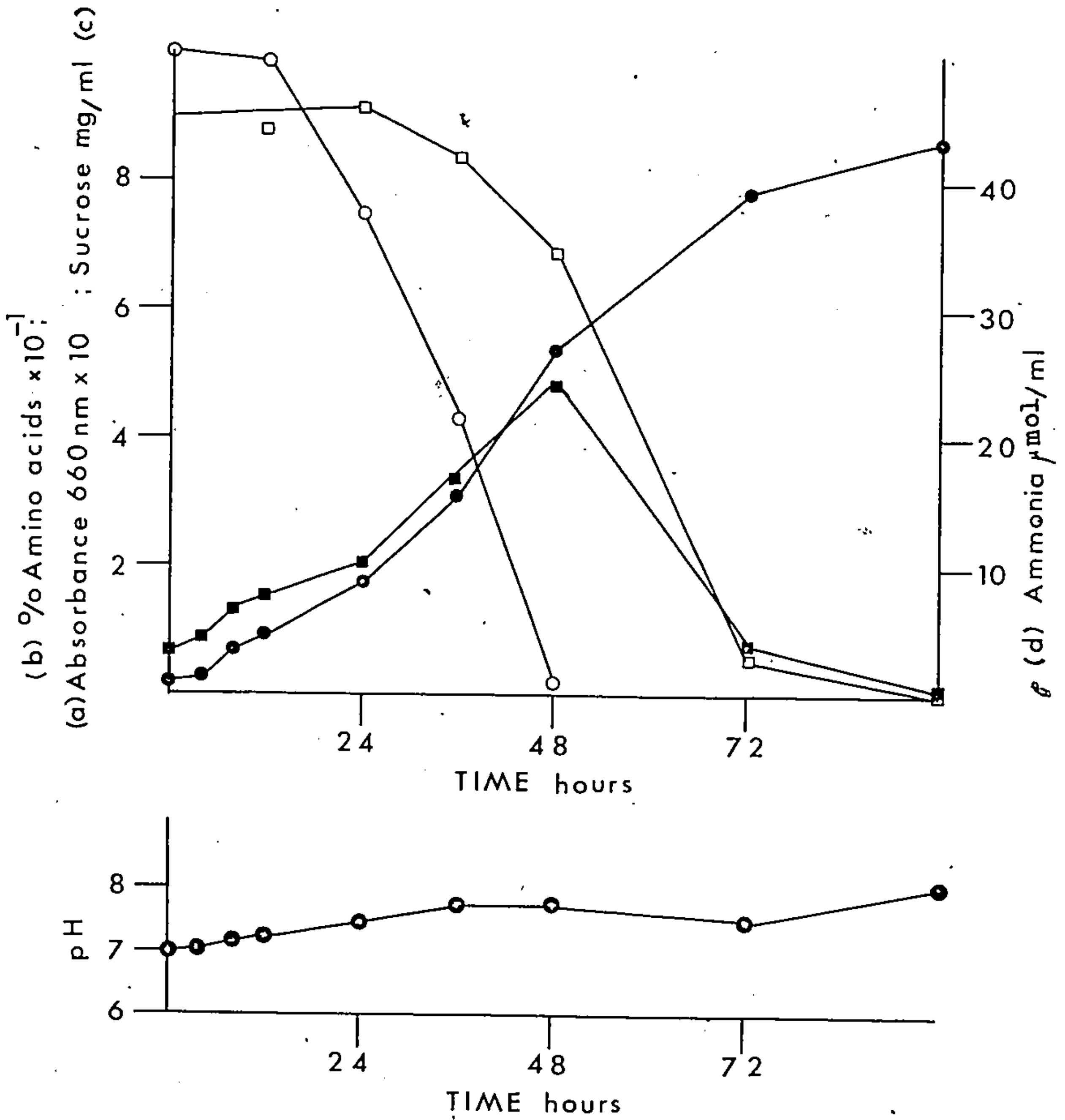


FIGURE 16

FIGURE 17

Growth parameters for 8 litre 1% casamino-1% sucrose culture aerated at 8 l/min (vvm) in a 14-litre fermenter: the symbols and units are as in fig. 16.

(b) % Amino acids x 10^{-1}

(a) Absorbance 660 nm x10 ; Sucrose mg/ml (c)

8
6
4
2

(d) Ammonia μmol/ml

40
30
20
10

24
48
72
TIME (hours)

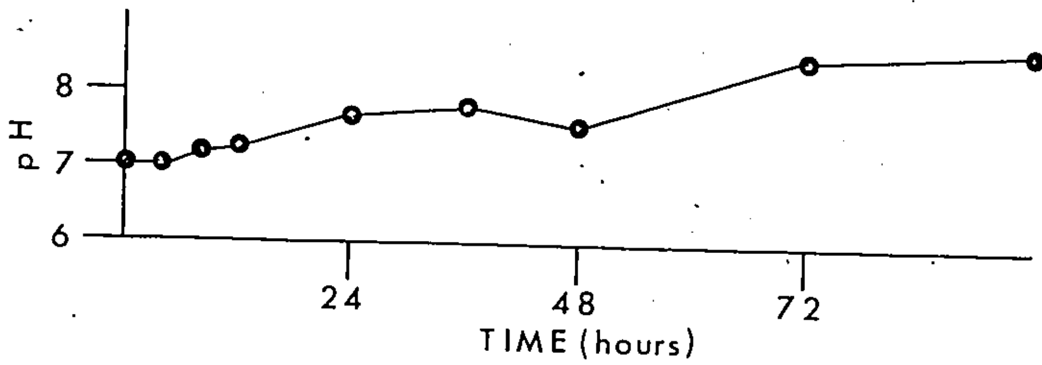


FIGURE 17

TABLE 3

COMPARISON OF ANTIBIOTIC PRODUCTION AT FOUR AERATION RATES: 8 LITRE 1% GLU-1% SUC CULTURES OF MYXOBACTER 495

AERATION RATE	TIME (HOURS)				
	0	24	48	72	96
4 l/min; ½ vvm	21/31	43/80	294/378	525/651	441/525
8 l/min; 1 vvm	16	70/80	341/451	378/441	341/451
12 l/min; 1½ vvm	16/21	80/90	231/275	257/341	198/231
16 l/min; 2 vvm	16	231/275	451/671	451/671	451/671

Figures are given in units of antibiotic activity per ml of culture filtrate; details of the assay are as for Table 1.

Data for the antibiotic activity of culture filtrates from 8 litre fermentations on 1% casamino-1% sucrose medium at ½ vvm and 1 vvm are shown in Table 4. Included in this table are the results for a culture grown at 1½ vvm. Peak activity was reached between 72 and 96 hours.

The most active filtrates of the small fermenter study were three times as active as the best from the shake flask study. Excellent antibiotic activity could be obtained by aerating the medium at ½ or 1 vvm with either the glutamate or the casamino medium. The optimal time for harvesting the culture appeared to be about 72 hours. Foam control was easily accomplished with the mechanical foam breaker when agitation was by sparged air alone.

(c) Growth in Large Fermenter

The 1% glutamate-1% sucrose medium was chosen for a large scale batch fermentation in a 150 litre fermenter. The culture was grown in 50 litres of medium following inoculation with 5 litres of a mid-log phase culture grown in the small fermenter. An approximation to the aeration of 8 litres of medium at ½ vvm in the small fermenter to a similar condition for 50 litres of medium in the large fermenter was made using the following equation:[39]

$$\frac{\frac{Q}{V}\text{ Large Ferm.}}{\frac{Q}{V}\text{ Small Ferm.}} = \left[\frac{H_{\text{Large Ferm}}}{H_{\text{Small Ferm}}}\right]^{-2/3}$$

TABLE 4

COMPARISON OF ANTIBIOTIC PRODUCTION AT THREE AERATION RATES: 8 LITRE 1% CAS-1% SUC CULTURES OF MYXOBACTER 495

AERATION RATE	TIME(HOURS)				
	0	24	48	72	96
4 l/min; ½ vvm	21/31	80/90	205/305	205/305	275/341
8 l/min; 1 vvm	16	155/205	341/451	671/851	671/851
12 l/min; 1½ vvm	16	16/21	125/155	125/155	231/275

Figures are given in units of antibiotic activity per ml of culture filtrate; details of the assay are as for Table 1.

In the above equation Q is the aeration rate in litre/min, H is the depth of the medium in the fermenter vessel, and V is the volume of medium in litres. For a Q/V ratio of ½ vvm in the small fermenter the Q/V ratio in the large fermenter was 0.33 vvm. Therefore an aeration rate of 17 l/min in the large fermenter approximated 4 l/min in the small fermenter.

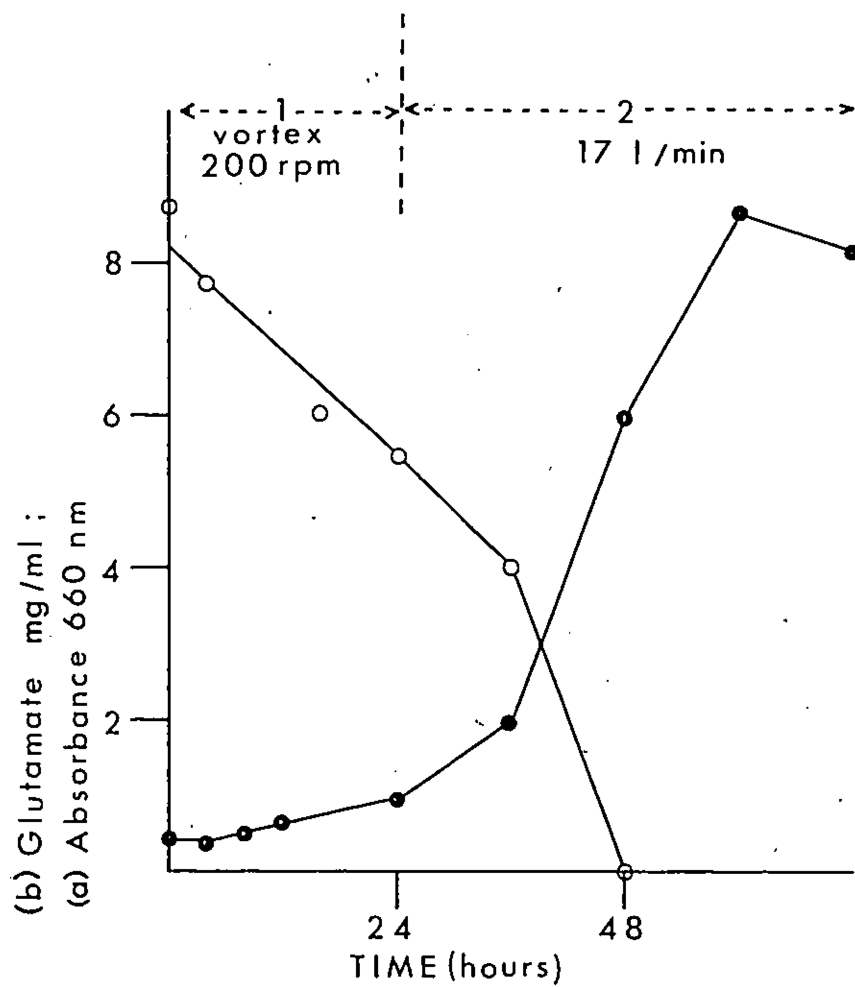
Turbidity increase, glutamate consumption, and pH changes are illustrated in fig. 18. The culture was harvested at 72 hours. Antibiotic activity was the best of any culture in the growth study: 883/1300 dilution units/ml at 48 hours, 1300/1716 dilution units/ml at 72 hours. After passage through a CEPA continuous centrifuge, 36 litres of culture supernatant assayed at 1716/2350 dilution units/ml was obtained for further processing.

E. DISCUSSION:

Cultures of Myxobacter 495, grown in shake-flasks on casamino-sucrose media, exhibited the phenomenon known as diauxie. This type of microbial growth was defined by Monod as "a double growth cycle consisting of two exponential phases separated by a phase during which the growth rate passes through a minimum, even becoming negative in some cases."(40) Monod accounted for this type of growth as being the result of either "the exhaustion of a compound partially covering an essential nutritional

FIGURE 18

Growth parameters for a culture of Myxobacter 495 grown on 50 litres of 1% glutamate-1% sucrose in a 150-litre fermenter: (a) —●—●— turbidity measured at 660 nm; (b) glutamate —○—○— concentration mg/ml. The lower graph illustrates pH changes in the same culture. The culture was stirred at 200 rpm with the sparger turned off and air passing over-top of the medium (vortex aeration) for the first 24 hours following inoculation. From 24 to 72 hours the culture was aerated by sparged air at 17 l/min.



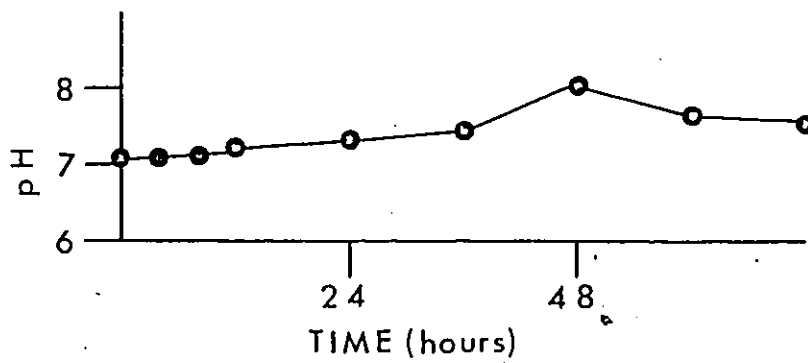


FIGURE 18

requirement, or from the transitory accumulation of a metabolite, which will eventually serve as a secondary nutritional source."(41) The example of diauxie seen in fig. 2, a shake-culture grown on 1% casamino-1% sucrose, appeared to be of the former type. The first phase of growth exhausted the free amino acids of the medium, some of which may have been essential to the cells' metabolism. During the second phase of growth the cells used sucrose as a substrate. A diauxic effect was not apparent in the growth curve of cultures grown on 1% glutamate-1% sucrose medium.

The results from the shake flask study indicated that good growth could be obtained on glutamate and casamino media with either 1% or 0.1% sucrose. However, cultures grown on media with the lower sucrose concentration tended to autolyze rapidly. The filtrate from autolyzed cultures was found to be very difficult to work up for isolation of the desired product. Since autolysis was undesirable, 1% glutamate-1% sucrose and 1% casamino-1% sucrose media were used in order to study the effect of various aeration conditions on the growth of the producer organism in a small fermenter.

Foam control was found to be an important factor in the fermenter studies. Too much foaming, just after inoculation, led to a longer lag-phase and poor growth.

Cultures grown on glutamate medium foamed less than cultures grown on casamino medium, at a given aeration rate. The lowest aeration rates tried, $\frac{1}{2}$ vvm and 1 vvm, were sufficient for good growth and production of the antibiotic on these media.

Monosodium-L-glutamate is an inexpensive and easily obtained substrate compared to casamino acids. For a series of large scale fermentations, including the inevitable aborted runs, the glutamate medium was much more economical than the casamino medium. The growth study culminated in a successful production run on 50 litres of glutamate medium. About 36 litres of supernatant from the culture was harvested at 72 hours and was assayed at 1300/1716 dilution units/ml or 5×10^7 dilution units in total. This represented approximately 5 grams of the antibiotic substance or 140 mg/litre of culture. In comparison, the same culture filtrate had 394 mg/litre of α-lytic protease. Thus, determination of the growth parameters for a variety of different media, and for several different aeration conditions, made possible a large scale fermentation which resulted in an excellent yield of antibiotic.

CHAPTER III

ISOLATION PROCEDURE

The isolation procedure used in this investigation was devised by Dr. D.R. Whitaker. The original procedure is outlined in point form in Table 5. The rationale is as follows.

The output from the 50 litre culture, grown as described in Chapter II, was freed of cells with a continuous flow centrifuge (Step 1). The effluent was then passed through a mixed bed ion-exchange resin, which, in effect, replaced the effluent's complex mixture of cations and anions with NH_4^+ and acetate.

At this stage, the antibiotic activity, as assayed against Sphaerotilus natans, was associated with a complex having a fairly low sedimentation coefficient. The complex could be spun down in a preparative ultracentrifuge, but the separation required high-speed runs lasting several hours and was not practicable for large scale isolation. The complex was, therefore, concentrated by an ultrafiltration procedure (Step 3), using an Amicon assembly with a capacity of 20 litres. The ultrafilters, Amicon XM-100A, were rated as permeable to solutes with molecular weights up to 100,000. Concentration of the solution was accompanied by an increase in its viscosity, with a consequent falling-off of the rate of ultrafiltration. The polysaccharides responsible for the high viscosity were removed by

precipitation with 2 volumes of acetone after the addition of potassium phosphate to the concentrate. The precipitate was removed by a glass-wool filter. The filtrate was diluted with water, in order to reduce the acetone concentration to a level not injurious to the ultrafilters, reconcentrated, treated again with acetone to remove any residual polysaccharide, and again reconcentrated. By the end of this extended treatment, the complex containing the antibiotic activity had undergone extensive aggregation, and could be collected by centrifugation at low speeds.

The product from Step 3 was readily soluble in ethanol but not in water, readily soluble in concentrated (4.2 M) guanidine hydrochloride, but not in dilute (0.5 M) guanidine hydrochloride, and, on extraction with chloroform from a 4.2 M solution of guanidine hydrochloride, yielded large amounts of antibiotically-inactive, lipid-like material in the chloroform extract. These observations are compatible with the dissociation of a peptide-lipid complex by concentrated guanidine hydrochloride and are exploited in Step 4.

In the final step, the Myxosidin was freed of salt. The most obvious methods--gel-permeation chromatography on Sephadex or cross-linked polyacrylamide gels or ion exchange chromatography with volatile acids, bases, or salt solutions as eluents--proved to be inapplicable as Myxosidin is

strongly adsorbed by Sephadex, cellulose, and cross-linked methacrylate and polystyrene ion-exchange resins. The procedure adopted exploited the solubility of Myxosidin in ethanol-chloroform (Step 5). The elution with isopropanol in a silica gel column removed a coloured impurity.

TABLE 5

MYXOSIDIN: ISOLATION PROCEDURE

Preliminary: Input to fermentor - 50 l medium + 5 l inoculum

Harvested after 3 days growth

Output - 35 l fermentor broth

<u>Step 1</u> Continuous centrifugation to remove cells - 3 passages through CEPA 41G continuous-flow centrifuge.

<u>Step 2</u> Ion-exchange - 6 l bed of Amberlite IR120 (NH_4^+) - Amberlite IR45 (Acetate$^-$).

Effluent - 36.5 l. Recovery - quantitative.

TABLE 5 (continued)

MYXOSIDIN: ISOLATION PROCEDURE

Step 3 Concentrate active material and remove solutes with molecular weights below ca 10^5 by ultrafiltration; remove solutes precipitated by acetone; centrifuge active material from dispersion in water.

(a) Concentrate on Amicon XM-100A ultrafilters 36.5 l. to 2 l; dilute with water to 12 l reconcentrate to 4 l. Add K-phosphate (pH 7) to a concentration of 0.02 M.

(b) Add 2 volumes of acetone. Filter (12 l. of filtrate).

(c) Repeat (a) after addition of 3 volumes of water (48 l. to 0.5 to 1.2) to the filtrate.

(d) Repeat (b) (3.6 l. of filtrate) and (d) (14 l. to 0.5).

(e) Centrifuge final concentrate; wash sediment with 3 x 250ml water washes on filter unit; wash sediment twice with 60 ml 0.02 M K-phosphate (pH 7).

Output: 27 g of washed sediment

Recovery: ca 70%.

TABLE 5 (continued)

MYXOSIDIN: ISOLATION PROCEDURE

Step 4 Remove: Products insoluble in 6 M Guanidine hydrochloride (GHC), products soluble in 0.5 M GHC, products insoluble in 4.2 M GHC, products extracted by $CHCl_3$ from 4.2 M GHC.

(a) Centrifuge 4% solution of sediment in 6 M GHC. Discard sediment.

(b) Dilute the supernatant to 0.5 M GHC. Centrifuge and discard the supernatant.

(c) Redissolve sediment in 6 M GHC. Dilute to 4.2 M GHC, centrifuge and discard sediment.

(d) Extract supernatant twice with one third its volume of $CHCl_3$; wash first chloroform extract twice with half its volume of 4.2 M GHC. Combine aqueous phases.

Output: active product in 4.2 M GHC.

Recovery: 95%.

TABLE 5 (continued)

MYXOSIDIN: ISOLATION PROCEDURE

Step 5 Desalt with organic solvents and elute from silica gel with isopropanol.

(a) Saturate the 4.2 M GHC solution with $(NH_4)_2SO_4$ (50 g/100 ml) and extract with EtOH-$CHCl_3$ (1:1; 200 ml/100 ml GHC solution). Upper phase evaporated to dryness; residue extracted with EtOH; EtOH extract concentrated and mixed with 2 volumes of $CHCl_3$. Precipitated salts removed; solution evaporated to dryness.

(b) Isopropanol extract of residue eluted from column of silica gel (Sigma type 1). Myxosidin, monitored by its absorbance at 240 nm, is eluted immediately after the effluent from the void-volume of the column. Eluate evaporated to dryness.

Recovery: 95%

Yield: as dried product - 409 mg/ 5 g washed sediment; as leucine residues: 96 mg/ 5 g washed sediment.

CHAPTER IV

STRUCTURAL CHARACTERIZATION

A. OBJECTIVES

The purified antibiotic substance isolated from culture filtrates of Myxobacter 495 was found to be ninhydrin-positive. Upon acid hydrolysis 11 different amino acids could be distinguished in the residue. A test for carbohydrates (hexoses and pentoses) carried out on the hydrolysate was negative. The ultraviolet spectrum of the product in methanol showed no chromophores apart from a mono-substituted benzene pattern. Natural abundance ^{13}C nmr spectroscopy of Myxosidin showed peaks corresponding to a benzene ring bearing five hydrogens. It was concluded that the inhibitory agent was a peptide or a closely related set of peptides.

Peptide antibiotics of bacterial origin have the following features in common: (1) they often occur as a set of peptides differing in the nature of the amino acid residue at one or more positions in an otherwise identical sequence; (2) they are usually cyclic; (3) they often contain unusual amino acids, for example amino acids with α-carbons in the D-configuration, amino acids derived from intermediary metabolism, or amino acids with modified side-chains; (4) they are resistant to degradation by the proteases and peptidases normally employed in the sequencing of proteins; (5) N- and C-terminals are often blocked by cyclization or as N-terminal

amide or C-terminal ester groups; (6) the upper limit of molecular weight appears to be around 3000 daltons; (7) a great variety of compounds such as amines, sugars, fatty acids, hydroxyacids, and heterocyclic carboxylic acids may be attached covalently to the peptide. Structural features peculiar to peptides of bacterial origin require a different approach to sequencing than that used for the sequencing of peptides obtained from proteins.

The objectives of the study of Myxosidin described in this chapter were (1) to determine the amino acid composition including identification of any 'unusual' amino acid residues and assignment of D- and L- configurations; (2) to estimate the molecular weight of the peptide; (3) to determine the sequence of amino acid residues; (4) to propose a complete model of the macrocyclic structure of the peptide.

B. MATERIALS

(a) Chemicals

All common reagents and solvents were of reagent grade. Some special reagents were used. For amino acid analyses these were: 1, 2, 3-triketohydrindene monohydrate (ninhydrin, Pierce), 0.2 N pH 3.25 sodium citrate buffer, 0.2 N pH 4.25 sodium citrate buffer, 0.35 N pH 5.26 sodium citrate buffer (all from Beckman), polyoxyeth ylene lauryl ether (Brij 35, Pierce), titanous ch loride solution (Pierce), and methyl cellosolve (Pierce). For Edman sequencing reactions, the following

compounds were used: phenylisothiocyanate (Pierce), trifluoroacetic acid (Pierce) and 5-dimethylaminonaphthalene-1-sulfonyl chloride (dansyl chloride, Pierce). For enzymic determinations of D- and L-configurations of α-amino acids, the enzymes horseradish peroxidase (Sigma), hog kidney D- amino acid oxidase (Cal. Biochem.), and the co-factor β-nicotinamide adenine dinucleotide (Sigma), were used.

(b) Apparatus

Absorbances were measured with a Beckman DB-G grating spectrophotometer. Ultra-violet spectra were recorded with a Unicam SP 1800 scanning spectrophotometer. Infrared spectra were recorded with a Pye Unicam Scanning infrared spectrophotometer. Amino acid analyses were done with a Beckman model 121 amino acid analyzer. Mass spectra were determined with a DuPont 90 Mass Spectrometer equipped with a variable-temperature, direct insertion probe. A water-cooled plate system (Locarte) was used for high voltage electrophoresis.

C. METHODS

(a) Hydrolyses

A portion of a solution of the peptide to be hydrolyzed, containing a known amount of phenylalanine as an internal standard, was placed in a glass hydrolysis tube and evaporated to dryness in a heated vacuum desiccator. Two hundred to 500 μl of 6 N HCl (Aristar) was added to the dried residue. The tubes were evacuated with a mechanical vacuum pump and the contents were frozen in a dry ice-acetone bath.

The evacuation was continued with thawing until no more dissolved gas was evolved. The tubes were then sealed *in vacuo* and were left in an oven at 110°C overnight. At the end of the hydrolysis period, the tubes were cooled, opened, and the hydrolysate was evaporated to dryness in a heated vacuum desiccator. Distilled water was added to the dried hydrolysate and the evaporation was repeated as often as necessary in order to remove the last traces of HCl. For amino acid analyses, the residue was redissolved in distilled water.

(b) Amino Acid Analysis

Samples for amino acid analyses were injected into a Beckman 121 analyzer and were eluted with 228 ml 0.2 N sodium citrate pH 3.20 followed by 87.5 ml 0.2 N sodium citrate pH 4.05 buffer. Hydrolysates of Myxosidin contained an amino acid which was not resolved from glycine in the above system. Addition of benzyl alcohol (0.5%) to the buffers shifted the position of the unknown amino acid so that it was completely resolved from glycine. The basic amino acids were determined on a short column using 0.32 N sodium citrate pH 5.25 buffer. Norleucine was used as an internal standard.

(c) Column Chromatography for Estimation of Molecular Weight

Sephadex G50 (ultrafine) was washed three times with distilled water, degassed, equilibrated with column buffer, and poured into a column with a glass bead support. The dimensions of the settled resin bed were 28.1 cm x 1.0 cm. The column was eluted with 4.5 M guanidine-HCl-0.02 M phosphate, pH 5.44.

The column output was continuously monitored by a 280 nm detector and flow cell apparatus (LKB).

The standard peptides used to calibrate the column are listed in Table 10. The peptides from β-lytic protease were obtained by cyanogen bromide degradation.[42]

(d) High Voltage Electrophoresis

The mobilities of various samples of peptides and amino acids were determined on a Locarte water-cooled plate, high voltage electrophoresis (HVE) apparatus. Buffers used were: pyridine-acetic acid-water (80:2.4:720) for electrophoresis at pH 6.5; and 85% formic acid-acetic acid-water (16:64:720) for electrophoresis at pH 2.1. Conditions for HVE at pH 6.5 were 50 volts/cm for 90 minutes. Conditions for HVE at pH 2.1 were 50 volts/cm for 2½ hours. Serine, aspartic acid, leucine, glycine, and arginine were used as standards. Samples were spotted on sheets of Whatman 3MM paper. At the completion of a run the sheet was air dried, and in the case of preparative runs, sidestrips were removed for staining in a cadmium-ninhydrin bath. The composition of the staining solution was as follows: solution A, 5 g cadmium acetate in 500 ml water and 250 ml glacial acetic acid; solution B, 1% ninhydrin (Pierce) in acetone; 15 ml of A was added to 100 ml of B just prior to use. After staining, sheets or sidestrips were allowed to air-dry and were stored in plastic bags in the dark.

(e) Edman Degradations

(i) Subtractive Edman

The method used for subtractive Edman degradations was essentially that of Konigsberg.[43] A sample of the peptide to be sequenced (about 500 nanomoles) was dried briefly in vacuo. Two hundred microlitres of distilled water and 200 μl of 5% phenylisothiocyanate (PITC) in pyridine were added to the sample. The reaction mixture was incubated at 45°C for a minimum of 1½ hours, removed and dried under a stream of nitrogen. The residue was dried briefly in vacuo and 400 μl of trifluoracetic acid (TFA) was added. The sample was incubated at 45°C for 1½ hours, evaporated with a stream of nitrogen, and extracted twice with 200 μl portions of ether. The residue was dissolved in ethanol and an aliquot was removed for hydrolysis and amino acid analysis. The remainder was evaporated to dryness and cycled back to the first step.

(ii) Dansyl Edman

The procedure followed for dansyl Edman degradations was essentially that of Gray.[44] Aliquots from solutions of peptides to be sequenced were placed in a graded series in small test tubes (6 mm x 50 mm) and evaporated to dryness in a vacuum desiccator at 45°C. The first tube was set aside for reaction with dansyl choloride and determination of the N-terminal residue. The rest of the tubes were reacted with

PITC as follows: 25 μl of water and 25 μl of 5% PITC in pyridine were added to each tube; the tubes were flushed with nitrogen, sealed, and incubated for 1½ hours at 45°C; the reaction mixture was dried *in vacuo* at 45°C. The second tube was set aside and the remaining tubes were cycled through reaction with PITC and cleavage with TFA for the number of required Edman cycles. One tube was set aside at the end of each cycle. If samples were not drying easily after several Edman cycles, they were extracted with 50 μl of 50% aqueous ethanol and 200 μl of butylacetate; the peptide remained in the lower, aqueous phase.

Dried residues set aside at each Edman cycle were reacted with 5-dimethylaminonapthalene-1-sulfonyl chloride (dansyl chloride) in alkaline solution: 10 μl of 0.5% dansyl chloride (Pierce) in acetone and 10 μl of 0.01 M sodium bicarbonate were added to the dried residue; the reaction mixture was incubated for 30 minutes at 45°C in sealed tubes under nitrogen. Samples were evaporated to dryness under a stream of nitrogen and the dried residue containing dansyl-peptide was hydrolyzed with 25 μl of 6 N HCl (Aristar) in sealed tubes for 18 hours at 110°C.

Dansyl amino acid derivatives from hydrolysates were identified by thin layer chromatography in three different solvent systems on polyamide sheets. Solvents used were: (1) H_2O: 85% formic acid (100:1.5); (2) benzene: glacial acetic acid (9:1); (3) ethyl acetate: methanol:

glacial acetic acid (20:1:1). Samples were spotted on polyamide sheets 7.5 cm x 7.5 cm (Cheng Chin Trading Co.) and chromatographed in three dimensions as shown in fig. 19.

(f) Partial Hydrolysis of Myxosidin

(i) Acid hydrolysis: 100°C 6 N HCl

A solution of Myxosidin, 2.00 mg in 500 µl of water, was heated to 100°C in a boiling water bath. A tube containing 500 µl of 12 N HCl (Aristar) was maintained at 100°C. Hydrolysis was initiated by pouring the acid into the tube containing the peptide solution and rapidly mixing with a pipet. The reaction was allowed to proceed and samples were withdrawn at 5, 10 and 15 minutes. The samples were cooled to 0°C in a water-ice bath and the acid was removed immediately using a rotary evaporator. The peptides thus obtained were separated by HVE at pH 6.5 into acidic, neutral, and basic zones. These were cut from the sheets and either eluted with 25% ethanol-2% acetic acid or resewn on to sheets of Whatman 3 MM for further separation by HVE at pH 2.1 or by descending chromatography in a solvent composed of butanol: glacial acetic acid: water: pyridine (60:12:48:40). Zones were located by staining sidestrips in a cadmium-ninhydrin bath prepared as described previously.

(ii) Acid Hydrolysis 110°C, 0.03 N HCl

Peptides were obtained from Myxosidin by treatment with 0.03 N HCl. The procedure for mild acid hydrolysis was

FIGURE 19

THIN LAYER CHROMATOGRAPHY OF
DANSYL AMINO ACID STANDARDS

Solvents used were: (1) H_2O:85% formic acid (100:1.5); (2) benzene: glacial acetic acid (9:1); (3) ethyl acetate: methanol: glacial acetic acid (20:1:1); Dns-"X-2" is the dansyl derivative of an unidentified aromatic amino acid present in acid hydrolysates of Myxosidin.

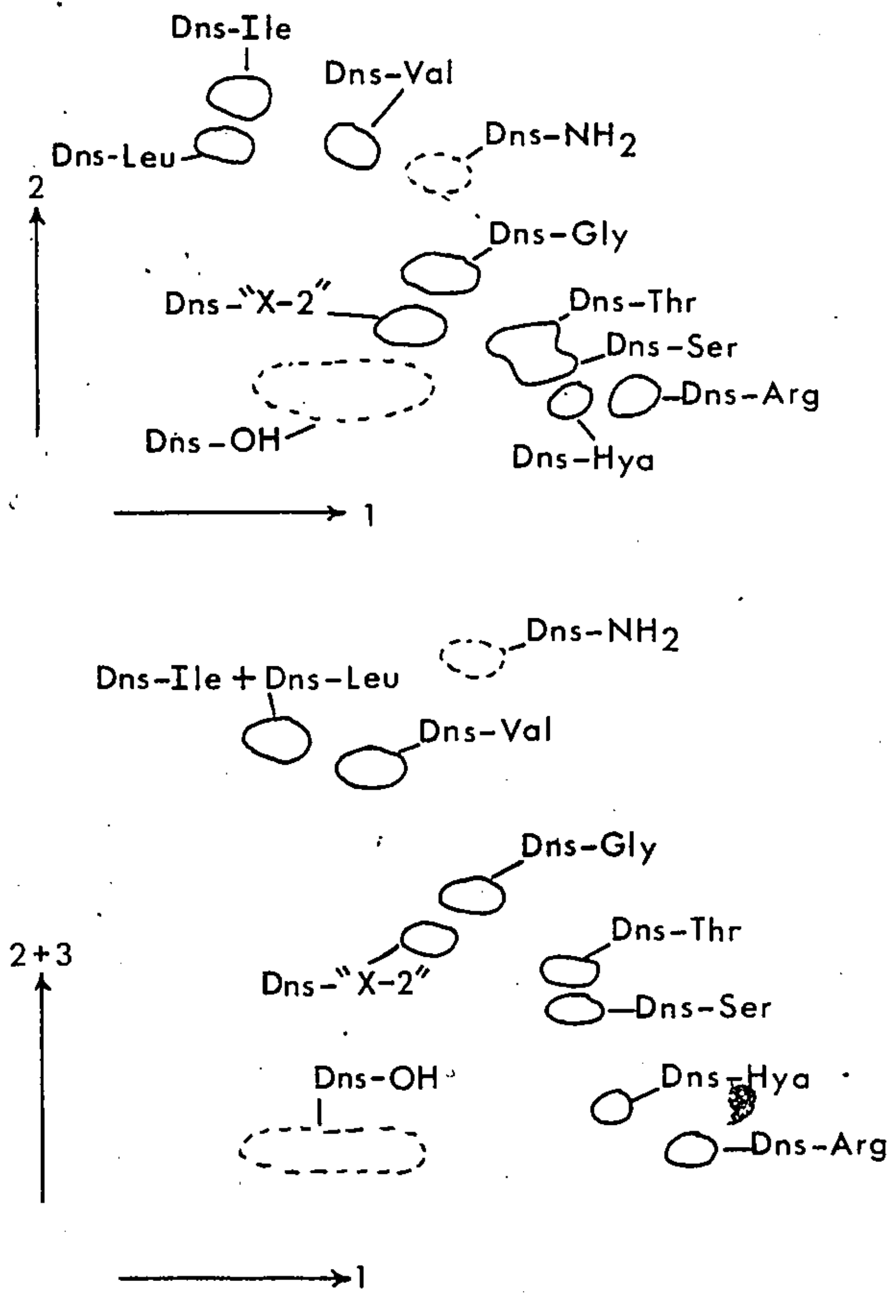
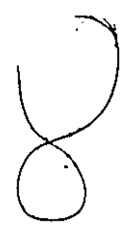


FIGURE 19



as follows: 100 μl of 0.06 N HCl was added to 100 μl of a solution of Myxosidin containing 0.60 mg of peptide; the resulting solution was made up to 1.00 ml by addition of 800 μl of 0.03 N HCl; the reaction mixture was sealed and incubated at 110°C for 24 hours; the hydrolysate was dried in a rotary evaporator and resulting peptides were separated by the techniques described above.

The time course of this reaction was followed by preparing the reaction mixture as above but with phenylalanine as an internal standard. The mixture was divided into four hydrolysis tubes and incubated at 110°C. At 5, 12, 17 and 24 hours a tube was removed, cooled, opened and evaporated to dryness. Relative amounts of a free amino acid and of a dipeptide were determined by amino acid analysis.

(iii) Cold Alkaline Hydrolysis: 0°C, 0.374 N $Ba(OH)_2$

Native Myxosidin was found to be a basic peptide by electrophoresis at pH 6.5. Following treatment with cold alkali, it migrated as a mixture of basic and neutral peptides. The two forms were separated by electrophoresis at pH 6.5 and were purified by re-electrophoresis at pH 2.1.

Myxosidin was treated with cold barium hydroxide as follows. To 1.0 mg of Myxosidin in a test tube, 2.0 ml of 0.374 N $Ba(OH)_2$ solution, chilled to 0°C, was added. Nitrogen was bubbled through the reaction mixture and the tube was sealed with parafilm and stored in an

ice-bath for 60 hours at 0^oC. At the end of the reaction period the mixture was diluted to approximately 10 ml by addition of 8 ml of water. A 3.0 ml portion of 0.2 N H_2SO_4 was added and the reaction mixture was titrated to pH 6.88. A dense white precipitate of barium sulfate formed. The precipitate was removed by centrifugation for 15 min at 10,000 rpm (Sorvall JA-14). The supernatant was evaporated to a clear glassy residue. The residue was redissolved in 50% ethanol and spotted on to sheets of Whatman 3 MM paper for HVE at pH 6.5. The basic and neutral zones were purified by electrophoresis at pH 2.1.

The amino acid composition of the neutral component differed from that of native Myxosidin in one respect. As discussed later, an amino acid (designated X-2) of Myxosidin is converted to another amino acid (designated X-3) on prolonged acid hydrolysis. Hydrolysates of neutral Myxosidin had higher X-3 contents than those of native Myxosidin. A small amount of an acidic tripeptide was also recovered.

(g) Derivatives for Mass Spectroscopy

(i) Acetylation

The dried peptide was dissolved in an excess of 1:4 (v/v) acetic anhydride: methanol and allowed to stand for 3 hours at room temperature in a tube sealed with parafilm. The solvent was removed by evaporation *in vacuo* at 40^oC.

(ii) Trifluoroacetylation

Trifluoroacetylation was accomplished by dissolving the peptide in 0.5 ml of trifluoroacetic acid anhydride (TFAA) and allowing it to stand for one hour before removal of the solvent by evaporation under a stream of nitrogen.[45] Alternatively, peptides were treated with TFA:TFAA 1:4 (v/v) at room temperature for one hour, according to the method of Priddle, Rose and Offord.[46]

(iii) Permethylation

Permethylations were by the method of Morris et al.[47] and followed his subsequent recommendation for short reaction times.[48] Sodium hydride (Baker) was washed with anhydrous ether (Mallinkrodt) and thoroughly dried. The basic reagent solution was prepared by gently heating sodium hydride, 50 mg/ml, in dimethylsulfoxide (Baker) in a sand bath maintained at 60°C until evolution of hydrogen ceased and a clear yellow solution was obtained. A ten-fold excess of this base was added to the dried residue of acetylated peptide (between 1 to 10 micromoles) followed by 0.3 ml of methyl iodide. One minute after addition of CH_3I the reaction mixture was diluted with water and extracted with 1 ml of chloroform. The chloroform extract was washed twice with water and evaporated to dryness *in vacuo* at 40°C.

(iv) Hydrazinolysis

Hydrazinolysis of arginyl containing peptides was by method of Morris, Dickenson and Williams.[49]

Hydrazine: water (1:1 v/v) was added to the peptide in a test tube. The mixture was heated for 15 min at 75°C. At the end of the reaction period the contents of the tube were diluted with water. The reagents were removed in a heated vacuum desiccator. The residue was dried *in vacuo*.

(v) Sample Volatilization

Samples were vaporized from a variable-temperature, direct insertion probe for electron-impact ionization in a DuPont 90 high resolution mass spectrometer at 70 electron volts. Data was collected on photographic chart paper with single ion focussing. In an attempt to increase sensitivity, a photographic plate recording was made with the instrument in the Mattauch-Herzog geometry. This method focusses and records all ions simultaneously, and is sometimes more sensitive for short-lived ions.

(vi) Derivatives for Gas Chromatographic Mass Spectroscopy (GCMS)

A sample of Myxosidin was hydrolyzed in 6 N HCl at 118°C for 18 hours. The hydrolysate was dried in a heated vacuum desiccator. The residue and samples of amino acid standards were reacted with TFAA and n-butanol to form N-TFA, n-butyl ester amino acids which can be readily separated by gas chromatography. Column materials and elution procedures were as in Summons et al.[50]

(h) Amino Acid Syntheses

(i) β-Hydroxyaspartic Acid

Hydroxyaspartic acid, 2-amino-3-hydroxybutanedioic acid, was prepared as a mixture of its four optical isomers by the method of Okai, Imamura, and Izumya.(51) Maleic acid, I (29g), was added to a solution of 20 g of NaOH and 12.9 ml of bromine in 1 litre of distilled water. The mixture was allowed to stand overnight at room temperature. To the reaction mixture, 50 ml of 10 N NaOH was added, followed by 275 ml of 1 M $BaCl_2$. A dense, white precipitate of barium transepoxysuccinate dihydrate (II) formed.

Product II (18 g air-dried) was suspended in 1 litre of concentrated aqueous ammonia containing 43.7 g of $(NH_4)_2SO_4$. The mixture was allowed to stand for 4 days at 40°C. At the end of the incubation period, precipitated barium sulfate was removed by filtration. The filtrate contained D, L-*erythro*-β-hydroxyaspartic acid (III) and D, L-*threo*-β-hydroxyaspartic acid (IV). The filtrate was evaporated to dryness and the residue was recrystallized from water to yield 1.29 g of IV (12%) as an ammonium salt. The mother liquor was evaporated to dryness and the residue recrystallized from water-methanol to yield 2.35 g of III (26%).

The ammonium salt of IV was applied to a column of Dowex 1 (OH^- form). The column was washed with water, in order to remove ammonium ions, and was eluted with 2 N acetic acid.

The eluate was evaporated to dryness in a rotary evaporator to yield 1.01 g of IV (11%). The synthesis is illustrated schematically in fig. 20.

(ii) β-phenylserine

The compound, 2-amino-3-hydroxy-3-phenylpropanoic acid or β-phenylserine, was synthesized according to the method of Erlenmeyer.[52] The procedure was as follows: 3.0 g of glycine (Analar) and 2.4 g of NaOH in 10 ml of water were chilled to 15°C. Benzaldehyde (8.5 g) was added with vigorous stirring. One hour later, 5.0 ml of concentrated HCl was added dropwise with stirring. After standing 2 hours at 5°C, the precipitate was removed by filtration. The precipitate was recrystallized from water to obtain <u>threo</u>-D, L-β-phenylserine. The filtrate was heated to boiling with an equal volume of dioxane. Upon cooling, <u>erythro</u>-D, L-β-phenylserine-dioxane addition product precipitated. This product was decomposed in hot water to yield the crystalline <u>erythro</u> diastereomer upon cooling.

(iii) α-phenylserine

The compound, 2-amino-2-phenyl-3-hydroxypropanoic acid or α-phenylserine, was synthesized by the method of Henze and Craig.[53] Phenacyl acetate (V), 2.0 g, was dissolved in 25 ml of 80% aqueous ethanol together with 1.0 g of KCN and 4 g of $(NH_4)_2CO_3$. The reaction mixture was diluted with 3 volumes of water and 1.7 g of 4-phenyl-2-oxazolidone-4-carboxamide (80%), VI, was recovered

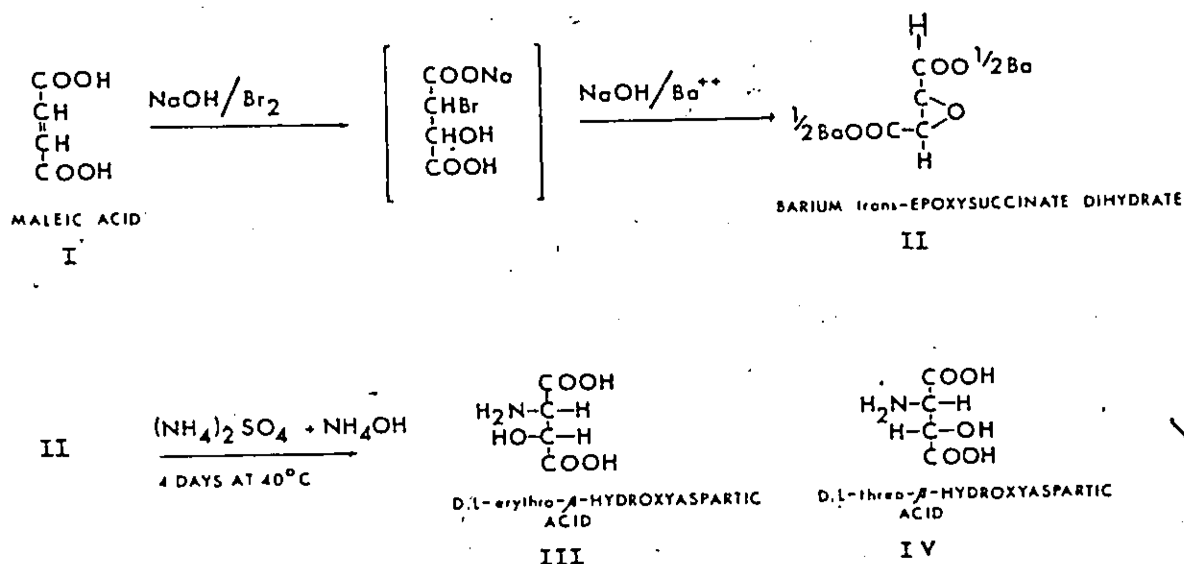


FIGURE 20

SYNTHESIS OF D,L-<u>ERYTHRO</u> D,L-<u>THREO</u>
HYDROXYASPARTIC ACIDS

(178-9°C obs. m.p.[1], 187-8°C lit. m.p.[1]) This amide was hydrolyzed in 6 N HCl to its corresponding carboxylic acid, 4-phenyl-2-oxazolidone-4-carboxylic acid, or under stronger conditions VI was hydrolyzed to 2-amino-3-hydroxy-2-phenylpropanoic acid (VII). The latter crystallized from aqueous solution as long, fine, white needles. The synthesis is illustrated schematically in fig. 21.

(i) Determinations of D and L Amino Acids

The method of Manning and Moore(54) was used to determine the configuration of the α-carbons of amino acid residue obtained from hydrolysates of Myxosidin. Standards were prepared from samples of D and L amino acids as follows: 20 μmoles of amino acid was weighed into a test tube and dissolved in 2.0 ml of 0.45 M borate buffer pH 10.2. The buffer was prepared by dissolving 13.9 g of boric acid in 497 ml of water and adjusting to pH 10.20 with 50% aqueous NaOH. The amino acid solution was cooled to 0°C in an ice-bath. A 10 to 20% excess of L-leucyl-N-carboxyanhydride (L-leu-NCA) was weighed out and added to the contents of the test tube with vigorous agitation by a Vortex mixer. After 2 min, 0.8 ml of 1 N HCl was added to stop the reaction. The final pH was 1 to 3 as estimated by pH paper. Cloudy samples were filtered prior to use as standards on the amino acid analyzer.

A sample of Myxosidin was hydrolyzed in 6 N HCl at

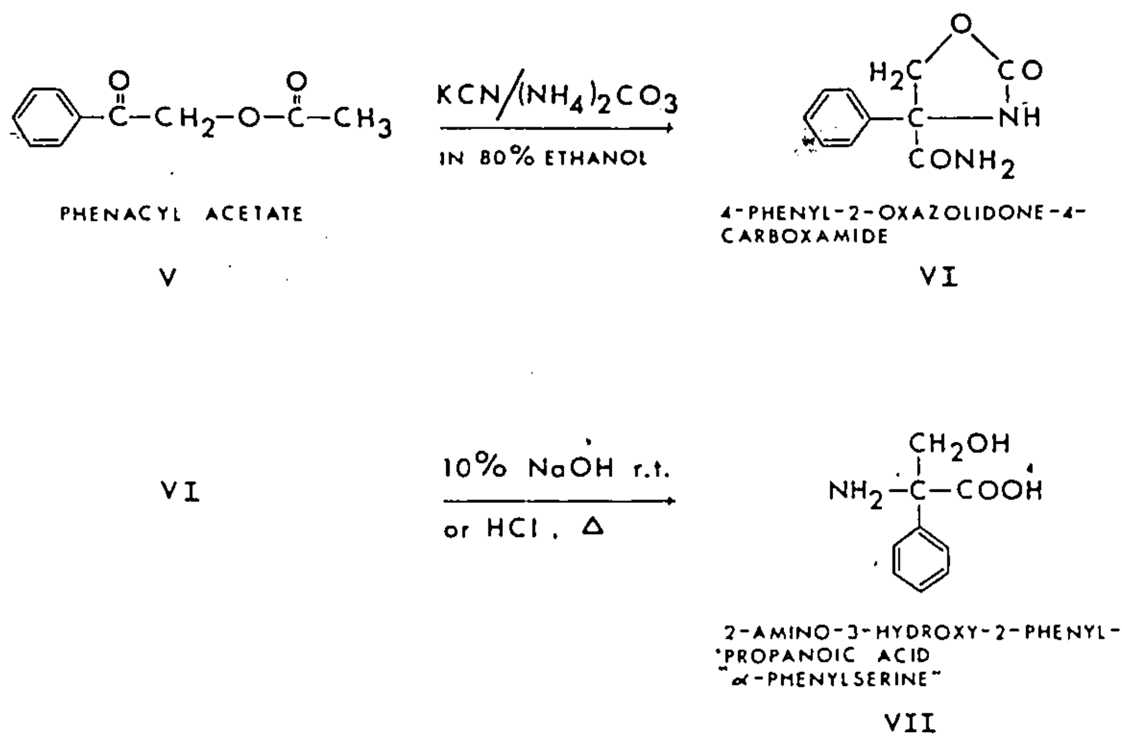


FIGURE 21

SYNTHESIS OF α-PHENYLSERINE

110°C for 18 hours and was reacted with L-leu-NCA as described above. The 0.32 N pH 5.25 buffer (Beckman) was diluted to 0.2 N (pH 5.42) and was mixed 1:1 with the pH 4.25 buffer for a buffer pH of 4.84, 0.2 N sodium citrate. The analyzer was operated exactly as before except that pH 4.05, 0.2 N sodium citrate buffer was the first buffer, followed by pH 4.84, 0.2 N sodium citrate as the second. With this system the standards eluted at the following volumes: threonine (60 ml), serine (66 ml), L-leucyl-D threonine (149 ml), L-leucyl-L-threonine (162 ml), L-leucyl-D-serine (133 ml), L-leucyl-L-serine (176 ml), and leucine at 131 ml.

Hydrolysates of Myxosidin were digested with L-amino acid oxidase and D-amino acid oxidase.(55) A sodium pyrophosphate buffer of pH 8.2 was prepared as follows: 2.23 g of $Na_4P_2O_7 \cdot 10H_2O$ was dissolved in distilled water and made up to 25 ml; 23 ml of 0.1 N HCl was added and final pH adjustment was made with 0.1 N HCl. Tris buffer of pH 7.7 was made from 0.606 g of tris (hydroxymethyl) aminomethane (Sigma) dissolved in distilled water and made up to 25 ml; 30 ml of 0.1 N HCl was added and final pH adjustment was made with 0.1 N HCl.

A solution of 1 ml of Myxosidin hydrolysate, 1 ml of pyrophosphate buffer, 10 μl of a solution of catalase, 50μl of a 10 mg/ml solution of β-NADH, and 10 μl of a 5 mg/ml solution of D-amino and oxidase (DAO) were mixed

together in a small vial. A second reaction mixture contained 1 ml of hydrolysate, 1 ml of Tris buffer, 10 μl of a 10 mg/ml solution of peroxidase and 100 μl of a 1 mg/ml solution of L-amino acid oxidase (LAO). Blanks contained each of the above components except 10 μl of distilled water for DAO instead of the D-oxidase solution and 100 μl of water for LAO instead of the L-oxidase solution.

The vials were incubated at 37°C for 18 and 48 hours and were slowly rotated on an inclined axis to promote oxygen exchange. At the end of the incubation period the contents of each vial were evaporated to dryness and redissolved in distilled water prior to amino acid analysis.

D. RESULTS

(a) Hexose Analysis of Myxosidin

A sample of Myxosidin was tested for the presence of hexoses and pentoses by the phenol-sulfuric acid method described in Chapter II. Hexoses and pentoses are degraded to furfural and related compounds when treated with strong acids. The breakdown products formed can combine with such reagents as phenol to give coloured addition complexes. A positive result for this test does not necessarily imply the presence of carbohydrates since other classes of compounds may yield products with a similar absorption, but a negative result virtually rules out their presence.

No carbohydrate could be detected in Myxosidin by this method. The lower limit, set by the sensitivity of the

analysis, was a carbohydrate content, by weight, of less than 3%.

(b) Amino Acid Analysis

(i) Initial Results

Myxosidin, obtained as described in Chapter III, yielded a mixture of 11 ninhydrin positive compounds when hydrolyzed in 6 N HCl at 110°C. These are listed in Table 6 which gives the results for an individual analysis of a 24 hour hydrolysate. Threonine, serine, glycine, valine, iso-leucine, leucine, and arginine accounted for 7 of them; in a 24 hour hydrolysate, their molar ratios were approximately 3:3:3:1:2:9:3, respectively. Two other major components were designated X-1a and X-2. The amino acid X-1a eluted well ahead of aspartic acid; if its colour yield factor were taken to be the same as that of threonine, then its molar ratio in a 24 hour hydrolysate was approximately the same as that of threonine. Component X-2 eluted as a shoulder at the leading edge of the glycine peak when conventional buffers were used in the analyzer. It was eluted as a separate peak when the buffers were made up with the addition of 0.5% benzyl alcohol. If its colour yield factor were taken to be the same as that of threonine, then its molar ratio in 24 hour hydrolysates was also approximately the same as that of threonine.

The tenth component, designated X-1b, was eluted after X-1a but before aspartic acid. It was a minor component

TABLE 6

AMINO ACID COMPOSITION OF MYXOSIDIN

24 HOUR ACID HYDROLYSATE

Amino Acid	Count	Colour Constant counts/nmol	nmol/250μl	Ratio	Elution Volume ml
X-1a	215225	5057	42.57	2.44	46
X-1b	8665	5057	1.71	0.10	64
THR	248265	5057	49.09	2.81	104
SER	239741	4944	48.48	2.78	112
X-2	219675	5057	43.44	2.49	160 (172)
GLY	268292	5122	52.38	3.00	174
X-3	33100	5057	6.55	0.38	193 (205)
VAL	81299	4891	16.62	0.95	226
ILE	150324	5420	29.73	1.70	296
LEU	829714	5229	164.07	9.40	304
ARG	311743	5110	47.08	2.70	352

Analysis was made in standard sodium citrate buffers (Beckman) made up to 0.5% benzyl alcohol. Figures in brackets in the tabulation of elution volumes are volumes in the absence of benzyl alcohol. Thus only X-2 and X-3 were shifted by this reagent.

in 24 hour hydrolysates but increased in amount with increasing duration of hydrolysis. The increase in X-1b was accompanied by a decrease in X-1a. In a 72 hour hydrolysate, X-1a and X-1b were present in approximately equal amounts. This is illustrated in fig. 22 . If the colour yield factor of X-1b were taken to be the same as that of threonine, then the molar ratio of the sum (X-1a + X-1b) remained approximately constant for various durations of hydrolysis.

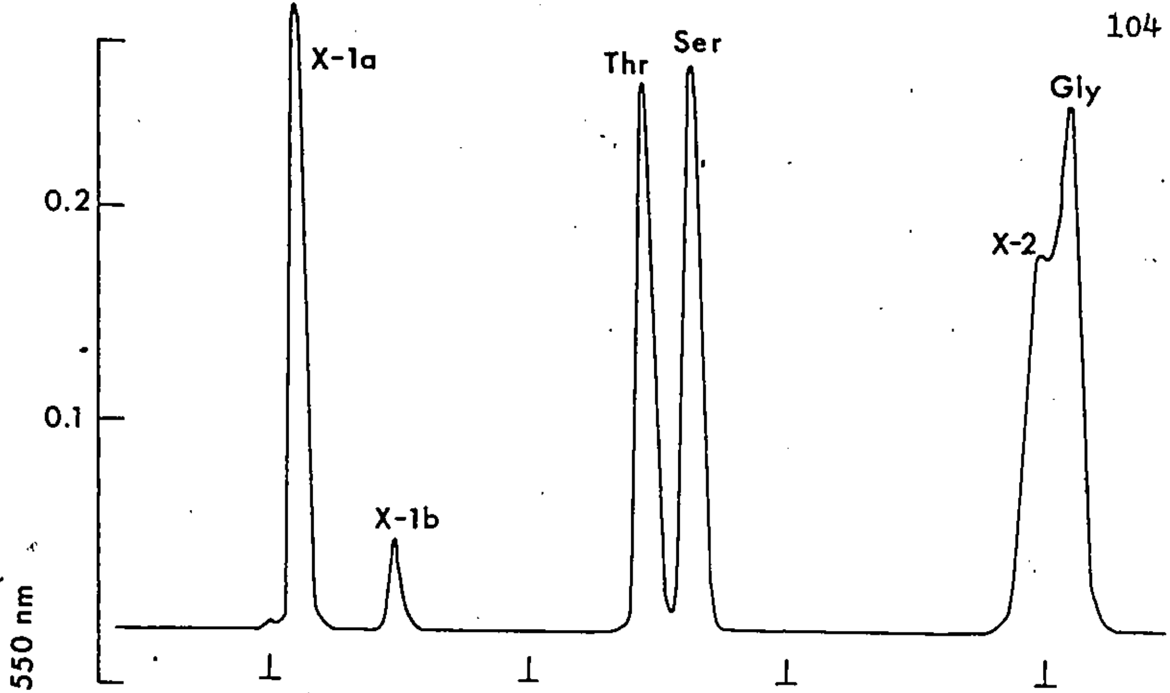
The remaining component of Myxosidin hydrolysates, designated X-3, was eluted after glycine and before valine with conventional citrate buffers. Its elution volume was reduced when the citrate buffers were made up with the addition of 0.5% benzyl alcohol. However, it was still eluted between glycine and valine. Component X-3 was a minor constituent of 24 hour hydrolysates but it increased in amount with increasing duration of hydrolysis, although not as quickly as did component X-1b. The increase in peak area of X-3 was accompanied by a decrease in the peak area of X-2, as shown in Table 7 . In a 72 hour hydrolysate, the area of X-3 was about half that of X-2. If the colour yield factor of X-3 were taken to be the same as that of threonine, then the molar ratio of the sum (X-2 + X-3) remained constant for various durations of hydrolysis.

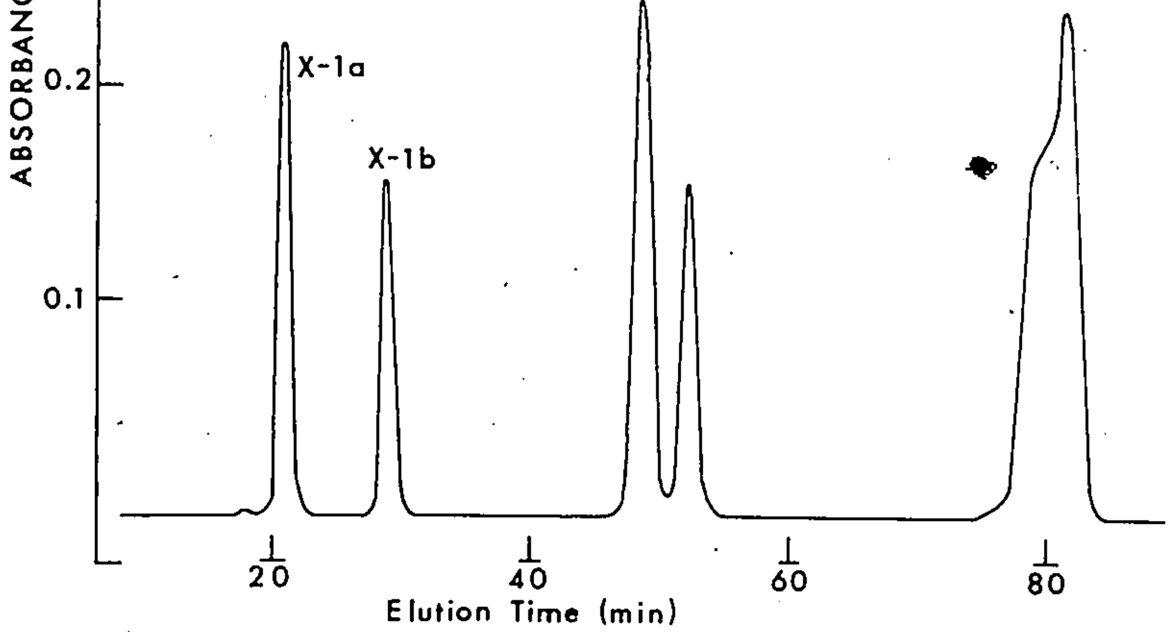
A sample of the hydrolysate of Myxosidin was run

FIGURE 22

MYXOSIDIN: ANALYSES OF THERMOLABILE COMPONENTS

Samples were hydrolyzed in 6N HCl at 110°C for the times indicated; 24 hour hydrolysates were observed to have only a small amount of component X-1b, extended hydrolysis times led to increasing amounts of X-1b and decreasing amounts of X-1a.





MYXOSIDIN: ANALYSES OF THERMOLABILE COMPONENTS (Beckman Model 121)

FIGURE 22

TABLE 7

PEAK AREAS OF X-2 AND X-3 AS A FUNCTION OF CONDITIONS OF HYDROLYSIS

TIME(HOURS)	COUNT (X-2) + (X-3)	COUNT GLY	RATIO GLY: (X-2) + (X-3)
12½	176161 + 32364	257081	1.2
18	232193 + 32166	283022	1.1
24	116346 + 13566	140572	1.1
72	87063 + 44916	177062	1.3

Samples were hydrolyzed in 6N HCl at 110°C for the times indicated. Conditions for amino acid analyses were as for Table 6

at pH 6.5 on the high voltage electrophoresis apparatus. The components were thus separated into acidic, neutral, and basic zones. The acidic zone was found to contain X-1a and X-1b and had a mobility with respect to aspartic acid of 0.93. The ninhydrin stain of the acidic zone was bright orange, easily distinguished from the pink colouration of the aspartic acid standard. The neutral zone contained threonine, serine, X-2, glycine, X-3, valine, isoleucine, and leucine. The basic zone contained arginine.

(ii) Identification of X-1a and X-1b

The compounds X-1a and X-1b are strongly acidic amino acids as indicated by their elution volumes in the amino acid analyzer and by their electrophoretic mobilities on paper at pH 6.5. The observation that extended acid hydrolysis seemed to convert X-1a into X-1b led to the speculation that they may be a diastereomeric pair. It was suspected that X-1a and X-1b were the <u>threo</u> and <u>erythro</u> diastereomers of β-hydroxy-aspartic acid. The two diastereomers were therefore synthesized for comparison with X-1a and X-1b obtained from hydrolysates of Myxosidin by electrophoresis.

Hydroxyaspartic acid (both <u>threo</u> and <u>erythro</u>) and X-1a had the same electrophoretic mobilities at pH 6.5 (R_{Asp}= 0.93). As shown in fig. 23, dansyl X-1a had the same mobility in the two solvents as dansyl-hydroxyaspartic acids, although the chromatograms did not unequivocally distinguish between the diastereomers. Similarly X-1a had the same

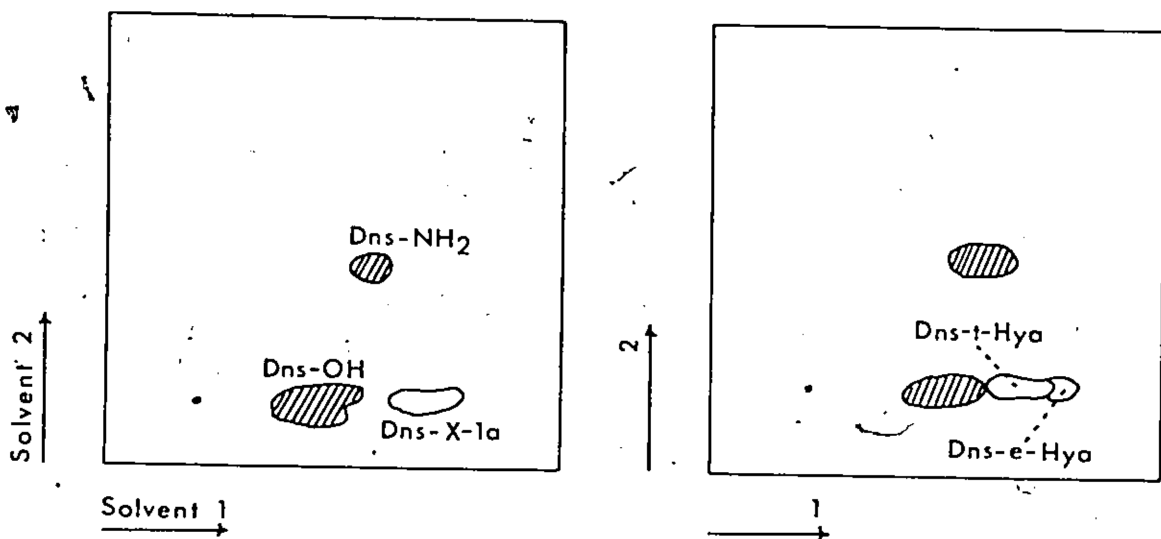


FIGURE 23

COMPARISON OF DANSYLATED HYDROXYASPARTIC ACID AND X-1a ISOLATED FROM 6 N HCl HYDROLYSATES

Solvents as for Figure 19.

mobility as the hydroxyaspartic acids on descending chromatography in BAWP (butanol-acetic acid-water-pyridine 60:12:48:40), R_{Leu}= 0.31, although again the *threo* and *erythro* forms were not separable. It was noted that when stained in a cadmium-ninhydrin bath, zones from the hydroxyaspartic acids and from X-1a progressed through colour changes from orange to brown in an identical fashion.

The two diastereomers were clearly separated by ion-exchange chromatography in the amino acid analyzer. The elution volumes are shown in Table 6 for the products of a Myxosidin hydrolysate. This table includes the data for X-1a and X-1b. By comparison with synthetic standards, it was determined that X-1a had the same elution volume as *threo*-hydroxyaspartic acid. The *erythro* diastereomer was found to have the same elution volume as X-1b. It was concluded that X-1a and X-1b were the *threo* and *erythro* diastereomers of hydroxyaspartic acids respectively, and that the *threo* isomer was present in the peptide prior to hydrolysis.

(iii) Identification of X-2 and X-3

The peak which represented the unknown amino acid X-2, overlapped the glycine peak when amino acid analysis was carried out with 0.2 N citrate buffer, pH 3.25. Addition of benzyl alcohol to the buffer (0.5%), shifted the X-2 and X-3 peaks by reducing their elution volumes and completely resolved the former from glycine. Benzyl alcohol is a reagent which is known to shift selectively the positions of

aromatic amino acids on ion-exchange columns.[56] As mentioned in the introduction to this chapter, an aromatic compound was indicated by nmr and U-V spectra of Myxosidin. It was suspected therefore, that X-2 and X-3 were aromatic amino acids.

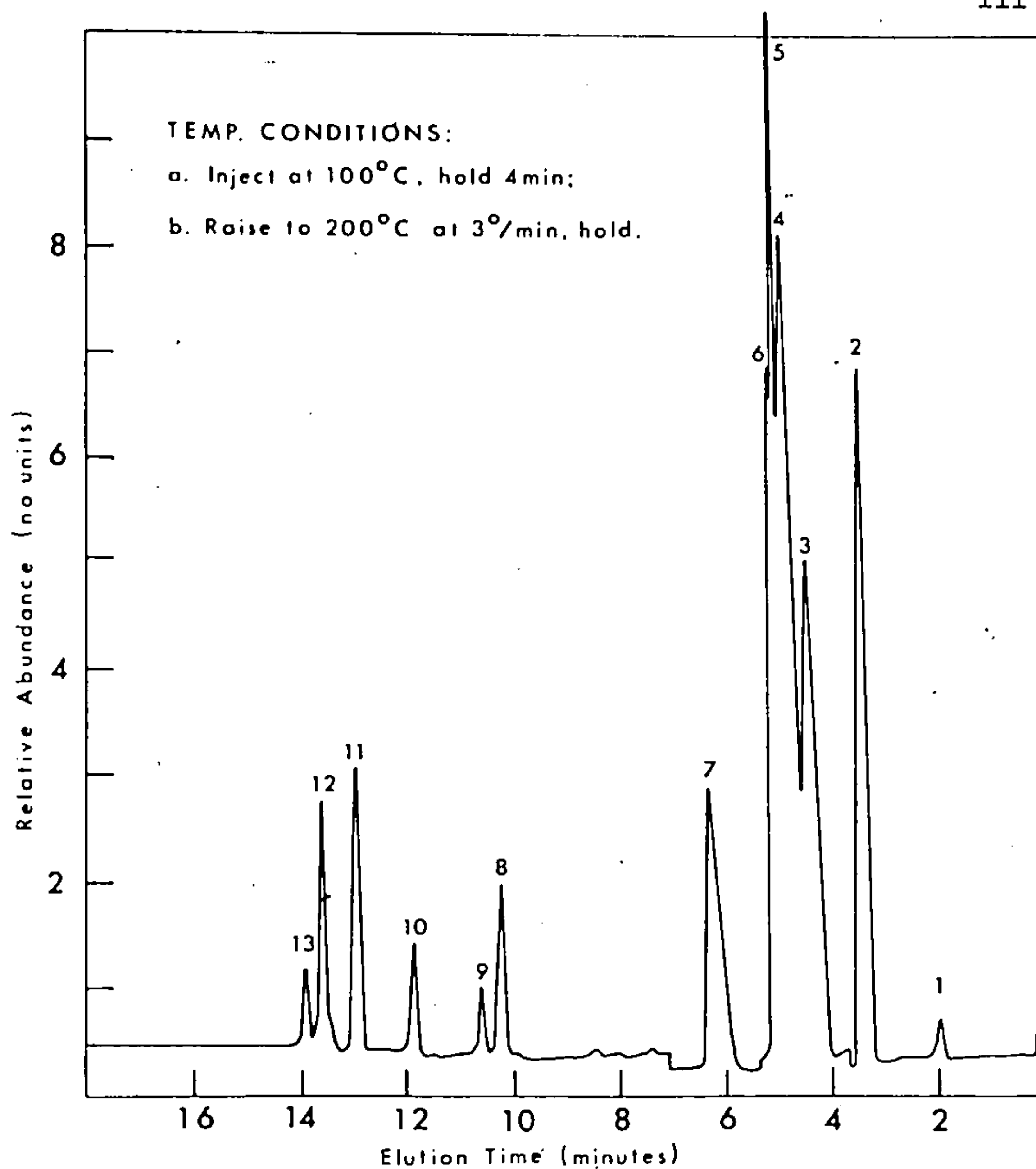
Micromolar amounts of component X-2 were isolated from 18 hour acid hydrolysates of Myxosidin as follows. The hydrolysate was fractionated first by high voltage paper electrophoresis at pH 6.5. The neutral zone was sewn onto a second sheet of paper for descending chromatography in BAWP solvent. This separation gave 4 zones composed of (a) glycine and serine, (b) threonine, (c) X-2 and valine, and (d) iso-leucine and leucine. Zone (c) was then refractionated twice by high voltage electrophoresis at pH 2.1 in order to separate X-2 from valine. This procedure provided the reference samples used for MS and GCMS analyses.

Figure 24 shows the gas chromatogram for the N-trifluoroacetyl, n-butyl ester derivatives of amino acids present in an 18 hour hydrolysate of Myxosidin. The elution time of peak 8 matched that of the X-2 standard. The mass spectrum of peak 8 is shown in fig. 25 . The important features of this spectrum are the ion at 333 mass units, representing a molecular ion or possibly the molecular ion with a loss of one or two hydrogens,a common loss from amino acid and peptide derivatives in MS , and the ion at 107 mass units,

FIGURE 24

GAS CHROMATOGRAM OF N-TFA, n-BUTYL DERIVATIVES OF MYXOSIDIN HYDROLYSATE

Peaks were identified by comparison with standards and with literature values as: (1) dehydroalanine; (2) valine; (3) glycine; (4) isoleucine; (5) leucine; (6) threonine; (7) serine; (8) X-2; (9) phenylserine (X-3); (10) ?; (11) hydroxyaspartic acid; (12) hydroxy-aspartic acid; (13) ?. The unidentified peaks appeared to be related to Hya. The column used was a six foot, 4 mm (id) glass column, packed with Tabsorb. The sample was injected at an oven temp. of 100°C, held 2 min and increased to 220°C at 4°C/min. The carrier gas was He at 60 ml/min.

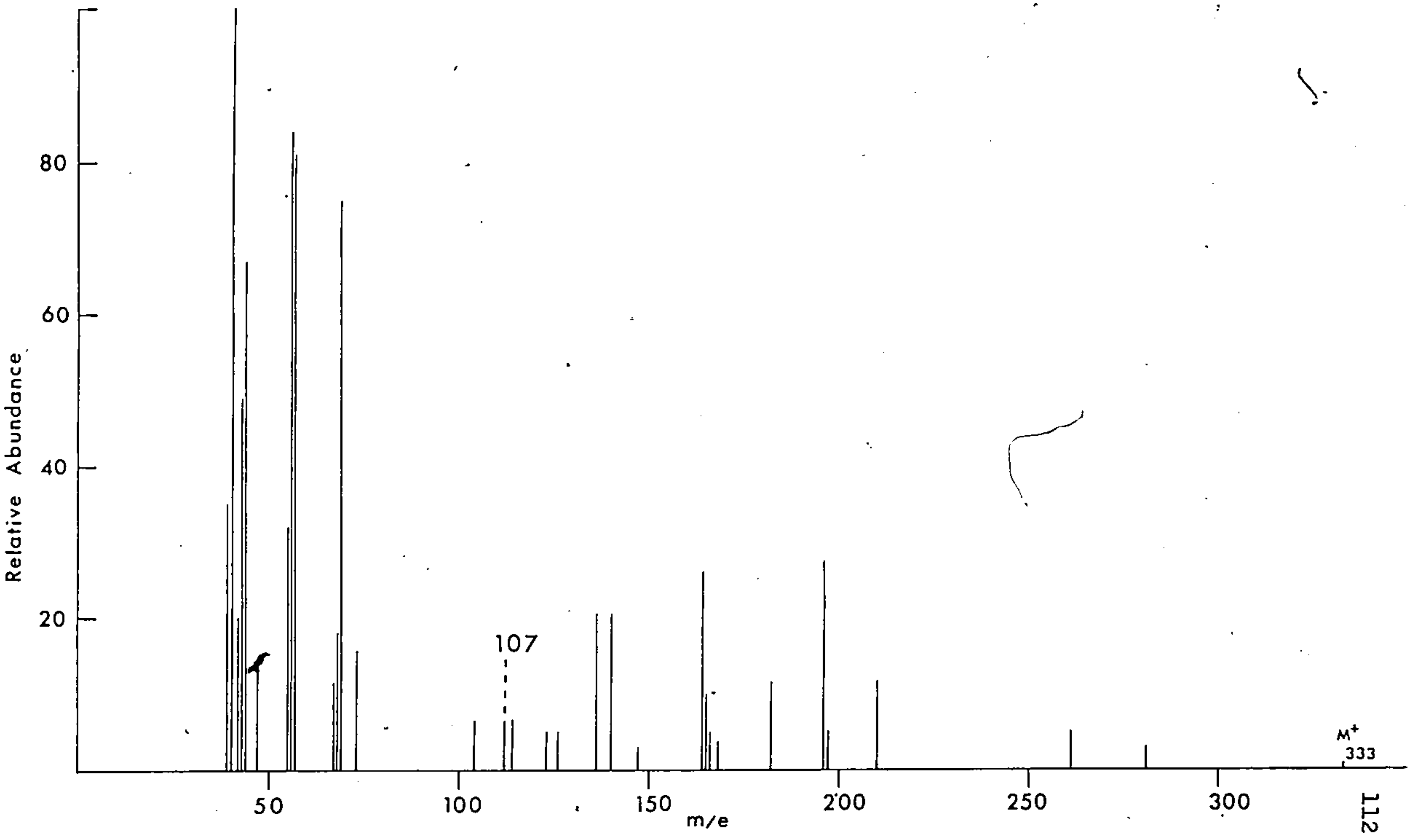


GAS CHROMATOGRAM OF N-TFA, n-BUTYL DERIVATIVES OF MYXOSIDIN HYDROLYSATE

FIGURE 24

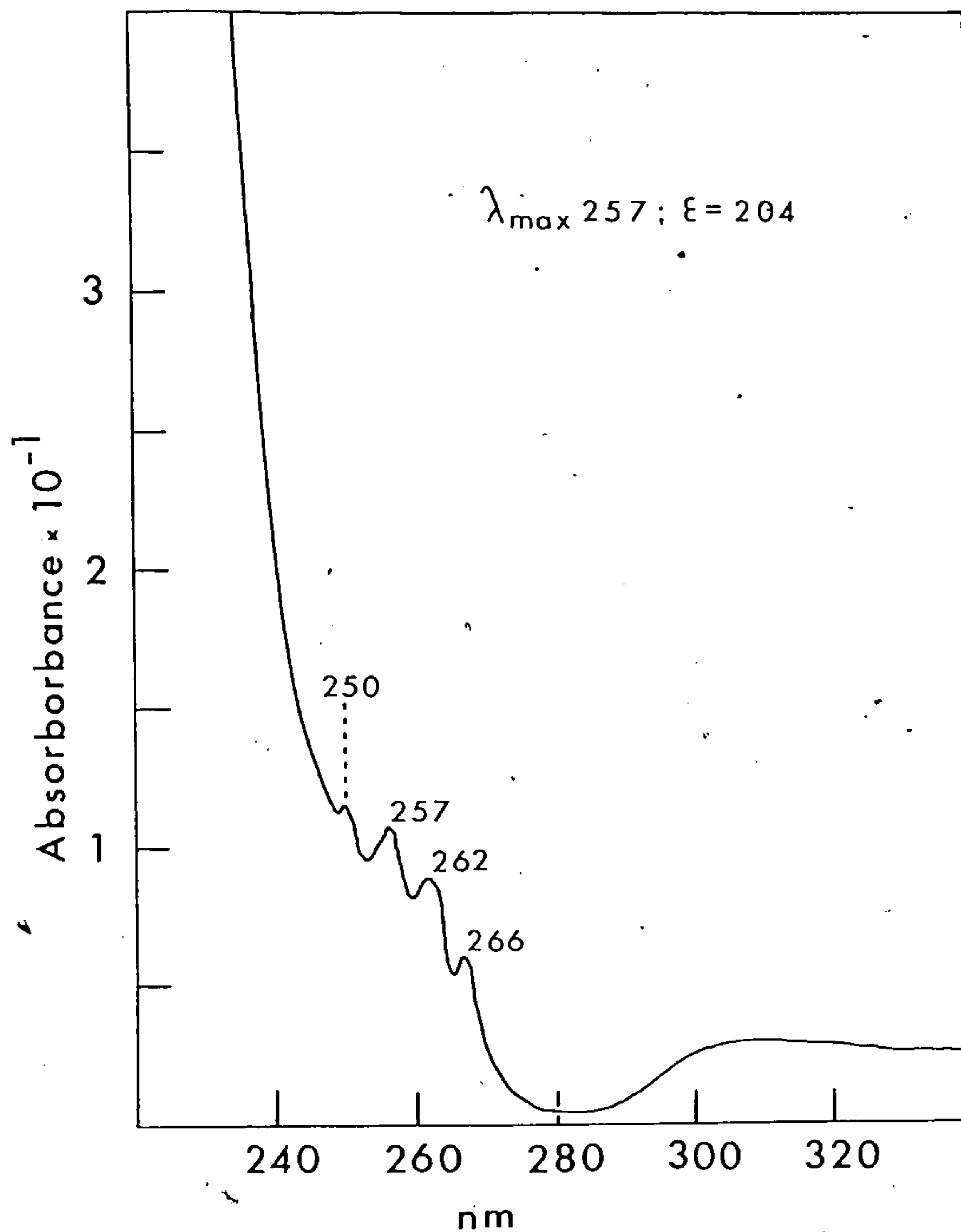
FIGURE 25

PEAK 8: GC/MS OF N-TFA, n-BUTYL ESTERS OF MYXOSIDIN HYDROLYSATE



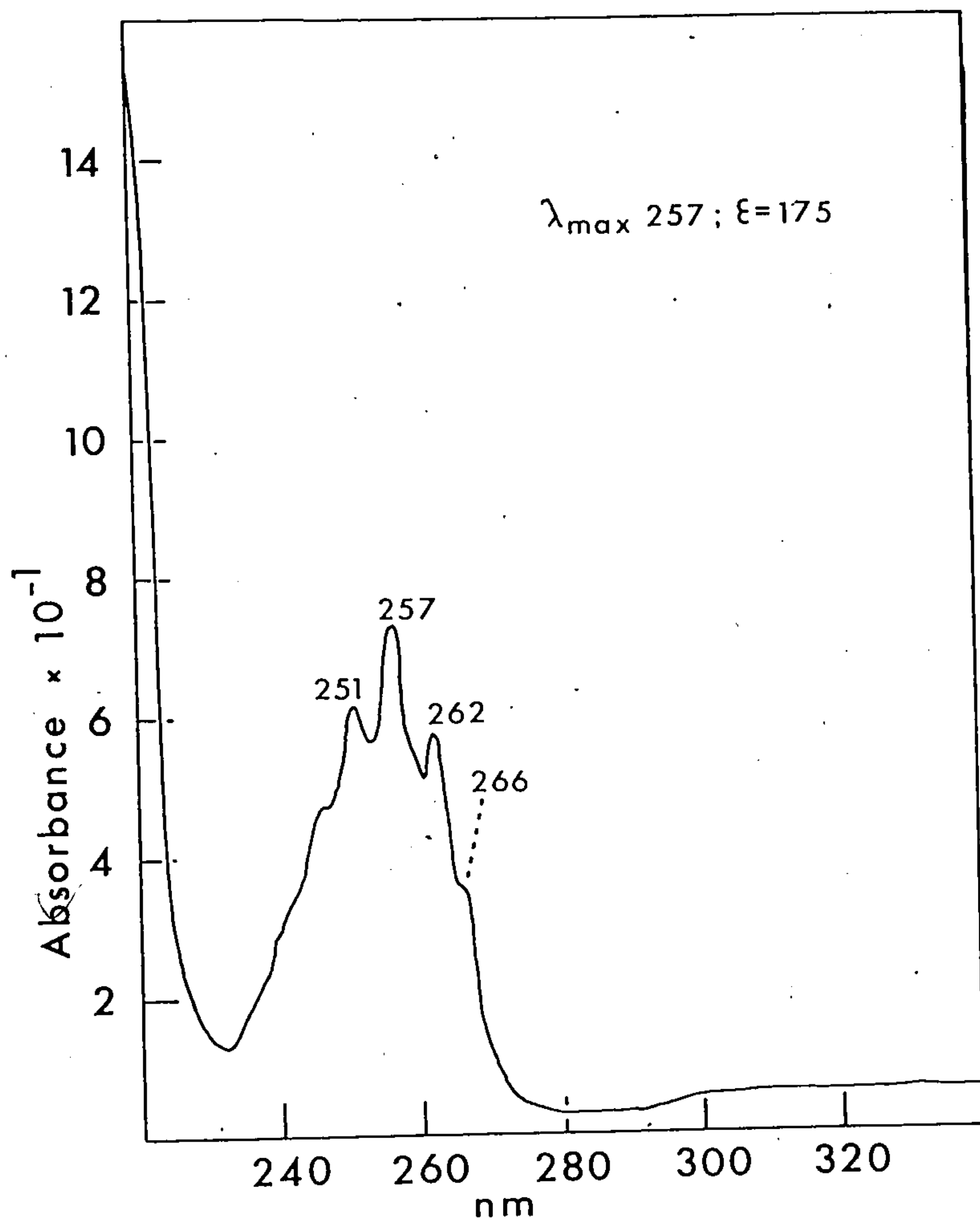
a possible $C_7H_7O^{+\cdot}$ ion or $C_7H_9N^+$ ion, both indicating an aromatic compound. A molecular ion of 333 implies a molecular weight for the free amino acid of 179 to 181 daltons. These figures are obtained by deducting the weight of the N-TFA group and the n-butyl group (97 and 57 mass units respectively) and adding 2 hydrogens. The ^{13}C nmr spectrum of the aromatic group of Myxosidin showed that it was a six-membered carbon ring with five of the carbons attached to hydrogen atoms. A search of formula lists showed that the molecular formula $C_9H_{11}NO_3$ was very likely to be the formula of X-2.

The U-V spectrum of Myxosidin is shown in fig. 26. The absorption spectrum between 240 and 270 nm indicated the presence of an aromatic chromophore of the phenyl type. The model compound, benzene, for example, absorbs in the region of 180 to 210 nm, and in the 250 to 255 nm region. Absorption in these two bands is common to all benzenoid compounds. They are termed the E band and B band of the chromophore, respectively. Typically the molar extinction of the B band is 10^2 to 10^3, and that of the E band 2 to 6 x 10^3. These points are illustrated by the U-V spectrum of the amino acid β-phenylserine shown in fig. 27. The B band pattern is evident from 240 to 270nm. Fine structure is apparent in



U-V Spectrum of Myxosidin (methanol)

FIGURE 26



U-V Spectrum of t-β-d,l-phenylserine (methanol)

FIGURE 27

this region with local maxima occuring at 251, 262 and 266 nm. The B band λ_{max} is at 257 nm. The molar extinction of this model compound in methanol at the B band λ_{max} was found to be 175 l/cm-mole. The E band of the chromophore in Myxosidin is obscured by the strong absorption of the peptide bonds below 240 nm. The B band pattern can be seen and some fine structure is apparent. The peak at 257 nm represents the λ_{max} of the B band of Myxosidin's chromophore. It was assumed that this absorption pattern was due to the side chain of the X-2 residue. The local maxima at 262 and 266 nm are characteristic of a monosubstituted benzenoid compound. The molar extinction coefficient was calculated to be 204 l/cm-mole at 257 nm. This calculation is based on a molecular weight estimate for Myxosidin of 1.14×10^3 daltons. The estimate of molecular weight is derived from evidence presented in the next section of this chapter. The absorption spectra of X-2 and X-3 were not determined as the amounts of these two amino acids purified from Myxosidin hydrolysates were insufficient for U-V spectroscopy by methods available to the author.

The B band of the absorption spectrum of Myxosidin has two significant features. Firstly, the value of λ_{max}, 257 nm is close to that of benzene and typical of a phenyl group which is not substituted by an hydroxyl or other electron-donating group. As a contrast, tyrosine, for

example, has a phenolic hydroxyl and an absorption spectrum with λ_{max}= 278nm for the B band. Secondly, the value of the molar extinction coefficient at λ_{max} is only 204 l/cm-mole for Myxosidin. Hydroxyl-substituted aromatic chromophores have much higher extinction coefficients, in the range of 7×10^2 to 3×10^3 l/cm-mole.

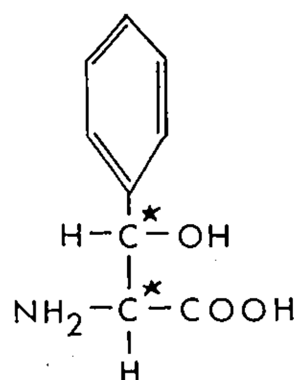
The foregoing evidence suggests that X-2 has an unsubstituted phenyl group. The elution volume on the amino acid analyzer also suggests that X-2 is considerably more polar than phenylalanine. The molecular formula of X-2 inferred from GCMS data led to the speculation that X-2 might contain a hydroxyl group on either its α or β-carbon. Amino acids with the molecular formula $C_9H_{11}NO_3$ and which fit the above clues are illustrated in fig. 28 . The amino acid α-phenylserine and the two β-phenylserines were synthesized by methods found in the literature.

The amino acid X-3, which is formed from X-2 on prolonged acid hydrolysis, was found to match the β-phenylserines in the following respects: the location on paper chromatograms after development in BAWP solvent (R_{Leu}= 0.74); the elution volumes in the amino acid analyzer before and after the addition of benzyl alcohol to the buffers (205ml and 193 ml, respectively); location of dansyl derivatives on polyamide thin layers following development

FIGURE 28

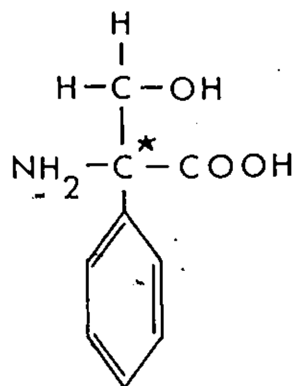
AROMATIC HYDROXY α-AMINO ACIDS OF THE MOLECULAR FORMULA $C_9H_{11}NO_3$

Assymetric centers are indicated as C^*; β-phenylserine has two assymetric centers, and exists as a set of four optical isomers; α-phenylserine has one assymetric center.



2-amino-3-hydroxy-3-phenylpropanoic acid

"β-phenylserine"



2-amino-2-phenyl-3-hydroxypropanoic acid

"α-phenylserine"

FIGURE 28

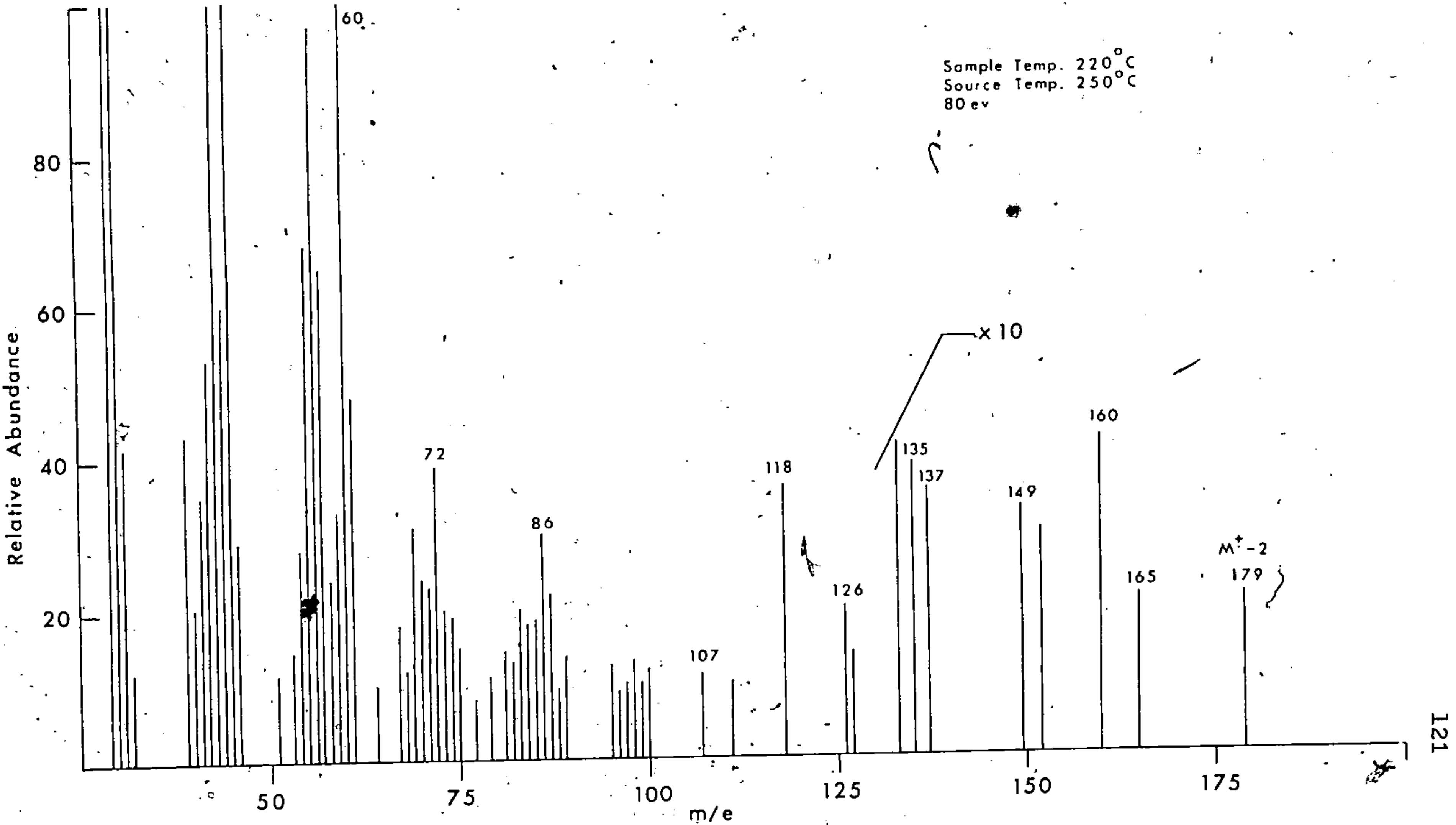
in two dimensions with solvents 1 and 2. It was concluded that X-3 was either threo-β-phenylserine, erythro-β-phenylserine, or a mixture of both. None of the above separation methods resolved the two diastereomers unequivocally. Thus, extended acid treatment of X-2 resulted in its partial conversion to β-phenylserine.

The unknown amino acid X-2 was easily distinguished from the β-phenylserines by all the criteria listed above. The amino acid α-phenylserine was synthesized and compared to X-2. The following results were found for α-phenylserine: the electrophoretic mobility at pH 2.1 was $R_{Arg} = 0.48$ ($R_{Arg} = 0.57$ for X-2); the location on paper chromatograms after development in BAWP solvent was $R_{Leu} = 0.86$ ($R_{Leu} = 0.82$ for X-2); elution volumes before and after the addition of benzyl alcohol to the buffers of the amino acid analyzer were 182 ml and 166 ml (the corresponding volumes for X-2 were 172 ml and 160 ml). Furthermore dansyl derivatives of X-2 and of α-phenylserine were separable by two dimensional polyamide thin layer chromatography in solvents 1 and 2. Clearly X-2 is not α-phenylserine. The remaining possibility in fig. 28 is α-hydroxyphenylalanine, a geminal amino alcohol which cannot exist as a free compound at room temperature.

A purified sample of X-2 isolated from hydrolysates of Myxosidin was used to obtain the mass spectrum shown in fig. 29. This spectrum was obtained without derivatization of X-2 to the usual ethyl ester. Amino acids are not very

FIGURE 29

MASS SPECTRUM OF "X-2" AS FREE AMINO ACID



volatile and they usually do not have a well defined melting point since they tend to decompose on heating. If a free amino acid is heated quickly to 250°C in the sample probe, it will rapidly decompose or pyrolyse. However, a sufficient amount of the compound may vaporize into the source chamber and if the detecting equipment is sensitive enough a spectrum can be recorded.

In the spectrum shown for X-2, the most important features are: the ion at 179, identified as the molecular ion missing two hydrogens; and three ions at 133, 135 and 137 which were identified as amine fragments; and the ion at 107 identified as the $C_7H_7O^+$ ion or $C_9H_9N^+$ ion, indicating an aromatic compound.

The mass spectra of amino acids are often missing the molecular ion, represented symbolically as $M^{+\cdot}$, since by interaction with other molecules on the probe, or by internal reactions, one or two hydrogen atoms may be abstracted. The ion at 179 was recognized as implying a molecular ion of mass 181 because, from other spectral data, a minimum of 8 carbons, 8 hydrogens, two oxygens, and one nitrogen were required. A computer listing of all possible molecular formulae for CHNO atoms between 179 and 181 showed that $C_9H_{11}NO_3$ at 181 daltons was the most likely formula for X-2. Thus the ion at m/e 179 was the $M^{+\cdot}$-2H ion of X-2.

Amino acids tend to fragment initially in such a fashion as to retain charge on the amino group. Cleavage of

the molecule at the carbon-carbon bond and the resulting elimination of neutral molecules such as CO_2, HCO_2, and H_2CO_2 forms a series of ions referred to as amine fragments. The series seen in the spectrum of X-2 at 133, 135, and 137 seems to be of this type. The formation of amine fragments from amino acids is illustrated in fig. 30.

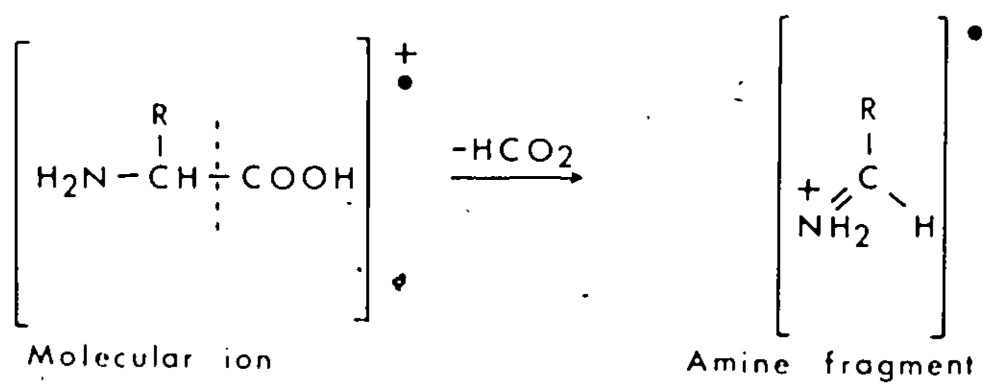


FIGURE 30

FORMATION OF AMINE FRAGMENTS:
MASS SPECTROSCOPY OF AMINO ACIDS

The cyclic peptide ergotamine, illustrated in fig. 31, contains the unusual amino acid residue 2-amino-2-hydroxy-propanoic acid. This α-hydroxy-α-amino acid is not stable as a free amino acid at room temperature. Acid hydrolysis of the peptide releases an α-keto acid, pyruvic acid, and ammonia. In the peptide it is stabilized by covalent bonds to each of its three functional groups. An analogous aromatic compound with a phenyl group on carbon 3 may be considered as a possibility for the structure of the aromatic residue of Myxosidin if its three functional groups are covalently bonded as in the ergotamine structure. However, it cannot be considered as a possibility for X-2, as isolated from acid hydrolysates, since it would appear as phenylpyruvic acid and ammonia. Phenylpyruvic acid is only weakly reactive with ninhydrin. The ammonia content of Myxosidin hydrolysates was not examined in a systematic manner.

In addition to the β-amino acids of fig. 32, similar geminal amino alcohol structures could be considered for the aromatic residue as existing in the intact peptide. They are subject to the same restrictions as the above mentioned compound (the α-hydroxy-α-amino acid residue of ergotamine) and cannot be considered as possibilities for X-2 of hydrolysates.

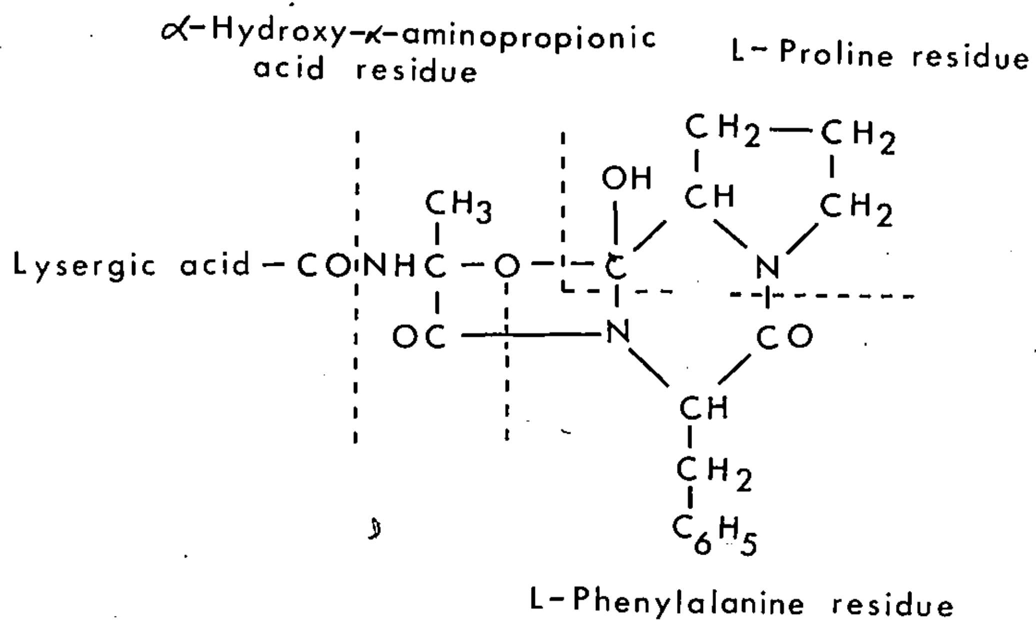
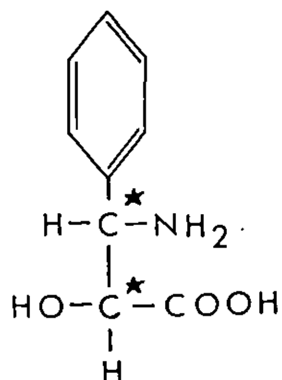
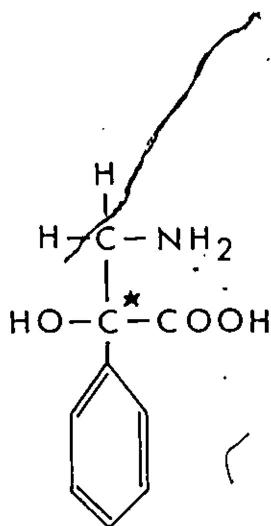


FIGURE 31

MOLECULAR STRUCTURE OF ERGOTAMINE



2-hydroxy-3-amino-3-phenylpropanoic acid



2-hydroxy-2-phenyl-3-aminopropanoic acid

FIGURE 32

AROMATIC HYDROXY β-AMINO ACIDS
OF THE MOLECULAR FORMULA $C_9H_{11}NO_3$

Note: C^* denotes an assymetric carbon.

Two further structures may be drawn (fig.32) which fulfil the requirements for X-2. These are 2-hydroxy-3-amino-2-phenylpropanoic acid and 2-hydroxy-2-phenyl-3-amino-propanoic acid. Both are β-amino acids. The former has two assymetric centers and thus would exist as a set of four optical isomers. The latter has only one assymetric center. A β-amino acid is generally less polar than its corresponding α-amino acid and this is reflected in their relative order of elution from amino acid analyzers. For example, β-alanine is eluted from the ion-exchange column of the analyzer well after alanine. The unknown X-2 is eluted from the analyzer before both α-phenylserine and the β-phenylserines, an indication that it is a more polar compound than these α-amino acids.

(iv) Amino Acid Composition of Myxosidin

Some amino acids, especially serine and threonine, are partially destroyed by acid hydrolysis. In order to establish a true ratio of these amino acids a timed series of hydrolyses of a peptide or protein are made. The data in Table 8 for the labile compounds were determined at an early stage in this investigation, before conditions were available for separating glycine and X-2 on the amino acid analyzer. It is evident that serine and hydroxyaspartic acid are released from the peptide extremely quickly. After 12 hours of hydrolysis at 110°C in 6 N HCl the ratio for these thermolabile components reached near integral values: Thr, Ser, Hya, (Gly + X-2) being 3:3:3:6 respectively.

The thermostable amino acids of Myxosidin are shown in Table 9. The data for arginine is incomplete. The ratios found for these components were: Thr, Arg, Val, Ile, and Leu 3:3:1:2:9 respectively. As pointed out previously, erythro-β-hydroxyaspartic acid and β-phenylserine were found to be

TABLE 8

AMINO ACID ANALYSES OF THERMOLABILE COMPONENTS OF MYXOSIDIN AFTER BRIEF PERIODS OF HYDROLYSIS

COMPONENT	COMPOSITION AFTER HYDROLYSIS FOR					
	15 min	1 hr	2 hr	3 hr	4 hr	12 hr
THREONINE	0.13	0.86	1.49	2.17	2.48	3.00
SERINE	0.99	2.37	2.64	3.03	2.95	3.00
HYDROXYASPARTIC ACID	0.79	2.28	2.64	2.97	2.99	3.08
GLYCINE + X-2	0.43	1.61	2.65	-	3.30	5.98

Figures are expressed as a ratio of threonine = 300 residues at 12 hr; the colour constants of hydroxyaspartic acid and X-2 were assumed to be equal to that of threonine; hydrolysis conditions were 6N HCl at 110°C

TABLE 9

AMINO ACID ANALYSES OF THERMOSTABLE COMPONENTS OF MYXOSIDIN UPON EXTENDED HYDROLYSIS

COMPONENT	COMPOSITION AFTER HYDROLYSIS FOR		
	24 hr	48 hr	72 hr
THREONINE	2.83	-	-
ARGININE	2.66	-	-
VALINE	0.85	1.20	0.91
ISOLEUCINE	1.92	2.02	1.72
LEUCINE	9.00	9.00	9.00

Figures are expressed as a ratio of leucine = 9.00 residues at 24 hr.

artefacts of the hydrolysis conditions. They were derived from *threo*-hydroxyaspartic acid and X-2, their forms as present in native Myxosidin.

It was concluded that Myxosidin had the following amino acid composition relative to valine, its least abundant residue: threonine (3), serine (3) glycine (3) valine (1), isoleucine (2), leucine (9), arginine (3), and *threo*-hydroxyaspartic acid + *erythro*-hydroxyaspartic acid (3), X-2, + β-phenylserine (3).

The above result is consistent with the hypotheses that Myxosidin is a single peptide of 30 residues with a ratio of leucine to valine of 9:1, or that it is a mixture of two kinds of decapeptide, one with a ratio of leucine to valine of 3:1, the other with a ratio of leucine to isoleucine of 3:1. If the isoleucine-containing peptide were designated Myxosidin A and the valine-containing peptide were designated Myxosidin B, then a ratio of two peptides of type A to one of type B would be consistent with the amino acid ratios of Myxosidin. The choice between decapeptides or 30-membered peptides can be made by a determination of molecular weight.

(c) Molecular Weight

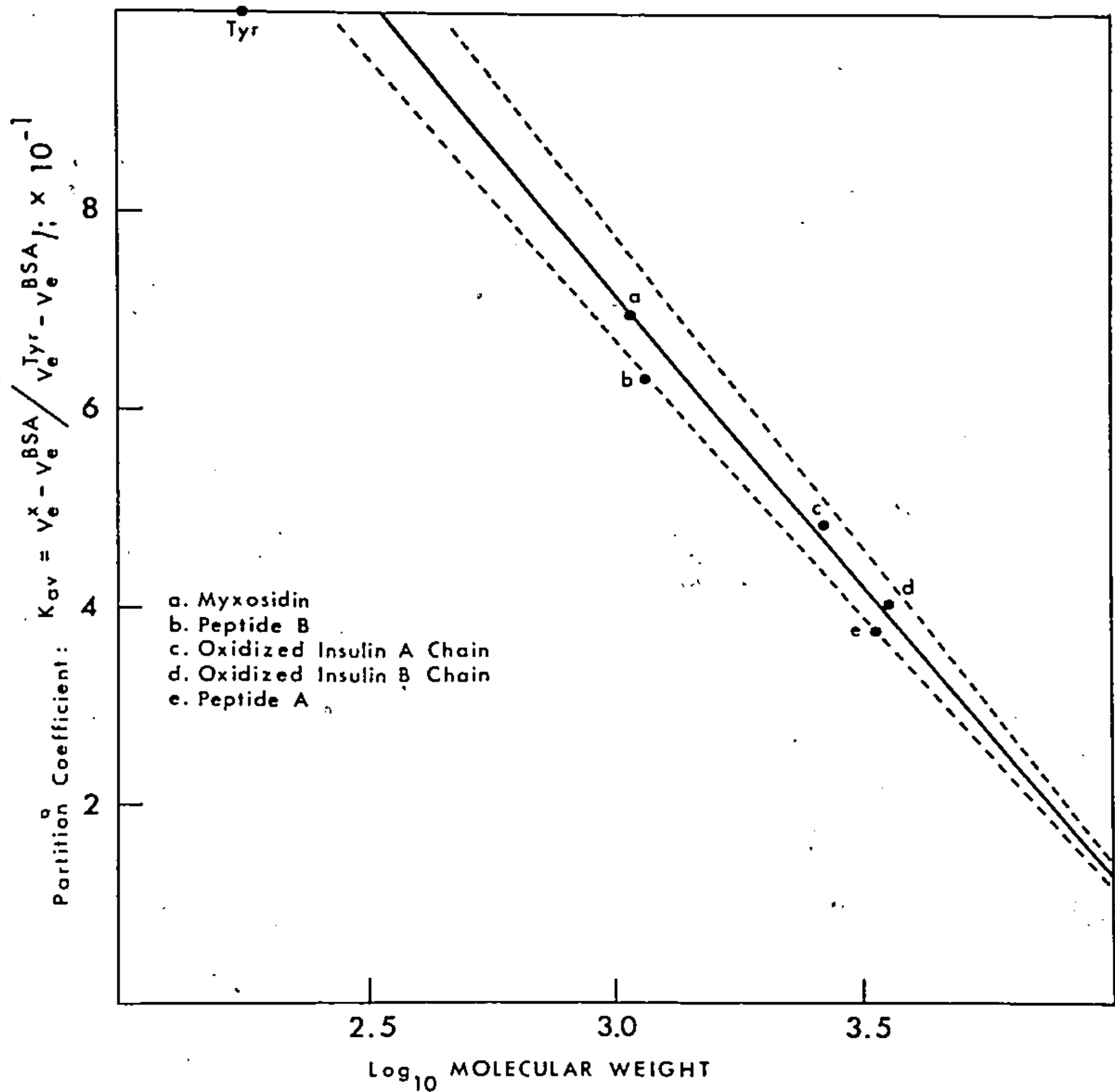
The molecular weight of Myxosidin was determined by gel filtration column chromatography and by high voltage paper electrophoresis.

The data for column chromatography of Myxosidin on

Sephadex G50 superfine gel are shown in fig.33. The partition coefficient, K_{av} was plotted against Log_{10} of the molecular weight for standards of Table 10 and for Myxosidin. A linear relationship was assumed and a straight line was drawn by eye through the experimental points. The dashed lines represent an approximate graphical interpretation of the error in measurements. The buffer used had a high concentration of guanidine-hydrochloride (4.5 M) in order to prevent adsorption of Myxosidin to the gel. The molecular weight found for Myxosidin by this technique was $1.07 \times 10^4 \pm 0.24 \times 10^3$ daltons. This is within the range expected for a decapeptide.

Table 11 gives estimates of molecular weights from electrophoretic data. The estimates were made from Offord's nomographs relating electrophoretic mobilities at pH 6.5 and 2.1 to the net charge and molecular weight of a peptide. The relationships are totally empirical and are based on the distribution of a large number of different peptides. (63) In the case of Myxosidin at pH 6.5, a net charge of +1 is assumed. The alkaline treated peptide was neutral at pH 6.5. Both forms were assumed to have a net charge of +1 at pH 2.1. The set of values derived for molecular weight based on these assumptions is consistent with the estimate from column chromatography. The significance of the net charges on Myxosidin is discussed in a later section.

It was apparent that the data for the molecular weight



ESTIMATION OF MOLECULAR WEIGHT OF MYXOSIDIN ON SEPHADEX G-50

FIGURE 33

TABLE 10

STANDARDS FOR DETERMINATION OF MYXOSIDIN'S MOLECULAR WEIGHT BY COLUMN CHROMATOGRAPHY

STANDARD	MOLECULAR WEIGHT (DALTONS)	RESIDUES
BOVINE SERUM ALBUMIN	67,000	
OXIDIZED BOVINE INSULIN B CHAIN	3573	30
β-LYTIC PEPTIDE A	3326	32
OXIDIZED BOVINE INSULIN A CHAIN	2684	21
β-LYTIC PEPTIDE B	1282	21
β-LYTIC PEPTIDE C	522	12
TYROSINE	181	1

β-Lytic peptides A,B and C were all derived from cyanogen bromide cleavage of the enzyme β-lytic protease.[42]

TABLE 11

MOLECULAR WEIGHT OF MYXOSIDIN

METHOD	MOLECULAR WEIGHT
COLUMN CHROMATOGRAPHY	1072 ± 235
HVE pH 6.5	1250 ± 150
HVE pH 2.1	856 ± 160
HVE pH 2.1 ALKALINE TREATED	1015 ± 174
CALCULATED FROM AMINO ACID COMPOSITION	1143

Column chromatography was on Sephadex G50 resin (see fig.33); HVE results are estimates based on the nomographs of Offord (63) and assume a net charge of +1 at pH 6.5 for the native peptide, +1 at pH 2.1, and +2 at pH 2.1 for the alkaline treated peptide.

of Myxosidin were consistent with the hypothesis that the antibiotic is a mixture of two kinds of decapeptide, possibly homologous in sequence. The amino acid compositions of the two are: Leu (3), Ser (1), Thr (1), Hya (1), Gly (1), Ile (1), X-2 (1), and Arg (1) for Myxosidin A; and Leu (3), Ser (1), Thr (1), Hya (1), Gly (1) Val (1) X-2 (1), and Arg (1) for Myxosidin B. The numbers in brackets indicate the number of moles of residue per mole of peptide. If two iso leucine-containing peptides, Myxosidin A, were present for each valine-containing peptide, Myxosidin B, then the leucine: isoleucine: valine ratio would be 9:2:1. This was the ratio found for acid hydrolysates of Myxosidin. The value of molecular weight calculated from amino acid composition given in table 9 uses 181 daltons as the molecular weight of the amino acid X-2 and is the number-average arithmetic mean of the molecular weights of the two forms of Myxosidin.

(d) N-Terminal Sequence by Edman Degradations

The result of three subtractive Edman cycles is illustrated in Table 12. After the first cycle, the leucine to valine ratio (9:1) had dropped to 6:1. After the second cycle the ratio had dropped to 3:1. Three or more cycles the Edman reactions did not lead to conclusive results. The third residue appeared to be resistant to further subtractive Edman degradation. The loss of three N-terminal leucines supports the hypothesis that Myxosidin is a mixture of two

TABLE 12

ETHOSIDIN: ANALYSES AFTER SUCCESSIVE EDMAN DEGRADATIONS

COMPONENT	COMPOSITION AFTER		
	1 cycle	2 cycles	3 cycles
HYA	3.2	2.8	2.7
THR	3.0	3.0	3.0
SER	2.6	2.4	2.3
GLY + X-2	6.2	5.5	5.2
VAL	0.82	0.86	0.90
ILE	2.0	1.7	1.7
LEU	6.2	3.1	2.7
ARG	-	-	2.8

Results are expressed as a ratio of the amount of threonine in each sample with threonine = 3.00 residues; the degradation was stopped after two cycles, the third and subsequent cycles were not conclusive.

decapeptides, the N-terminal sequence of both decapeptides being H -Leu-Leu.

The dansyl Edman method gave the same result as above. The N-terminal sequence of two leucyl residues was detected, but after the third and succeeding cycles, no further dansyl amino acid derivatives were noted.

As discussed previously, treatment of Myxosidin with cold barium hydroxide resulted in a product, designated "neutral Myxosidin" which gave hydrolysates with higher X-3 content (β-phenylserine) than native Myxosidin. Table 13 shows the results of three subtractive Edman cycles on this product. The analysis prior to treatment illustrates the high β-phenylserine: X-2 ratio. After the first two cycles, three residues of leucine were lost in each. At the third cycle all of the β-phenylserine was removed although X-2 was unaffected. This result showed that the third residue of the sequence was the aromatic amino acid. In the native peptide the third residue is resistant to Edman degradation. In neutral Myxosidin, the third residue is partially removed by the subtractive Edman procedure indicating an N-terminal sequence of H -Leu-Leu- X-2 (PheSer).

(e) C-Terminal Tritiation

The method of Matsuo et al.[64] was used in an attempt to determine the C-terminal residue of Myxosidin. In this

TABLE 13

ALKALINE-TREATED (NEUTRAL) MYKOSIDIN:
ANALYSES AFTER SUCCESSIVE EDMAN DEGRADATIONS

COMPONENT	COMPOSITION AFTER			
	0	1 cycle	2 cycles	3 cycles
HYA	2.9	3.5	2.0	2.0
THR	2.6	2.9	2.7	2.4
SER	2.8	2.5	2.9	2.5
X-2	2.1	2.7	2.0	1.8
GLY	3.0	3.0	3.0	3.0
PheSer	1.1	0.76	0.97	0
VAL	0.88	1.2	0.97	0.92
ILE	1.4	1.7	1.3	1.1
LEU	8.5	7.4	2.6	2.3
ARG	2.5	-	-	-

Results are expressed as a ratio of the amount of glycine in each sample with glycine = 3.00 residues. Samples were hydrolyzed in 6 N HCl at 110°C for 18 hours.

procedure the hydrogen atom of the α-carbon of the C-terminal residue is substituted with tritium. The peptide is hydrolyzed and the radioactively labeled amino acid is identified.

The hydrolysate was divided into two portions. One was treated with dansyl chloride and separated on polyamide sheets by ascending chromatography in chloroform-methanol-glacial acetic acid (95:5:1) as shown in fig. 34 . The zones were scraped from the plastic support and their radioactivity was measured by liquid scintillation counting (LSC). A second portion was fractionated by HVE at pH 6.5 which separated the amino acids into acidic (hydroxyaspartic acid), basic (arginine), and neutral zones. Hya and Arg could be eluted directly from the paper with 25% ethanol-2% acetic acid and counted by LSC.

The distribution of radioactivity found for the dansyl amino acids of native Myxosidin is shown in Table 14 A certain amount of non-specific labeling was expected[65] and each zone had some background radioactivity. The levels of the dansyl-hydroxyaspartic acid (Dns-Hya) and dansyl-arginine (Dns-Arg) zones are clearly well above the background. As can be seen from the chromatogram shown in fig. 34, the Dns-Arg zone is at the origin and Dns-Hya is only slightly ahead of it. There is, therefore, a possibility that the activity present in the arginine zone is due to tailing of the Dns-Hya zone or to partial retention of Dns-Hya in the Dns-Arg zone as an ion-pair between the

FIGURE 34

PREPARATIVE CHROMATOGRAM OF DANSYL DERIVATIVES OF C-TERMINAL TRITIUM-LABELLED MYXOSIDIN

The mixture was spotted on a polyamide thin layer and separated by ascending chromatography in solvent 2; zones numbered 1 through 14 were outlined and scraped from the plastic support for measurement of radioactivity.

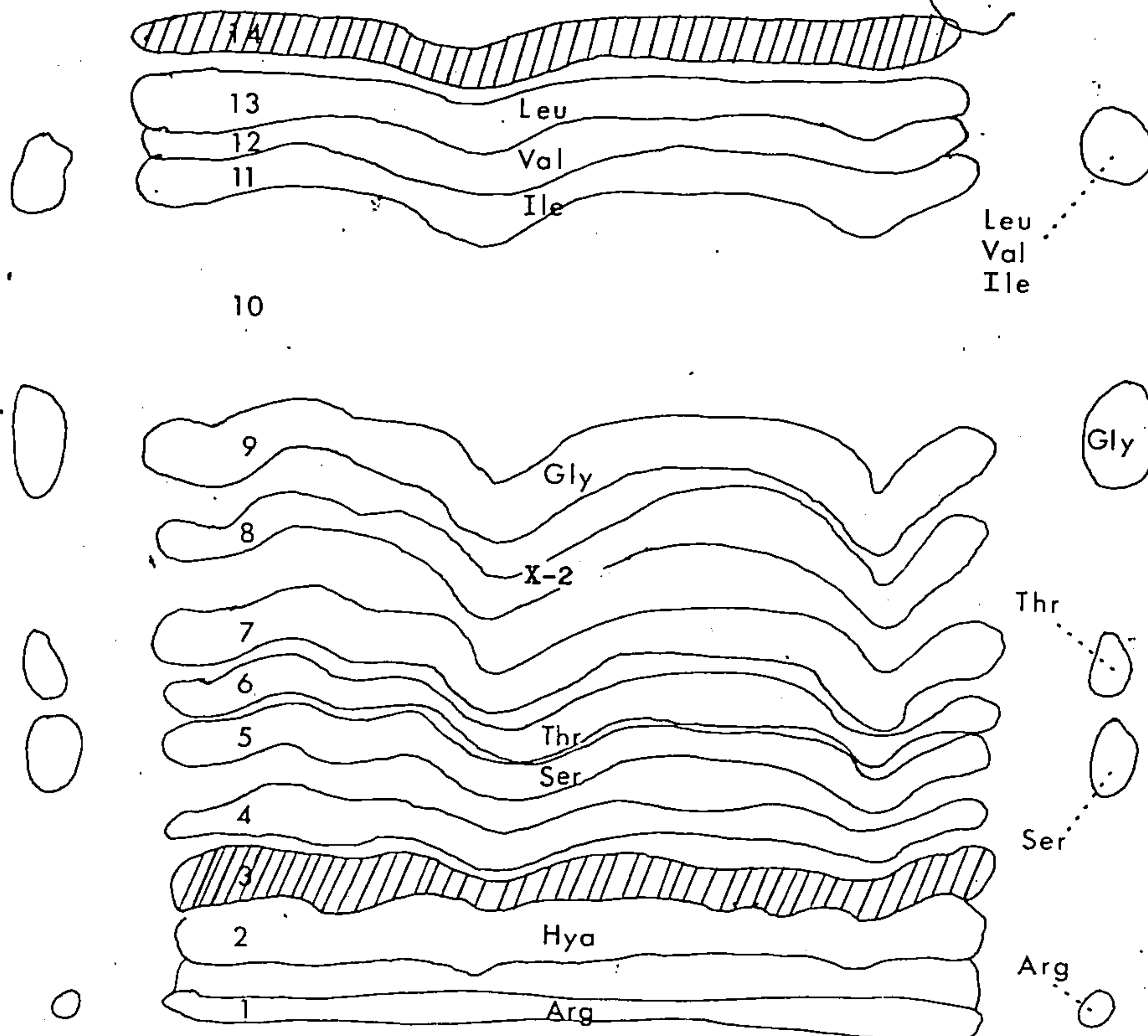


FIGURE 34

PREPARATIVE CHROMATOGRAM OF DANSYL DERIVATIVES OF C-TERMINAL TRITIUM LABELLED MYXOSIDIN

TABLE 14

DISTRIBUTION OF RADIOACTIVITY IN DANSYL DERIVATIVES OF C-TERMINAL LABELLED MYXOSIDIN

	ZONE	COUNTS cpm A	COUNTS cpm B	SCR (A/B)
1	Dns-Arg	679	1372	.495
2	Dns-Hya	900	1805	.498
3	Dns-OH	190	384	.494
4		223	447	.498
5	Dns-Ser	201	404	.497
6	Dns-Thr	166	326	.509
7		134	257	.519
8	Dns-X-2	186	344	.541
9	Dns-Gly	242	475	.509
10		376	752	.499
11	Dns-Ile	119	222	.536
12	Dns-Val	104	189	.549
13	Dns-Leu	99	181	.545
14	Dns-NH_2	99	188	.524
Blank		26	38	.693

Samples were counted for ten minutes each on an Isocap 300 liquid scintillation counter. Channel A was 0.5 to 3.6 keV lower and upper limits respectively; channel B was 0.5 to 18 keV lower and upper limits respectively. The approximate maximum counting efficiency for unquenched ^{3}H samples was 60% in channel B. Comparison of the arithmetic mean of the SCR for the above samples (0.515 $\pm$ 0.018) with a standard plot showed that these samples were counted at about 43% efficiency. Thus the presence of dansyl compounds caused considerable quenching of the tritium label.

extremely acidic dansyl-hydroxyaspartic acid and the strongly basic dansyl-arginine. The high voltage electrophoretic separation of Arg and Hya as free amino acids was aimed to eliminate this uncertainty. The results were as follows: the Hya zone was found to have 28.1 net cpm/nanomole; the Arg zone was found to have 4.0 net cpm/nanomole; that is, the activity of Arg was at the background level. The ratio of Hya to Arg activity was about 7:1. If the total radioactivity of the Dns-Arg and Dns-Hya zones of the dansyl derivatives is compared to the background levels of the neutral amino acids it is found to be in approximately a ratio of 7:1.

Thus the only residue labeled in native Myxosidin was hydroxyaspartic acid. However, dicarboxylic acids such as aspartic and glutamic acids are known to be labeled by this technique whether or not they are C-terminal.[65] The evidence presented in the next section on sequence showed that Hya-Ser and Gly-Hya-Ser peptides could be isolated from Myxosidin digests. Clearly Hya is not a C-terminal residue, thus no residue could be identified as C-terminal in native Myxosidin by this technique.

(f) Peptides Obtained from Digests of Myxosidin

Several attempts were made to digest Myxosidin with proteolytic enzymes. The enzymes employed were: pepsin (Nutritional Biochem.), 70 μg/ml in 2% formic acid, pH 2.10: β-lytic protease, 70 μg/ml in 0.1 M pyridine acetate buffer, pH 6.4; pronase (Cal. Biochem.), 70 μg/ml in 0.1 M pyridine

acetate buffer, pH 6.91; thermolysin (Cal. Biochem.), 70 μg/ml in 0.1 M pyridine acetate-0.002 M $CaCl_2$, pH 6.89; papain (Nutritional Biochem.), 61μg/ml in 0.05 M Tris-0.005 M mercaptoethanol-0.002 M EDTA-0.005 cysteine-HCl, pH 8.00; leucine amino peptidase (Worthington), 75 μg/ml in 0.05 M Tris, pH 7.50; carboxypeptidases A and B (Sigma), 50 μg/ml of A, 75 μg/ml of B, in 0.05 M Tris, pH 7.50. The substrate, Myxosidin, was incubated for 2 hours in the above enzyme solutions. The antibiotic was found to be very resistant to enzymic attack as evidenced by retention of antibiotic activity and by the lack of other peptides or free amino acids when checked by HVE at pH 6.5 and pH 2.1.

Peptide antibiotics are often found to be resistant to the enzymes used for sequencing.[66] The approach to sequencing such bacterial peptides is to produce peptide fragments by means of various chemical digests. Three such procedures were used to digest Myxosidin. These were: (a) brief acid hydrolysis in 6 N HCl for 5 min at 100°C; (b) cold alkaline hydrolysis in 0.327 M $Ba(OH)_2$ for 60 hours at 0°C; and (c) mild acid hydrolysis in 0.03 N for 24 hours at 110°C. These methods and the peptides obtained from each are shown in Table 15. In general, such chemical digests are less specific than enzymic digests. The peptide is cleaved into many different fragments with a low abundance of each type. An exception is dilute acid hydrolysis which can be very specific for cleavage at aspartic acid residues.[67]

TABLE 15

PEPTIDES FROM CHEMICAL DIGESTS OF MYXOSIDIN

TREATMENTS:				
	(a)	6 N HCl	100°C	5 min
	(b)	0.327 M $Ba(OH)_2$	0°C	60 hours
	(c)	0.03 N HCl	110°C	24 hours

PEPTIDE	SEQUENCE
a-1 and c-1	$\overrightarrow{\text{Hya}}$-$\overrightarrow{\text{Ser}}$ 1.0 1.0
a-2	$\overrightarrow{\text{Ile}}$-$\overrightarrow{\text{Thr}}$-$\overrightarrow{\text{Gly}}$ 0.7 0.9 1.0
a-3	$\overrightarrow{\text{Val}}$-$\overrightarrow{\text{Thr}}$-$\overrightarrow{\text{Gly}}$ 0.6 0.9 1.0
a-4	$\overrightarrow{\text{Ser}}$-$\overrightarrow{\text{Leu}}$ 1.0 1.0
b-1	$\overrightarrow{\text{Gly}}$-Hya-Ser 1.0 0.8 1.0
c-2	$\overrightarrow{\text{Leu}}$-$\overrightarrow{\text{Leu}}$-(X-2, $\binom{\text{Ile}}{\text{Val}}$, Thr, Gly, Leu, Arg)
c-3	$\overrightarrow{\text{Leu}}$-$\overrightarrow{\text{Leu}}$-(X-2, $\binom{\text{Ile}}{\text{Val}}$, Thr, Gly, Arg)
Model:	Leu-Leu- X-2-$\binom{\text{Ile}}{\text{Val}}$-Thr-Gly-Hya-Ser-Leu-Arg

Arrows denote sequence from N-terminus by Edman degradation; subscript numbers are molar ratios. The ratios of peptides C-2 and C-3 are explained in the text.

As shown in Table 15, brief acid hydrolysates yielded the two neutral tripeptides, a-2 and a-3, Ile-Thr-Gly and Val-Thr-Gly respectively. These peptides complete the evidence that Myxosidin is a mixture of two homologous decapeptides with one valine and one isoleucine in corresponding locations in their sequences. Although the above pair of peptides could be separated by descending BAWP chromatography, the two types of Myxosidin, A (with Ile) and B (with Val) would differ by only one CH_2 group and are very likely to be virtually inseparable. The neutral peptide, a-4, seryl-leucine, was also recovered from brief acid hydrolysates. The acidic dipeptide hydroxyaspartyl-serine (a-1 or c-1) was recovered from both brief acid hydrolysates and from dilute acid hydrolysates. The acidic tripeptide from cold alkaline treatments, b-1, was found to be Gly-Hya-Ser. These peptides establish the sequence of Myxosidin through six residues as Ile-Thr-Gly-Hya-Ser-Leu and Val-Thr-Gly-Hya-Ser-Leu.

The dilute acid hydrolysis of Myxosidin led to a very specific cleavage compared to brief acid treatments in 6 N HCl. The hydrolysate was fractionated first by HVE at pH 6.5 into four zones: two basic zones, a neutral zone, and an acidic zone. Each of these was rerun at pH 2.1 and except for the neutral zone each found to be homogenous. The acidic zone contained the dipeptide Hya-Ser which was removed from Myxosidin quite selectively.

A study of the time course of this reaction showed that at 24 hours 95.7% of the total hydroxyaspartic acid and 93.7% of the total serine was released from Myxosidin. At 36 hours 97.5% of the Hya and 100.8% of the serine was released. Some free glycine and free threonine appeared at 36 hours. The neutral zone of preparative scale dilute acid hydrolysates was found to contain small amounts of free glycine and threonine.

Two large basic peptides were recovered from dilute acid digests, peptides c-2 and c-3. Peptide c-2 had an HVE mobility with respect to arginine of 0.61. Following acid hydrolysis its composition was found to be: Leu(5.9), X-2 (1.7), Ile(1.7), Val(0.9), Thr(2.3), Gly(3.0), Arg(3.4), when expressed in terms of glycine= 3.0 moles of residue. Thus one leucine and the dipeptide Hya-Ser were missing from the sequence. About one third of the X-2 residues appeared as PheSer in hydrolysates of this peptide. Peptide c-3 had an HVE mobility with respect to arginine of 0.40. Its amino acid composition was found to be: Leu(10.1), X-2 (3.0), Ile(2.0), Val(1.1), Thr(2.9), Gly(3.0), Arg(3.5). This was equivalent to Myxosidin missing the Hya-Ser dipeptide. Very little PheSer was found in hydrolysates of this basic peptide. As with the native peptide subtractive Edman degradations of these peptides stopped after two cycles and indicated N-terminal Leu-Leu sequences.

Peptides c-2 and c-3, lacking the dipeptide Hya-Ser, suggest that Myxosidin has a cyclic structure in which the hydroxyl group of either X-2 or Thr is esterified by the carboxyl group of either Leu or Arg.

The lack of readily separable small basic peptides in the various chemical digests of Myxosidin leaves the assignment of arginine to the sequence ambiguous--it could be at residue 4, giving the sequence H-Leu-Leu-X-2 -Arg-Ile(Val)-Thr-Gly-Hya-Ser-Leu with leucine in position ten forming a lactone ring, or alternatively, the arginine residue could be at position ten of the sequence, H-Leu-Leu- X-2 -Ile(Val)-Thr-Gly-Hya-Ser-Leu-Arg- , with the carboxyl group of Arg in a lactone ring. The ambiguity might have been resolved by identification of the C-terminal residue of the "neutral Myxosidin", obtained by treatment of Myxosidin with cold alkali. However, a C-terminal tritiation of "neutral Myxosidin" failed to give a conclusive result--neither arginine nor leucine were labelled above the rather high level of non-specific labelling of other components. It should be noted that C-terminal amino acids vary in their relative reactivities towards C-terminal tritium labelling. Arginine is an amino acid which is labelled less efficiently than most other C-terminal residues. The ambiguity regarding the arginine location was resolved by mass spectroscopy.

The IR spectrum of Myxosidin, a portion of which is shown in the appendix, has an absorbtion peak at 1750 cm^{-1}, indicating a lactone carbonyl.

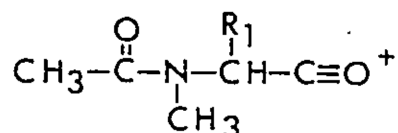
(g) Mass Spectroscopy of Myxosidin Derivatives

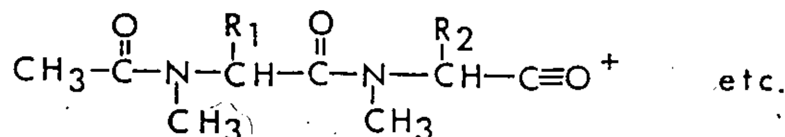
Myxosidin was converted to derivatives suitable for mass spectroscopy by a sequence of three reactions. The first was N-acylation to either the N-acetyl or N-trifluoroacetyl derivative. In the case of Myxosidin the N-terminal leucine residue is the only target for this reaction. The second reaction was permethylation. The technique used in peptide chemistry is based on a reaction from carbohydrate chemistry, developed by Hakamori,[68] to methylate hydroxyl groups. As applied to peptides, the reaction results in all hydrogens of the peptide bonds being replaced by methyls, all hydroxyls, carboxyls, amides, and other reactive side-chain groups, being methylated. Arginine-containing peptides such as Myxosidin, tend to be insufficiently volatile for a good mass spectrum even after permethylation. A third reaction was used in order to improve the spectrum of Myxosidin. Prior to the above two reactions a sample of the peptide was reacted with hydrazine to convert arginyl residues to ornithyl residues.

The goal of these reactions is to improve the volatility of peptides. N-acylation of amino groups prevents the formation of quaternary amonium salts. The permethylation reaction is believed to reduce the strength and number of interchain, non-covalent bonds between molecules of the derivative.[69] The first step of the permethylation reaction

is production of an extremely powerful base, CH_3-SO-CH_2^-, by mixing NaH and dimethylsulfoxide. Any hydrogens on a peptide with a slightly acidic nature will be removed by this base. The second step is reaction with the methylating reagent, methyl iodide, which forms N-methyl, O-methyl, and O-methyl ester bonds with the peptide. The resulting permethyl-peptide is much more volatile than a native peptide.

Permethylated peptides tend to fragment into a series of ions known as sequence ions. These ions are of the following general type:

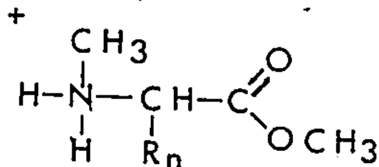


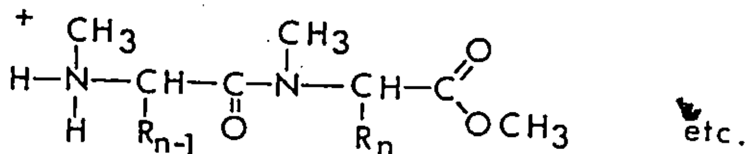


etc.

The mass difference between each successive ion is characteristic of a particular amino acid residue. The N-terminal residue is seen at the low mass end of the spectrum. Cleavages within the amino acid side chain may occur, resulting in non-sequence ions, which are difficult to interpret. Occassionally charge is retained on the nitrogen side of the permethylated bond resulting in a

sequence of ions with the C-terminal residue at the low mass range:





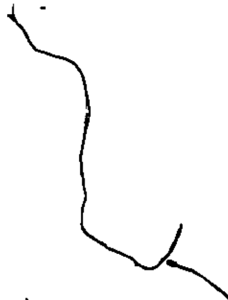
etc.

The spectrum of N-acetylated, permethylated Myxosidin is shown in fig. 35. The N-terminal amino acid residue was N-acetylleucine at 170 mass units. The loss of 28 mass units (CO) from the N-terminal residue was observed, m/e 142. The peak at 297 represented an N-terminal sequence of Leu-Leu. The peak at 488 mass units indicated that the third residue had a mass of 191 units.

The third residue appeared to be "X-2". The peak at 456, a loss of CH_3OH, indicated that the third residue contained an hydroxyl group in its side-chain. Elimination of methanol is observed for tyrosine, threonine and serine.[70] The peak at 581, a mass difference of 93

FIGURE 35

MASS SPECTRUM OF N-ACETYLATED, PERMETHYLATED MYXOSIDIN



100
AcLeu
Leu
80
60
40
20
Relative Abundance %
142
170
297
100
200
300
m/e

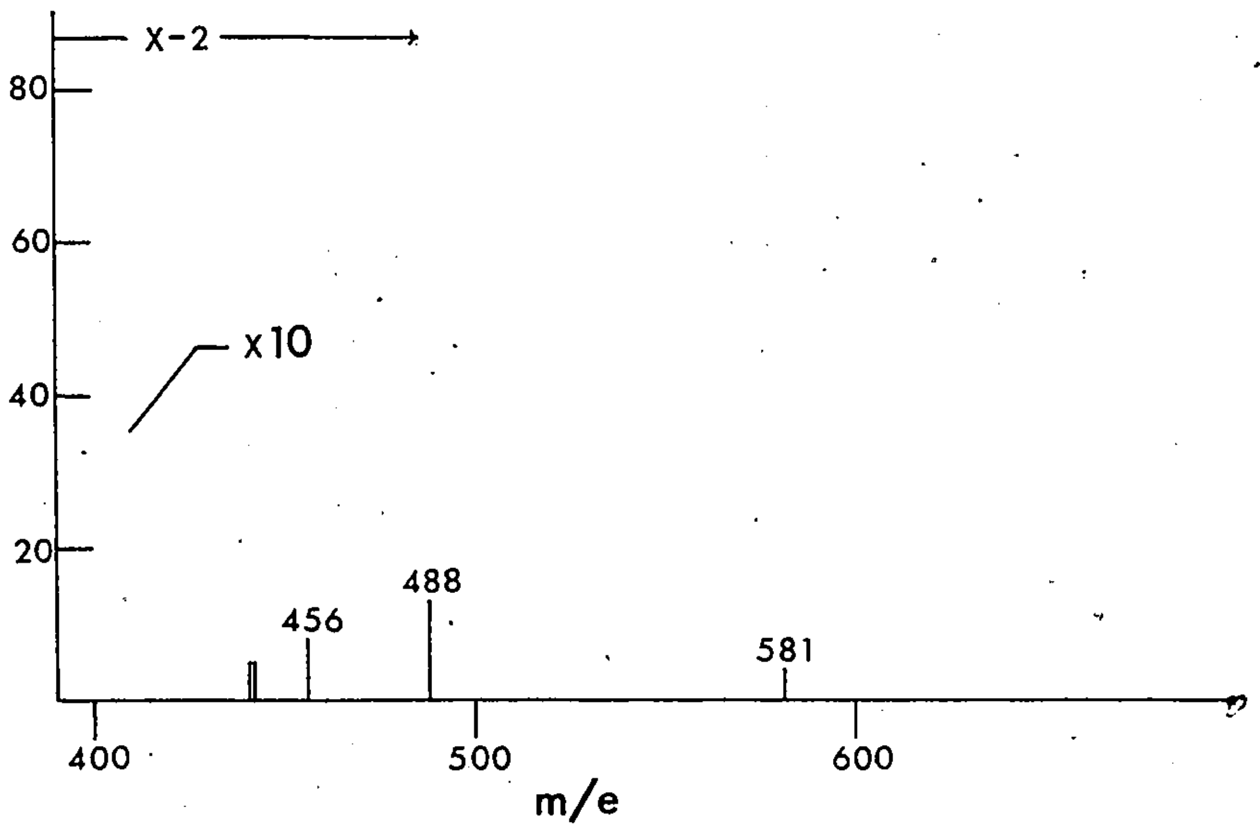
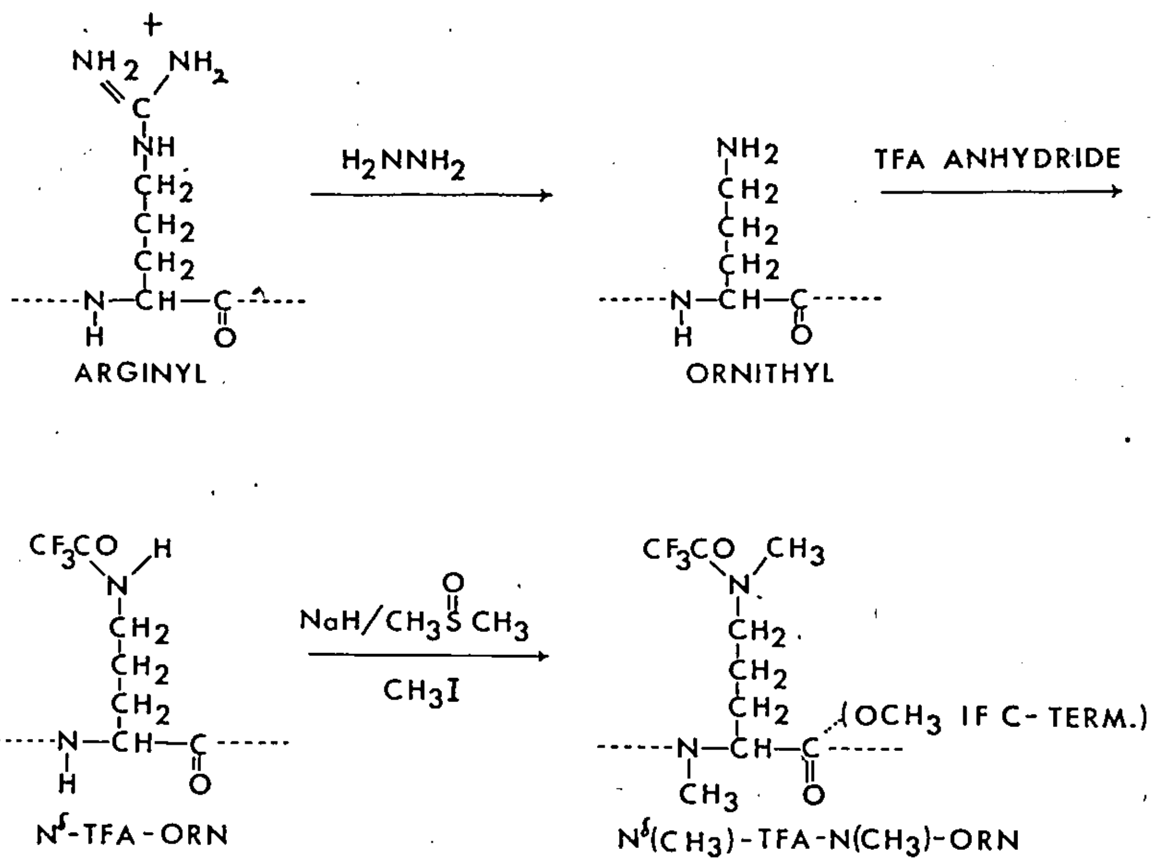


FIGURE 35

units, does not correspond to a complete amino acid residue, but must arise from fragmentation within the side-chain of the fourth residue of the sequence (note that glycine has an expected mass difference of 71 units and even if "over-methylated", or C-methylated to form alanine its mass difference would be 83 units). It was concluded that the N-terminal sequence of Myxosidin was Leu-Leu-"X-2", and that "X-2" was an amino acid, aromatic, with an hydroxyl group, and with a molecular weight of 181. The molecular weight was arrived at by deducting 30 units (two methyl groups) from the mass of the third residue, and adding 20 units (two hydrogens on the nitrogen atom, one hydrogen to form the hydroxyl, and one OH group to complete the carboxyl) for the molecular weight of a free amino acid.

An improved mass spectrum of Myxosidin was obtained by treatment of the peptide with hydrazine to convert arginine residues to ornithine. The ornithyl-Myxosidin derivative was treated with trifluoroacetic acid anhydride in order to acylate the N-terminal amino of the peptide. The δ-amino group of ornithine was trifluoroacetylated at the same time. Finally, the peptide derivative was permethylated. The fate of the original arginyl residues is illustrated in the following scheme of reactions:



DERIVATIZATION OF ARG PEPTIDES FOR MS

The mass spectrum obtained for this derivative is shown in fig. 36. The N-terminal leucine appeared as N-trifluoroacetyl leucine at 224 mass units. The loss of 28 units from 224 (peak at 196) represented elimination of CO from the N-terminal residue. The peak at 351 indicated the sequence Leu-Leu. The peak at 542 represented "X-2" in the third position, as before, with a loss of 32 units indicating an hydroxyl as a functional group. The peaks at 653 and 667 represented valine and either leucine or isoleucine. In view of the evidence of tripeptides with the sequence Val-Thr-Gly and Ile-Thr-Gly, it seemed reasonable to assign the N-terminal sequence of H -Leu-Leu-"X-2"-Ile and H -Leu-Leu-"X-2"-Val on the basis of this mass spectrum. The composition of this series of ions is indicated schematically in fig. 37.

The spectrum also contained evidence of a C-terminal sequence beginning with the peak at 180 mass units. The series of peaks at 180, 307, 422, are consistent with the sequence Ser-Leu-Orn (-91). A reaction scheme illustrating this fragmentation pattern is shown in fig. 38.

Thus a complete model of the sequence of Myxosidin A and B may be proposed:

H -Leu-Leu-X-2 -Ile-Thr-Gly-Hya-Ser-Leu-Arg- for Myxosidin A; and H -Leu-Leu- X-2 -Val-Thr-Gly-Hya-Ser-Leu-Arg- for Myxosidin B.

FIGURE 36

MASS SPECTRUM OF N-TRIFLUOROACETYLATED, PERMETHYLATED, HYDRAZINOLYZED MYXOSIDIN

100 196 160
AcLeu Leu
121
351
224
180
307
Relative Abundance %
80 60 40 20
100 200 300
m/e

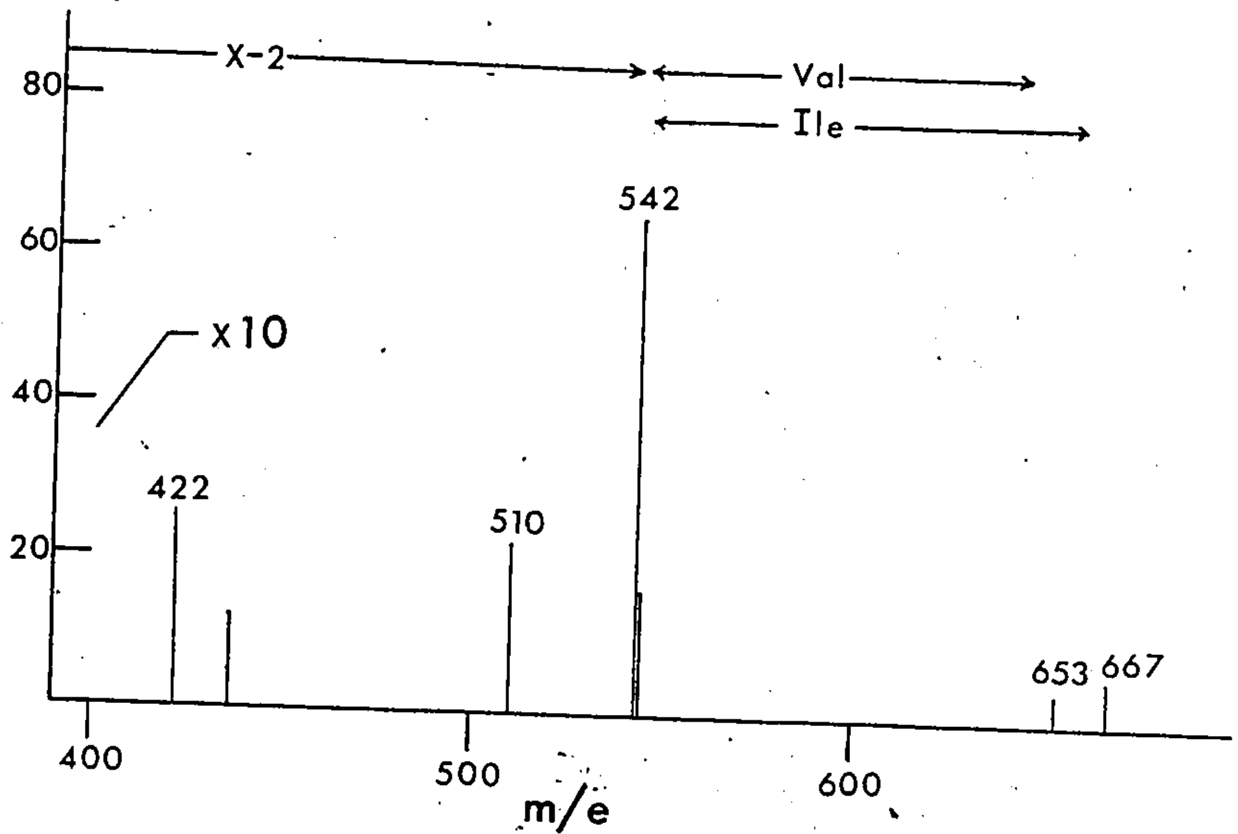


FIGURE 36

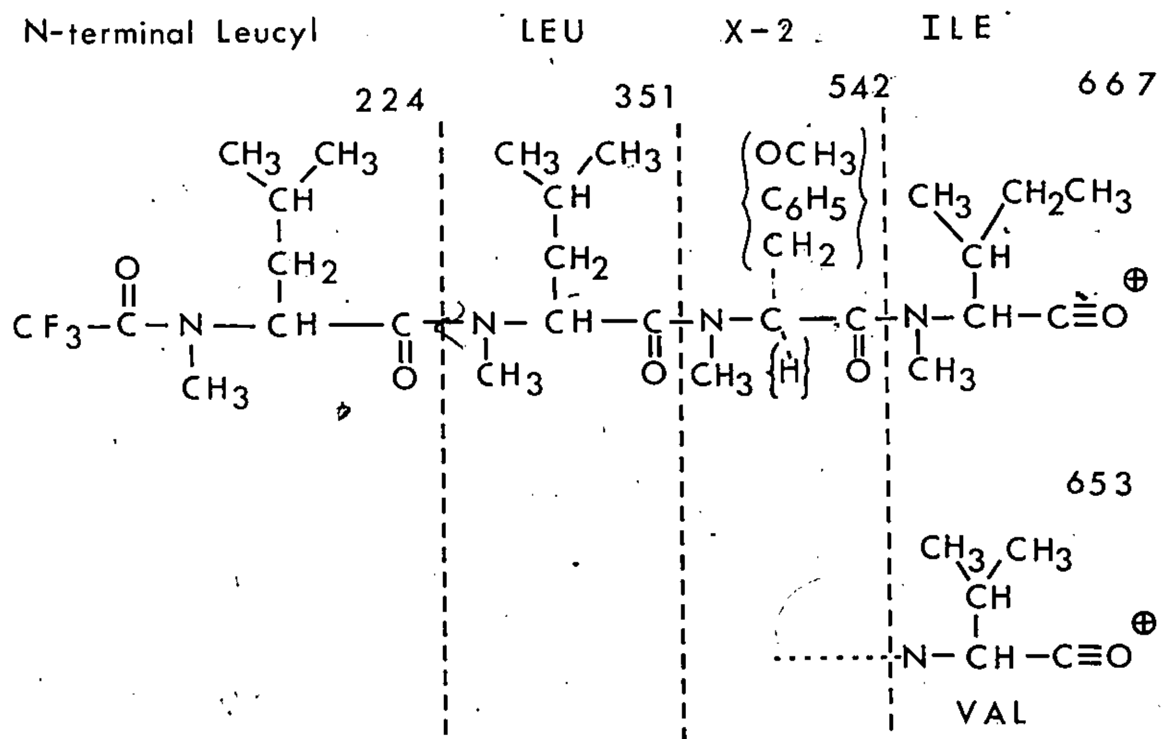


FIGURE 37

N-TERMINAL FRAGMENTATION OF N-TFA, PERMETHYLATED HYDRAZINOLYZED MYXOSIDIN

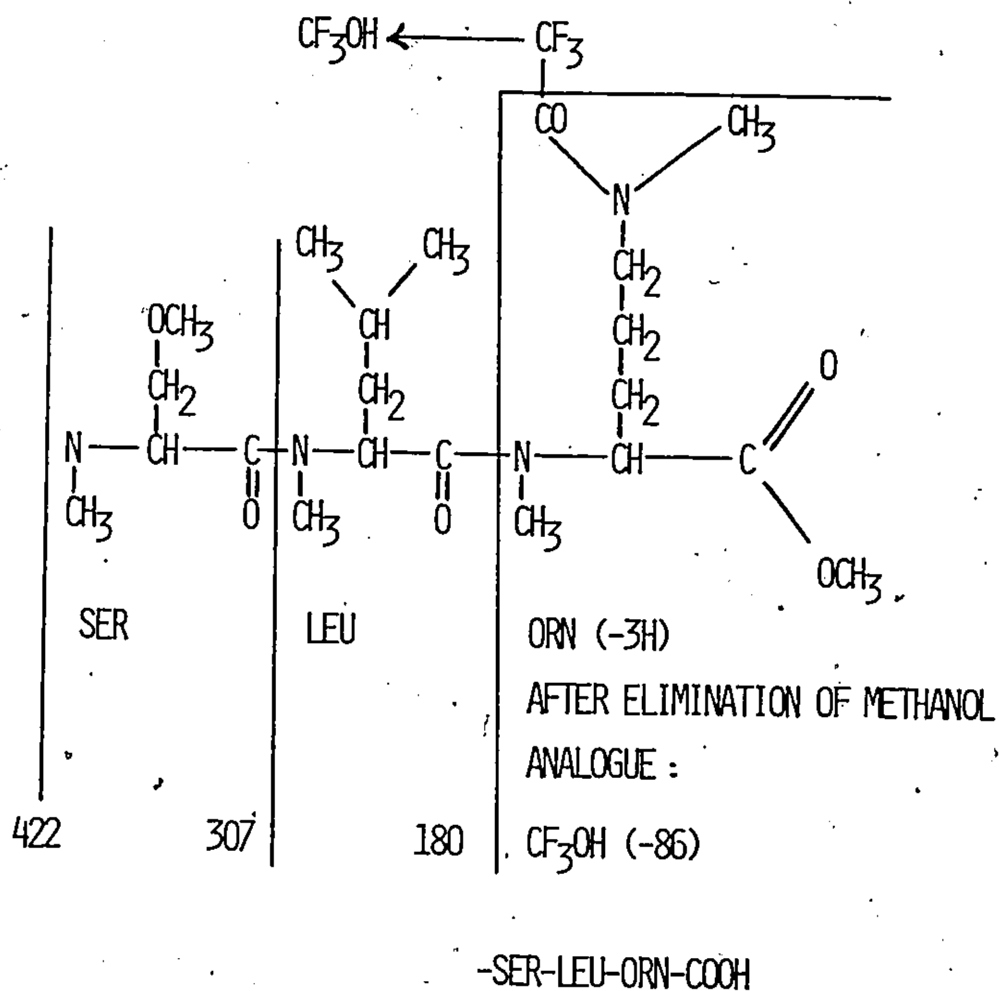


FIGURE 38

C-TERMINAL FRAGMENTATION OF N-TFA, PERMETHYLATED, HYDRAZINOLYZED MYXOSIDIN

(h) Chromic Acid Oxidation of Myxosidin

Myxosidin was oxidized with chromium trioxide following the method employed by Sheehan et al.[71] Amino acid residues with hydroxyl groups involved in ester links survive chromic acid oxidation, whereas amino acid residues with free hydroxyls are destroyed. By this technique it should be possible to identify the residue among the four hydroxy-amino acids of Myxosidin which is linked by an ester bond to the C-terminal carboxyl of the peptide.

Ten milligrams of Myxosidin was dissolved in 0.30 ml of glacial acetic acid containing 10 mg of CrO_3 and 10 μl of pyridine. After standing overnight at room temperature 5.0 ml of absolute ethanol was added and the mixture was evaporated to dryness, redissolved in 6 N HCl, hydrolyzed and analyzed for free amino acids.

The result is shown in Table 16. The chromium trioxide treatment destroyed hydroxyaspartic acid, threonine, serine, and X-2. The only hydroxyl-containing amino acid which survived was X-3 (β-phenylserine). This may indicate that the aromatic residue X-2 is protected in the native peptide. Some of the other amino acids were also affected by the oxidation.

TABLE 16

CHROMIC ACID OXIDATION OF MYXOSIDIN

AMINO ACID	BEFORE OXIDATION (nanomoles/250μl)	AFTER OXIDATION (nanomoles/250μl)
t-Hya	77.9	Trace
Thr	80.2	Trace
Ser	81.4	Trace
X-2	71.1	Trace
Gly	86.4	67.3
PheSer	12.7	18.8
Val	19.7	22.7
Ile	60.4	49.3
Leu	278	167
Arg	ND	ND

The figures above are given in nanomoles/250 μl sample of a hydrolysate of the native peptide (24hrs): "Before Oxidation", and of an hydrolysate of the chromic acid oxidized peptide: "After Oxidation". "Trace" means less than 1 nanomole/250μl. Arginine was not determined (ND). The only hydroxyl containing amino acid to survive the oxidation was β-phenylserine which may imply that X-2 is the residue involved in an ester link with the C-terminal.

(i) Determination of D and L-Amino Acids

The amino acids of hydrolysates of Myxosidin were reacted with L-leucine N-carboxyanhydride (L-Leu NCA) to form L-leucyl dipeptide derivatives. These were compared with standard L-leucyl dipeptides on the amino acid analyzer.

The following L-leucyl dipeptides were identified after reaction of L-Leu NCA with hydrolysates of Myxosidin: L-Leu-L-Thr at 159 ml, L-Leu-L-Ser at 172 ml, L-Leu-L-Val at 294 ml, L-Leu-L-Leu at 350 ml, L-Leu-D-Leu at 394 ml. Some of the L-Leu-L-Leu dipeptide may have arisen from the L-Leu NCA reagent itself, but the presence of both D and L-leucines in Myxosidin was confirmed by oxidations with amino acid oxidases. A sample eluted in 0.32 N sodium citrate buffer showed the presence of both L-Leu-L-Arg and L-Leu-D-Arg.

Table 17 illustrates the result of enzymic digests of an acid hydrolysate of Myxosidin with L-amino acid oxidase and D-amino acid oxidase. The stereochemical assignments which may be inferred from these data are: threo-L-hydroxy-aspartic acid; L-isoleucine; two moles of L-leucine for each mole of D-leucine. No conclusion was reached for the Arg or OHPhe residues. The positions of the D and L-leucines have not yet been determined. The stereoassignments of the amino acids of Myxosidin may be summarized as: threo-L-Hya; L-Thr; L-Ser; (?)- X-2 ; Gly; L-Val; L-Ile; two L-Leu and one D-Leu; (?)-Arg.

TABLE 17

OXIDATION OF AMINO ACID COMPONENTS OF MYXOSIDIN WITH LAO AND DAO

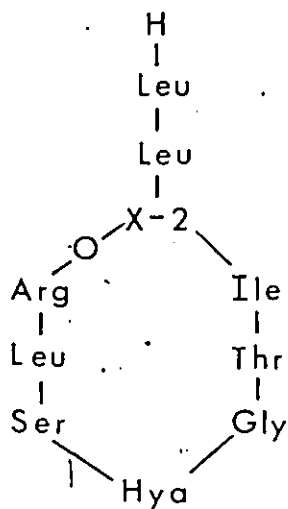
Amino Acid	Before Oxidation	LAO		DAO		Assignments
		18 hr	48 hr	18 hr	48 hr	
HYA	2.9	1.8	0	3.0	2.6	L
THR	2.8	2.4	2.3	3.2	2.3	L
SER	2.8	2.6	2.2	2.5	2.3	L
X-2	3.3	2.5	2.2	2.6	2.6	No conclusion
GLY	3.0	3.0	3.0	3.0	3.0	
VAL	0.9	0.7	0.6	1.1	1.0	L
ILE	1.9	0	0	2.4	2.1	L
LEU	9.0	3.6	3.4	6.9	5.7	Two L; one D
ARG	3.1	2.3	ND	2.5	ND	No conclusion

Results are expressed as ratios with respect to glycine = 3.00 residues; samples were hydrolyzed for 24 hours in 6 N HCl at 110°C; enzyme incubations were for 18 and 48 hours; results for arginine are incomplete (ND). Assignments for Thr, Ser, and Val are based on the data obtained for their dipeptides (see text).

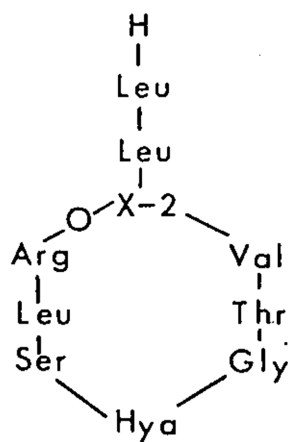
E. DISCUSSION

The antibiotic substance purified from filtrates of Myxobacter 495 was shown to be a mixture of two cyclic peptides: Myxosidin A and Myxosidin B. These differed in the nature of the residue at position four of their sequences, Ile in A and Val in B. Two peptides of Myxosidin A were present for each one of Myxosidin B. The sequence of Myxosidin was determined by a combination of methods, including subtractive Edman degradations, chemical digests, and mass spectroscopy. Two unusual amino acids were detected in hydrolysates; X-2 at position three, and threo-hydroxyaspartic acid at position seven. Consideration of the chromic acid oxidation result, peptides isolated from chemical digests, saponification with cold alkali, the net charge of the peptide at pH 6.5, and the lack of a free C-terminal led to the conclusion that Myxosidin contains an ester link between the carboxyl group of arginine in position ten and the hydroxyl of X-2 in position three of the sequence. A diagram illustrating this model of the structure of Myxosidin is shown in fig. 39.

A schematic representation of the ionic states of Myxosidin during electrophoresis at various pH values is illustrated in fig. 40. At pH 6.5 Myxosidin migrates as a basic peptide with a net charge of + 1. At pH 2.1 the net charge is approximately + 2, an estimate based



Myxosidin A



Myxosidin B

FIGURE 39

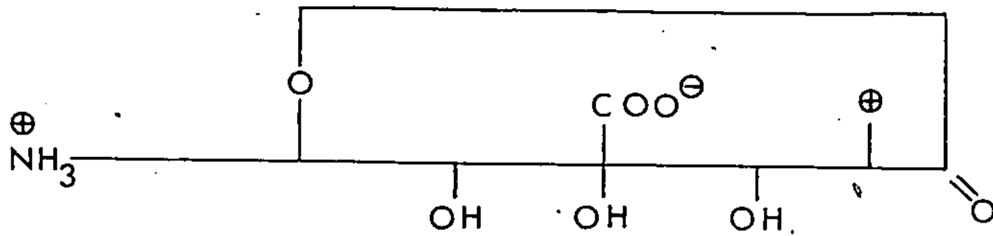
PROPOSED MOLECULAR STRUCTURE OF MYXOSIDIN

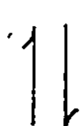
As isolated from culture filtrates of Myxobacter 495, Myxosidin is a mixture of two moles of the A-type for each mole of the B-type illustrated above.

FIGURE 40

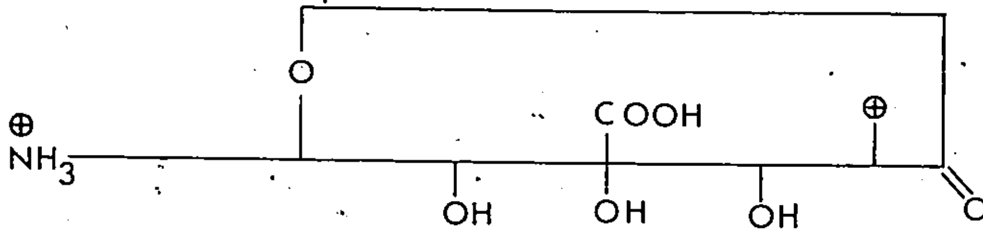
IONIC STATES OF MYXOSIDIN

A. Myxosidin at pH 6.5, ϵ = +1

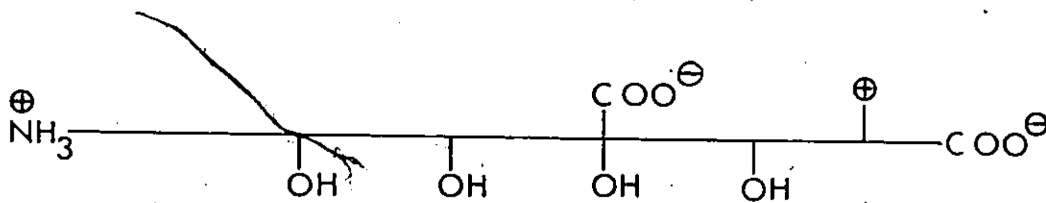


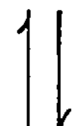


B. Myxosidin at pH 2.1, ϵ = +2

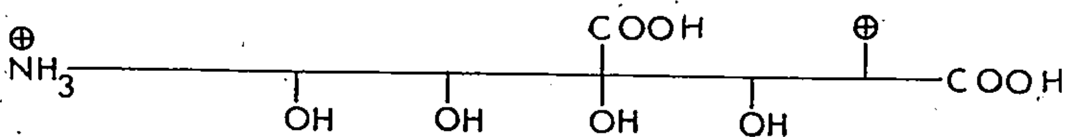


C. Alkaline-treated Myxosidin at pH 6.5, ϵ = 0





D. Alkaline-treated Myxosidin at pH 2.1, ϵ = +2



on Offord's nomographs.[63] This equilibrium is illustrated as parts A and B of the figure. Parts C and D are an interpretation of the electrophoretic data for alkaline-treated Myxosidin. This form of the peptide was neutral at pH 6.5 and basic at pH 2.1. The observed electrophoretic behaviour of Myxosidin is consistent with the proposed model of its structure.

The presence of two unusual amino acid residues in Myxosidin was determined by spectroscopy and by analysis of Myxosidin hydrolysates. An unidentified aromatic residue in the third position, X-2, created a problem in the sequencing of Myxosidin. Dansyl Edman and subtractive Edman reactions were stopped after two cycles. It appeared as though some property of the third residue was responsible. Evidence from the mass spectrum of a Myxosidin derivative indicated the presence of a hydroxyl group in X-2. Chromic acid oxidation indicated that the aromatic residue was spared to some extent. The presence of a lactone bond involving the C-terminal carboxyl of the peptide and the hydroxyl of the X-2 residue could account for failure of the Edman degradation. It was found that "neutral" Myxosidin, failed to undergo Edman degradation at the third cycle.

The amino acid L-threo-β-hydroxyaspartic acid was also identified in hydrolysates of Myxosidin. Hydroxyaspartic acid has been reported as a component of bacterial peptides.[72] It also appears in the toxic mushroom peptide, phalloidin, as the D-erythro-β-hydroxyaspartic acid diastereomer.[73] In this connection it is interesting to note that Bodanszky and Perlman have proposed a "rule of Epimerization" for amino acid residues of bacterial peptides.[66] According to these authors the D-amino acid residues of bacterial peptides arise as the result of an epimerase reaction at the α-carbon. Supporting evidence comes from a survey of 225 peptides of bacterial origin. The authors found no examples of D-isoleucine or D-threonine (except for D-threonine in telomycin, possibly a mistaken assignment). On the other hand, examples of D-alloisoleucine and D-allothreonine were identified. The allo compounds arise as the result of inversion at the α-carbon. Formation of D-threonine would require inversion at both the α-carbon and the β-carbon. The corresponding amino acid for the residue observed in Myxosidin, assuming epimerization at the α-carbon is D-erythro-β-hydroxyaspartic acid. Thus both members of this pair have been observed in natural products, but neither member of the pair: L-erythro-β-hydroxyaspartic acid and D-threo-β-hydroxyaspartic acid have been observed in peptides.

Myxosidin may be described as an antibiotic decapeptide with an eight-membered lactone ring--where eight-membered means eight amino acid residues (the actual nember of atoms in the lactone of Myxosidin is 22). It appears to meet Hook's definition as a member of the cyclodepsipeptides, the category he calls the peptide lactones. These are described as having a "lactone linkage through a hydroxy amino acid...either totally composed of amino acids or having a hetero moiety attached outside the lactone ring."(74) Fifteen examples are cited, all from the Streptomycetes. The same group of antibiotic is named the "peptolides" according to the classification of Asselineau and Zalta.(75) These authors divide the polypeptide antibiotics into three groups: "-- les antibiotiques polypeptidiques dont la chaîne principale ne contient que des liaisons amides; --les 'peptolides', dans lesquels la chaîne peptidique est fermée par une liaison ester, créant ainsi un macrocycle lactonique; --les 'depsipeptides', constitués par un enchaînement alterné d'acides α-aminés et d'acides α-hydroxylés."(76)

The properties of Myxosidin appear to fit very well with the general characteristics of the "peptolide" group. The peptolides are described as being "cyclisée par formation d'une liaison ester entre le carboxyle de l'acide aminé C-terminal et l'hydroxyle d'un acide aminé hydroxylé présent du côté de l'extrémité N-terminale."(77)

The authors list nine antibiotics or antibiotic families, all from the Streptomycetes. This list is equivalent to that of Hook for the peptide lactones.

The peptolides are also usually weakly basic and all of them contain at least one aromatic residue. Myxosidin shares these characteristics.

CHAPTER V

BIOLOGICAL PROPERTIES

A. OBJECTIVES

The study of the biological properties of Myxosidin had as objectives: determination of suitable conditions for biological assays by serial dilution and by disc assay techniques: determination of minimal inhibitory concentration (MIC); establishment of a spectrum of activity against a variety of bacteria, fungi, yeasts, and streptomycetes; determination of acute toxicity (LD_{50}) in mice; and of potential antitumor activity in mice.

The acute toxicity tests of Myxosidin were carried out by Dr. D.R. Whitaker and Dr. A. Jackson at the Laboratory Center for Disease Control, Tunney's Pasture, Ottawa. Tests for the acute toxicity and antitumor activity of Myxosidin were done at the Drug Evaluation Branch of the National Cancer Institute, Bethesda, Maryland.

The above tests allow a preliminary assessment of the potential usefulness of a new antibiotic. Desirable characteristics are: a low minimal inhibitory concentration; a low acute toxicity; and a useful spectrum of activity towards disease-causing organisms. Many factors besides these determine the eventual value of an antibiotic *in vivo*. Of the hundreds of antibiotics reported in the literature, less than a dozen are of major clinical importance.

B. MATERIALS

Stock solutions of Myxosidin in 50% aqueous ethanol (1.05 mg/ml and 5.25 mg/ml) were used to make Myxosidin-impregnated test discs with Whatman filter paper. The discs were 6 mm in diameter and contained approximately 1 μg/disc and 5 μg/disc of the antibiotic. Commercially available discs (Difco) were used for comparison: Streptomycin, 5 μg/disc; tetracycline, 30 μg/disc; neomycin, 5 μg/disc; and nystatin (mycostatin), 100 units/disc.

Media (Difco) used for preparation of slants and Petri plates were: Plate Count Agar; Penassay medium (Antibiotic Medium 1); Potato Dextrose Agar (PDA); and purified agar. Plates were poured in disposable 100 x 15 mm Petri dishes (Fisher). Cultures were propagated on slants in sterile screw-caped tubes.

The organisms used for a survey of the spectrum of activity of Myxosidin were obtained from Dr. E. A. Peterson of the Chemistry and Biology Research Institute, Canada Department of Agriculture, Ottawa. They comprised 8 bacteria, 9 fungi, 3 yeasts, and 3 actinomycetes, For routine assays of the antibiotic strength of Myxosidin preparations, the test organism was the strain of Sphaerotilus natans isolated at the beginning of this investigation as a contaminant of a culture of Myxobacter 495.

Microscopic examinations were made with a Reichert phase-contrast microscope, fitted with an oil immersion objective.

C. METHODS

(a) Cultures

Bacteria were propagated on slants of Penassay medium. Fungi and yeasts were propagated on slants of potato dextrose agar. Cultures from solid media were adapted to liquid media by subculturing at least twice in 1% casamino-1% glucose-0.1% yeast extract medium.

(b) Antibiotic Assays

(i) Disc assays

Plates for disc assays of antibiotics were composed of two layers of agar prepared as follows. A base layer of approximately 20 ml of sterile plate count agar was poured into 100 x 15 mm petri dishes. Inocula from bacterial cultures were prepared by adding 2 ml of 0.9% sterile saline to a 24 hour slant culture, dispersing with a loop, and adding 0.5 ml of the dispersion to 5.0 ml of melted, sterile Penassay agar maintained at 53°C. The inoculum was immediately poured on top of a layer of plate count agar. Duplicate plates were made from each slant.

The streptomycetes, fungi, and yeasts required a slightly modified procedure in order to get good suspensions for preparation of inocula. After addition of saline to the slants, the cultures were dispersed as well as possible with a loop or hook and then transferred to sterile screw-capped culture tubes containing large glass beads. These were shaken vigorously until all lumps were broken up. Duplicate

plates were prepared as above.

Antibiotic discs were placed on the surface of the plates with at least 20 mm between their edges. The plates developed a carpet of cells after incubation for 24 hours at 25°C (occasionally up to 48 hours for fungi) Zones of inhibition, recognized as areas free of growth around the disc, were measured to the nearest millimetre with a ruler after 24 hours of incubation. The discs were of 6 mm diameter; zones smaller than 7 mm in diameter were scored as zero, that is, indicating no sensitivity to the antibiotic.

(ii) Assays by Serial Dilution

Routine assays of Myxosidin were carried out as described in Chapter II. For a survey of bacterial and fungal sensitivity to Myxosidin, the assay was modified as follows. Each flask contained 5.0 ml of 1% casamino-1% glucose-0.1% yeast extract medium of 0.1 ml of inoculum from a 24 hour culture of the organism to be tested. Myxosidin, in 0.1 ml of sterile distilled water, at three concentrations, was added to each flask. The test was carried out in duplicate. Blanks for each different organism contained the same ingredients as above except that 0.1 ml of sterile distilled water replaced the 0.1 ml of antibiotic solution. Cultures were incubated at 25°C for 24 hours on a rotary shaker describing a circle 1 inch in diameter at 130 rpm. The flasks were scored in comparison with the growth of their corresponding blanks as negative

(no sensitivity) if equal in turbidity, as marginal if appreciably less turbid than the control, and as positive if growth was completely inhibited.

D. RESULTS

(a) Serial Dilution Assays of Myxosidin

A stock solution of Myxosidin containing 1.05 mg/ml of the purified antibiotic was tested against a variety of bacteria, fungi, and streptomycetes as shown in Table 18. This test was done in duplicate. The results in duplicate sets were usually identical but in two instances an organism was completely inhibited at a particular concentration in one set of flasks and not in the other at the same concentration. These were assigned as "marginals".

Sphaerotilus natans was found to be the most sensitive organism. It was totally inhibited at a concentration of 0.34 μg/ml. The least sensitive bacteria tested were *Agrobacterium tumifaciens* and *Staphylococcus aureus* which were inhibited at 0.81 μg/ml but not at 0.17 μg/ml. Thus, *Sphaer. natans* is a good choice for a test organism since it grows quickly to maximum turbidity (about 12 hours) and is sensitive to as little as 200 nanograms/ml of Myxosidin.

(b) Disc Assays of Myxosidin

Tests and comparisons of Myxosidin's spectrum of activity were done by the disc assay procedure which is more easily managed with a large number of different

TABLE 18

SERIAL DILUTION ASSAYS OF MYXOSIDIN: BACTERIA, FUNGI, AND ACTINOMYCETES

ORGANISM	Myxosidin: μg/ml 4.2	0.81	0.17	0.03
1. *Agrobacterium tumefaciens*	X	-	-	-
2. *Arthrobacter globiformis*	X	X	-	-
3. *Bacillus megaterium*	X	X	-	-
4. *Corynebacterium insidiosum*	X	X	-	-
5. *Pseudomonas phaseolicola*	X	X	-	-
6. *Staphylococcus aureus*	X	-	-	-
7. *Escherichia coli*	X	X	-	-
8. *Sphaerotilus natans*	X	X	X	-
9. *Streptomyces candidus*	X	X	-	-
10. *Streptomyces griseus*	X	X	-	-
11. *Streptomyces carneus*	X	X	-	-
12. *Botrytis cinerea*	-	-	-	-
13. *Fusarium oxisporum*	-	-	-	-
14. *Gliocladium roseum*	-	-	-	-

Each flask contained 5.0 ml of 1% casamino-0.1% yeast extract-1% glucose medium, 0.1 ml of inoculum, and 0.1 ml of Myxosidin solution. The final concentrations in g/ml are indicated. Flasks were scored as follows: X, no growth; X, marginal growth, not as turbid as the blank; and -, growth equal to that of the blank.

species of organisms and types of antibiotics. The bacteria used in this test included examples of both gram positive and gram negative species.

The relationship between concentration of Myxosidin and diameter of zones of inhibition of Sphaerotilus natans is shown in fig. 41. Zone diameter was found to be a logarithmic function of the dilution of the inhibitor solution in the range of about 10 mm diameter to 25 mm. The spectrum of antibiotic activity of Myxosidin against various species of bacteria is shown in Table 19. The results of these disc assays correspond well with results found by serial dilution assays. Sphaerotilus natans was again found to be the most sensitive of the organisms tested and no bias towards gram positive or gram negative organisms was noted.

The result shown in Table 20 for a survey of the same organisms with streptomycin, tetracycline, and neomycin indicates that Arthrobacter globiformis and Sphaer. natans were the most sensitive of these bacteria to all four antibiotics, including Myxosidin. It may also be concluded that Myxosidin had a comparable MIC for these organisms as the three standards used for comparison.

In Table 21 antibiotic activity of Myxosidin towards actinomycetes, fungi, and yeasts is compared with activity of tetracycline and nystatin for the same species. Myxosidin was found to have no activity towards any of the fungi at the

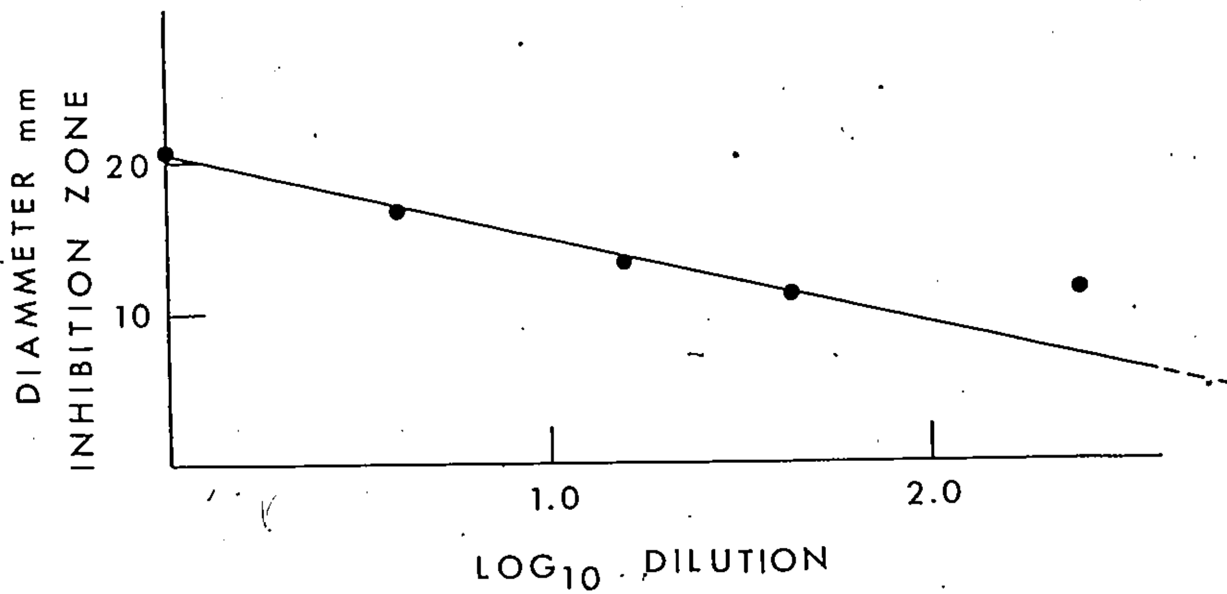


FIGURE 41

INHIBITION ZONE DIAMETER AS A FUNCTION OF DILUTION: ANTIBIOTIC DISC ASSAY OF MYXOSIDIN

TABLE 19

INHIBITION ZONE DIAMETERS FOR BACTERIA:
DISC ASSAY OF MYXOSIDIN

ORGANISM	Myxosidin: µg/disc 5.3	1.1	0.2
Agrobacterium tumifaciens	13	9	0
Arthrobacter globiformis	17	12	8
Bacillus megaterium	13	9	0
Corynebacterium insidiosum	14	10	0
Pseudomonas phaseolicola	13	9	0
Staphylococcus aureus	12	8	0
Escherichia coli	12	8	0
Sphaerotilus natans	18	13	11

Plates were in duplicate; results are given as diameters of inhibition zones in mm $\pm \frac{1}{2}$mm, and are the arithmetic mean of two plates; zero implies growth to the edge of the disc; zones were measured after 24 hours incubation at 25°C; a blank disc was included on each plate and showed no activity.

TABLE 20

INHIBITION ZONE DIAMETERS FOR BACTERIA:
DISC ASSAY OF STANDARD ANTIBIOTICS

ORGANISM	Streptomycin 5 μg/disc	Tetracycline 30 μg/disc	Neomycin 10 μg/disc
Agrobacterium tumifaciens	10	12	7
Arthrobacter globiformis	17	40	9
Bacillus megaterium	10	13	7
Corynebacterium insidiosum	11	12	7
Pseudomonas phaseolicola	10	14	7
Staphylococcus aureus	7	10	0
Escherichia coli	8	8	7
Sphaerotilus natans	14	30	12

The results shown are the arithmetic mean of two plates, the same plates as those of Table 19.

TABLE 21

DISC ASSAY OF MYXOSIDIN:
INHIBITION ZONE DIAMETERS FOR
ACTINOMYCETES, FUNGI, AND YEASTS

	ORGANISM	MYXOSIDIN 26	5.3	1.1	TETRACYCLINE 30	NYSTATIN 100
1.	Streptomyces candidus	28	23	14	30	0
2.	Streptomyces carneus	27	20	0	0	0
3.	Streptomyces griseus	18	14	8	24	0
4.	Botrytis cinerea	24	17	0	10	0
5.	Fusarium oxisporum	23	18	11	25	0
6.	Gliocladium roseum	0	0	0	0	14
7.	Cephalosporum acremonium	0	0	0	0	29
8.	Ceratocystis ulmi	0	0	0	0	44
9.	Helminthosporum sativum	0	0	0	0	50
10.	Aspergillus niger	0	0	0	0	30
11.	Paecilomyces marquandii	0	0	0	0	10
12.	Gliomastix murorum	0	0	0	0	23
13.	Rhodotorula mucilaginosa	21	17	11	13	20
14.	Saccharomyces cerevisiae	0	0	0	0	17
15.	Sacch. carlsbergensis	7	0	0	0	20

Conditions were as for Table 19, except that some of the slower-growing organisms were incubated up to 4 days. Numbers 1 to 3 inclusive are actinomycetes; 4 to 12 inclusive are fungi; and 13 to 15 are yeasts. Amounts of Myxosidin are μg/disc; Tetracycline is μg/disc; Nystatin or Mycostatin is in units/disc.

highest levels tested. All of the streptomycetes were inhibited by Myxosidin but only one of the yeasts. This pattern of activity was duplicated by tetracycline, except that no activity towards Strep. carneus was found. Nystatin was not inhibitory for any of the streptomycetes tested, but all of the fungi and yeasts were found to be sensitive. It was concluded that Myxosidin was not an anti-fungal agent, but rather was more like tetracycline in its spectrum of antimicrobial activity. Like tetracycline, Myxosidin can be described as a broad spectrum antibiotic.

A survey of activities against pathogenic organisms was conducted by Dr. J.R. Dillon of the Antimicrobial Section, Laboratory Centre for Disease Control, Canada, Department of Health and Welfare, Tunney's Pasture, Ottawa. Antibiotic discs of Myxosidin were prepared at ½ µg/disc and 2½ µg/disc concentrations. Sensitive organisms at these two levels were all gram positive. They were: Streptococci of types A, B, and D; Staphylococci; and Bacillus sp. Correlation with a gram positive spectrum is curious in that Sphaer. natans, the most sensitive of the bacteria tested in the survey detailed in Tables 18, 19, and 20, is a gram negative bacterium.

Myxosidin was tested for heat stability as follows. Six screw-capped culture tubes, each containing 3 antibiotic discs (5 µg/disc), were autoclaved at 121°C, 15 psi. One tube was removed and cooled after 15 min, and one at each

of 1, 2, 3, 4, and 6 hours in the autoclave. The discs were assayed on plates of Sphaer. natans as described above. The result is shown in fig. 42. The discs retained their biological activity for up to four hours of heat treatment.

The above result, indicating the excellent heat stability of Myxosidin, is consistent with other observations. For example, Myxosidin retains its activity as a dried powder at room temperature for at least three months, and as a powder stored in a freezer at -20°C for at least three years.

(c) Minimal Inhibitory Concentration

The relative efficiency of antibiotics may be compared by a determination of their minimum inhibitory concentrations (MIC) defined as the lowest concentration at which the antibiotic exerts its inhibitory action. The growth curves illustrated in fig. 43 set a range of concentrations for the MIC of Myxosidin for Sphaerotilus natans as a test organism. Low concentrations of the antibiotic, such as that illustrated for a concentration of 0.08 μg/ml, had relatively little effect on the rate of increase during log-phase growth but gave appreciably lower turbidities at the end of 24 hours. The growth curve for the intermediate concentration of 0.3 μg/ml shows a distinct lag-phase, of about six hours in this instance. This concentration appears to be at the lower limit of inhibitory effect for these experimental conditions. At 1.6 μg/ml the test cultures were completely inhibited. Microscopic examination

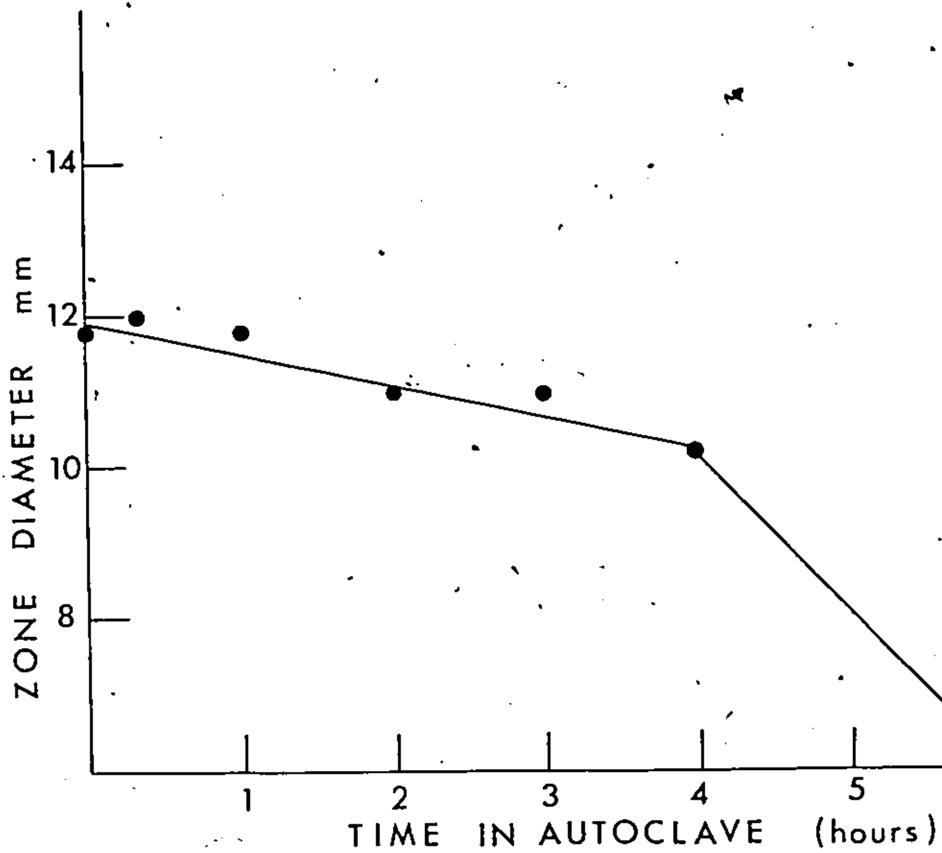


FIGURE 42

HEAT STABILITY OF MYXOSIDIN

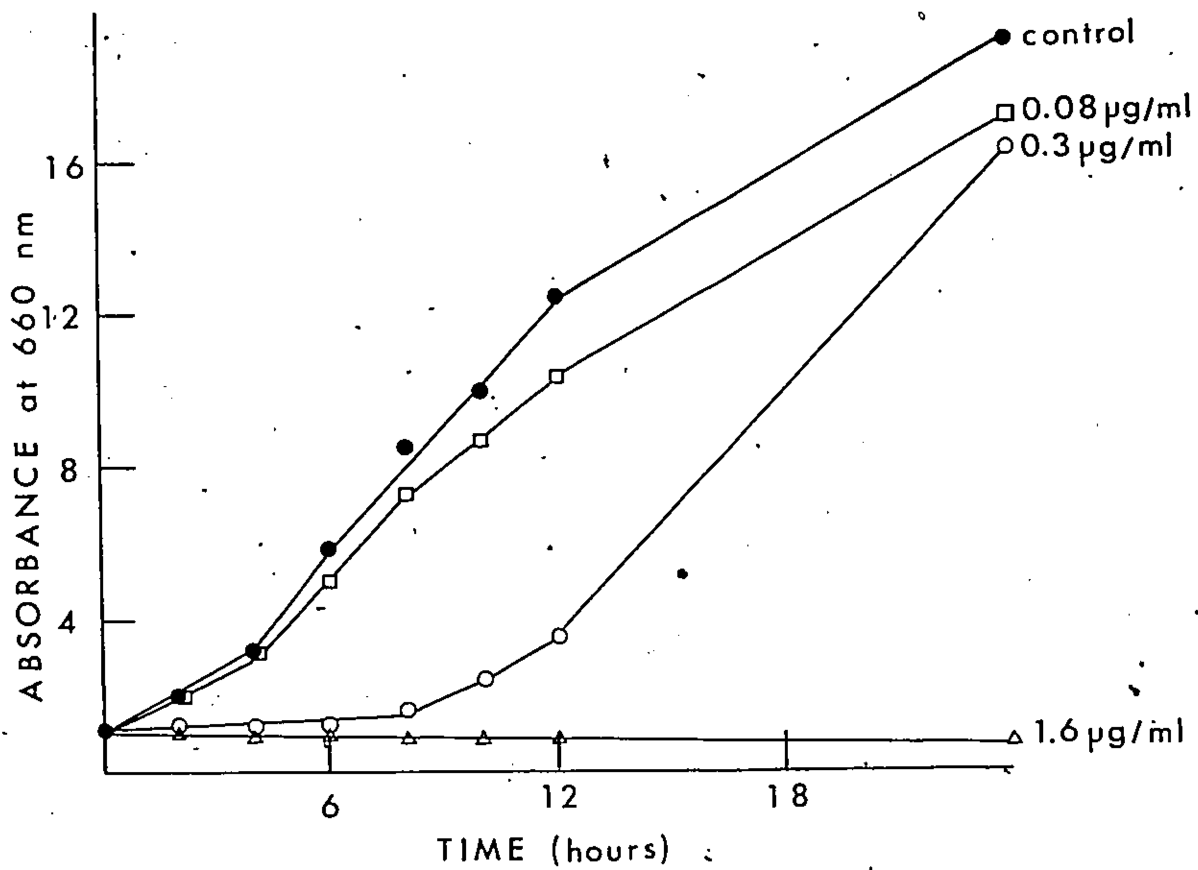


FIGURE 43

MINIMAL INHIBITORY CONCENTRATION OF MYXOSIDIN TOWARDS *Sphaerotilus natans*

of these cultures showed that apparently intact cells from the inoculum were still present; there were no indication of lysis. It was concluded that the MIC for Myxosidin against Sphaer. natans at 26°C lies between 0.3 μg/ml and 1.6 μg/ml. It is a desirable property for an antibiotic to have a low MIC since when used *in vivo* there are obvious advantages in being able to reach effective inhibitory levels at low concentrations. The MIC for Myxosidin is roughly comparable to that of tetracycline and other well known antibiotics.

(d) Acute Toxicity

The first set of tests were carried out by Dr. D.R. Whitaker and Dr. A. Jackson at the Laboratory Centre for Disease Control, Tunney's Pasture, Ottawa. Acute toxicity was assessed from the survival rate after single injections of Myxosidin into the peritoneal cavity of mice. The solutions for injection were prepared by diluting a concentrated ethanolic solution of the antibiotic with physiological saline and adjusting all solutions to an ethanol concentration of 1%. One ml of each solution was injected. The tests were made in triplicate for the highest concentration tested and in quadriplicate for all other concentrations. All mice injected with the highest concentration tested--260 mg/kg body weight--were dead within 6 hours. All mice injected at the lower concentrations tested--132, 88, 44, and 22 mg/kg body weight--were in

apparently good health until the end of the observation period (one week after injection). The LD_{50} for mice was taken to be between 130 mg/kg and 260 mg/kg.

A second set of tests was arranged by the Drug Evaluation Branch of the National Cancer Institute, Bethesda, Maryland, in conjunction with tests for anti-tumor activity. In these tests, groups of mice were given intraperitoneal injections of Myxosidin (in saline). None of the group receiving 150 mg/kg but all of the groups receiving 75 and 37.5 mg/kg survived the sixth day after injection. These results assign an LD_{50} between 75 mg/kg and 150 mg/kg. Both estimates are in the range for an antibiotic of low toxicity.

Tests of anti-tumor activity were arranged by the Drug Evaluation Branch of the National Cancer Institute, Bethesda, Maryland. Anti-tumor activity was tested in mice with P 38 lymphocytic leukemia tumor. No activity was indicated by these tests.

E. <u>DISCUSSION</u>

Antibiotics are divided into several broad categories on the basis of their anti-microbial spectrum.[78] Narrow spectrum antibiotics are active against a restricted group of bacteria or fungi. For example, penicillin is active against gram positive bacteria; polymyxin is active against gram negative bacteria.

Antibiotics of the peptolide class have been reported to be active against the tubercule bacillus and gram-positive bacteria (particularly against Staphylococci and Streptococci) but not against gram-negative bacteria.[79] Myxosidin may be exceptional in that, as indicated in Tables 18, 19, and, 20, it is active against various gram-negative organisms, one of these, Sphaerotilus natans, being the most sensitive organism tested. However, reports of the inactivity of other peptolides towards gram-negative bacteria may simply be a reflection of bias towards pathogens in the selection of test organisms.

The values obtained for the LD_{50} of Myxosidin, a measure of its acute toxicity, indicate that it is relatively non-toxic. All antibiotics have side-effects in at least some host individuals. The most frequently observed of such effects are allergic reactions, renal and liver damage, damage to the 8th cranial nerve (affecting balance and hearing) and nausea, vomiting, and diarrhea.[80] Antibiotics which are far too toxic for systemic use, may, however, be valuable as antiseptic creams or dressings. Bacitracin and neomycin are used for the treatment of infections of burns, although they are too toxic for use internally.

The above considerations suggest that Myxosidin, with its low acute toxicity, its significant spectrum of activity, its ease of production, and chemical and heat stability, may prove to be a useful agent.

CHAPTER VI

GENERAL DISCUSSION

Myxosidin is only the second antibiotic to be isolated and characterized from a member of the order Myxobacterales. The other, produced by a related organism,[23] is myxin which is a phenazine antibiotic and not a cyclic peptide. Most of the known peptide antibiotics are produced by streptomycetes or bacilli. For example, Bodanszky and Perlman's list[81] of 42 peptide antibiotics contains 35 which are produced by those two orders of bacteria. Myxosidin is the first example of a peptide antibiotic from the order Myxobacterales.

All other peptolide antibiotics reported so far are produced by species of the genus _Streptomyces_ (Table 22). It is of interest in this connection that _Myxobacter 495_ and a species of _Streptomyces_ are unique in another respect--both produce serine proteases with amino acid sequences which, unlike those of other bacterial proteases of known amino acid sequence, match the amino acid sequences of the pancreatic serine proteases.[82] Thus the production of a peptolide antibiotic by _Myxobacter 495_ may be another reflection of an evolutionary pathway shared with _Streptomyces_.

The peptolide, Myxosidin, produced by _Myxobacter 495_ has some distinctive structural characteristics when compared with other members of the group. One such difference is the presence of a free N-terminus in Myxosidin. The rest

TABLE 22

PEPTOLIDE ANTIBIOTICS

PEPTIDE	SOURCE	TYPE
STAPHYLOMYCIN	*Streptomyces virginiae*	CYCLIC HEXAPEPTIDE LACTONE
OSTREOGRYCIN	*Streptomyces ostreogriseus*	CYCLIC HEXAPEPTIDE LACTONE
ETAMYCIN	*Streptomyces griseus*	CYCLIC HEPTAPEPTIDE LACTONE
CYCLOHEPTAMYCIN	*Streptomyces sp.*	HEPTAPEPTIDE WITH 6 - MEMBER LACTONE
TELOMYCIN	*Streptomyces sp.*	NONAPEPTIDE WITH 8 - MEMBER LACTONE
MYXOSIDIN	*Myxobacterales sp.*	DECAPEPTIDE WITH 8 - MEMBER LACTONE
ECHINOMYCIN	*Streptomyces echineatus*	CYCLIC OCTAPEPTIDE DILACTONE
STENDOMYCIN	*Streptomyces sp.*	TETRADECAPEPTIDE WITH 7 - MEMBER LACTONE

of the peptolides are all isolated as N-terminal amides, including Echinomycin which is a cyclic dilactone with two N-terminal amides present in the same molecule. The most frequently seen group is an N-3-hydroxy-picolinyl moiety, but N-formyl, N-acetyl, and N-β-L-aspartyl amides have been reported for the peptolides. Myxosidin is also unusual in that it contains an arginine residue. Bodanszsky and Perlman have noted that "certain amino acids commonly found in proteins, for example, arginine, histidine, and methionine, apparently occur rarely in peptide antibiotics."(66) None of the other peptolides contain arginyl residues. Furthermore, among the peptolides only Myxosidin and telomycin do not contain any N-methyl amino acids (see Table 23).

The number of antibiotics reported in the literature is now in the thousands. It is quite unlikely that any totally new types remain to be discovered in the future. Almost every type of infectious disease is susceptible to some form of antibiotic treatment. The major exception is disease caused by small viruses. How can the search for new antibiotics be justified? While it is true that a handful of antibiotics are used to control the vast majority of diseases, these drugs may not be the optimum choices when considerations of toxicity, shelf-life, ease of manufacture, and other practical factors are taken into account. The rise of resistant strains of bacteria, since the advent of the antibiotic era, sometimes forces the use of less desirable

TABLE 23

AMINO ACID COMPOSITION OF PEPTOLIDE ANTIBIOTICS

ANTIBIOTIC	"USUAL" AMINO ACIDS	"UNUSUAL" AMINO ACIDS	OTHER COMPONENTS
STAPHYLOMYCIN	L-Pro L-Phe L-Thr	D-aminobutyric N-methyl-L-Phe N-methyl-p-dimethylamine-L-Phe	4-oxopipecolic acid
ETAMYCIN	L-Thr L-Ala	D-Leu D-allo-hydroxyproline sarcosine α-phenylsarcosine N-β-dimethyl-L-Leu	3-hydroxypicolinic acid
CYCLOHEPTAMYCIN	L-Thr	N-formyl-L-Val O-methyl-L-Tyr D-Ala N-methyl-L-Ile L-β-hydroxynorvaline N-methyl-5-methoxy-L-Trp	
TELOMYCIN	Gly L-Ala L-Thr L-Asp L-Ser L-Trp L-trans-hydroxyproline	L-cis-hydroxyproline β-hydroxy-L-Leu L-allothreonine L-β-methyl-Trp	

TABLE 23 (continued)

ECHINOMYCIN	L-Ala	D-SER N,N'-dimethylcystine N-methylvaline N-methylisoleucine N-dimethylleucine	2-carboxy-quinoxaline
STENDOMYCIN	L-Pro Gly	N-methyl-L-Thr D-Val D-allo-Ile D-Ala Δ-2,3-aminobutyric acid D-allo-Thr L-stendomycine	Fatty acid: $(H_3C)_2CH(CH_2)_{16}CO$
MYXOSIDIN	L-Leu Gly L-Ser L-Thr L-Arg L-Ile L-Val	D-Leu t-β-hydroxyaspartic acid "X-2", an aromatic amino acid	

alternatives. This trend can be expected to increase and can only be countered by an effort to develop new derivatives of known antibiotics and the careful development of new antibiotics, especially from novel sources.

An ideal in clinical applications of existing antibiotics, which is only recently gaining widespread attention,[83] is the use of narrow-spectrum drugs in order to control a given infecting organism while leaving the normal flora of the host as little affected as possible. This approach tends to supress the selection for resistant strains of bacteria, and to minimize the possibility of secondary infections overgrowing the original infecting species.

The study of new antibiotics as well as the older established ones, from a biochemical point of view, has contributed greatly to the understanding of protein biosynthesis, nucleic acid functions and cell wall structure. Antibiotics with no clinical significance at all have become invaluable tools for biochemical and molecular biological investigations. The search for new antibiotics is an important facet of biochemistry as long as their biosynthesis, structure and mode of action continue to provide insights into the nature of microorganisms.

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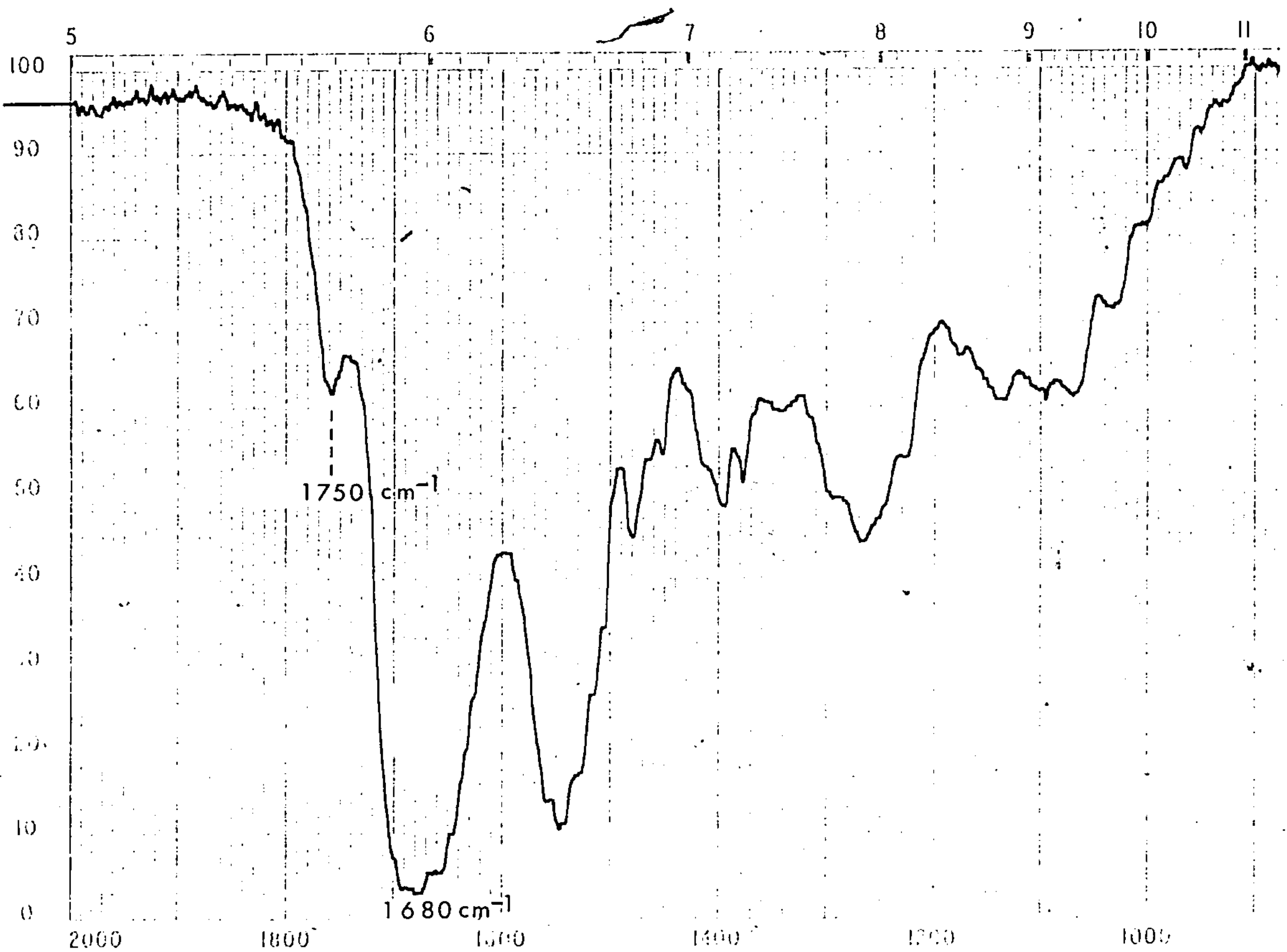
APPENDIX

INFRARED SPECTRUM OF MYXOSIDIN

The infrared spectrum of Myxosidin was recorded on a Pye Unicam SP 1800 scanning spectrophotometer as a KBr disc. A weak absorption maximum at 1750 cm^{-1} indicates a lactone carbonyl. The strong absorption at 1680 cm^{-1} is due to the carbonyls of peptide bonds. Thus the IR spectrum obtained for purified Myxosidin is consistent with a cyclic lactone peptide structure.



INFRARED SPECTRUM OF MYXOSIDIN

ABBREVIATIONS

BAWP butanol:glacial acetic acid:water:pyridine (60:12:48:40)

CAS hydrolyzed milk proteins, "casamino acids"

cpm counts per min

DAO D-amino acid oxidase

DFP diisopropylfluorophosphonate

Dns 5-dimethylaminonaphthalene-1-sulfonyl chloride

GCMS gas chromatography-mass spectrometry

GLU monosodium L-glutamic acid

H depth of medium in a fermenter vessel

HVE high voltage electrophoresis

Hya hydroxyaspartic acid

k second order velocity constant

K_{av} partition coefficient

LAO L-amino acid oxidase

LD_{50} acute toxicity in mg per kg body weight

L-Leu-NCA L-leucyl-N-carboxyamide

MIC minimal inhibitory concentration

MS mass spectrometry

NAD β-nicotinimide adenine dinucleotide

PDA potato dextrose agar

PheSer phenylserine

PITC phenylisothiocyanate

psi pounds per square inch

Q aeration rate in litres per min

rpm revolutions per min

SUC sucrose

V volume

V_e elution volume

vvm volume per volume per min (aeration rate per unit volume of medium in a fermenter)