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Title: INCIDENCE AND SIGNIFICANCE OF BACTERIAL CHITINASE IN THE MARINE ENVIRONMENT

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Richard Y. Morita

The marine vibrio ANT-500 is a typical psychrophile. The growth range of the bacterium at 35‰ S is from a maximum growth temperature of 13.5 C to a minimum growth temperature of -2.5 C or less with the optimum near 7 C. The bacterium elaborates its chitinase enzymes between the temperatures of 1.5 and 13.5 C. The enzyme system is composed of three separate enzymes, probably two chitinases and one chitobiase. The rate limiting step of the enzyme-substrate reaction appears to follow Mechaelis-Menton kinetics. Chitin is the only substrate that induces chitinase synthesis in ANT-500.

Chitinase activity was found in the digestive tracts of all species of fish sampled in Yaquina Bay, Oregon. Direct correlations were noted between gut content and chitinase activity in the gut, gut content and percent chitinoclasts in the gut, and chitinase activity and percent chitinoclasts. Fish with little or no chitinous material in their gut

contents showed low or no detectable levels of chitinase activity and a low percentage of chitinoclasts, while fish with predominantly chitinous material in their gut contents showed high chitinase activity and a large percentage of chitinoclasts. Neither hydrostatic pressure up to 1000 atm nor lack of oxygen had a detrimental effect on chitinase activity from the gut of Raja binoculata.

Sterile stomach fluid from Enophrys bison had no effect upon the growth rates of a pure chitinoclastic and a mixed culture of bacteria isolated from the gut of Enophrys bison; however, the gut fluid did allow for greater cell yield. N-acetyl-D-glucosamine, the end product of chitin decomposition, exhibited a "glucose effect" when added to culture medium. Both the pure and the mixed culture showed repression of growth in early log phase when grown in N-acetyl-D-glucosamine.

The chitinase isolated from the gut of Enophrys bison is bacterial in origin. Tests in which the bacterial flora of the gut of Enophrys bison was eliminated by use of chloramphenicol revealed no detectable chitinase activity and no chitinoclastic bacterial populations. Fish in the absence of the antibiotic showed both significant chitinase activity and predominating chitinoclast populations. Other data strongly indicate that the chitinase in many species of marine fishes may also be bacterial in origin.

Incidence and Significance of Bacterial Chitinase in the Marine Environment

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Professor of Microbiology and Oceanography
in charge of major

Redacted for privacy

Head of Department of Microbiology

Redacted for privacy

Dean of Graduate School

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INTRODUCTION

Chitin is a mucopolysaccharide. It is the major structural, or skeletal, component of most invertebrate species. It is the second most abundant organic polysaccharide. Cellulose, the structural component of plants, is more abundant. Few estimates of the amount of chitin production per year have been made. Johnstone (25) reported that copepods, a sub-class of crustaceans, produce several billion tons of chitin per year. Therefore, with the large number of invertebrate species containing chitin, the degradation of chitin is necessary for the re-cycling of the organic matter.

The biological degradation of chitin is carried out by a series of enzymes. These enzymes, referred to as chitinases and chitobiases, are produced by many organisms, including bacteria and fungi. Chitinases degrade large poly- and oligosaccharide fractions of chitin to three basic units, N-acetyl-D-glucosamine, chitobiose (the dimer of N-acetyl-D-glucosamine, β-1, 4 linked), and chitotriose (the trimer). The chitobiases hydrolyze the dimer and trimers to N-acetyl-D-glucosamine. Other enzymes are responsible for deamination and deacetylation as well as hydrolysis of the peptide cross-bridges which link the oligosaccharide chains and provide for structural strength;

however, these enzymes do not truly belong to the chitinase system.

There are many reports on the characteristics and properties of the chitinase system from specific bacterial, actinomycete and fungal sources. These characteristics and properties include pH range (40, 47), temperature range (42, 47, 60), specific ion requirements (42, 60), molecular weight estimates (42, 60), and homogencity and crystallizability of purified enzyme fractions (60).

ZoBell and Rittenburg (65) were the first workers to report on chitinoclastic bacteria of marine origin, although Benecke (6), Folpmers (13), Rammelberg (45), and Benton (7) had previously reported on bacteria which could lyse chitin. More recent reports relating chitinoclasts to the marine environment have dealt with taxonomy (4, 11, 29, 62), distribution in waters (11, 31, 51, 52, 53, 56, 57) and sediments (8, 11, 34, 62) and enumeration from environmental sources (1, 10, 15, 26, 27, 32, 36, 43, 44, 54, 55).

Seki (50), Chan (11), Liston et al. (34), and Hood (15) have each attempted to estimate rates of chitin decomposition in the marine environments. The estimates of Chan and Seki of 19-31 mg/day/10^{10} bacteria and 30 mg/day/10^{10} bacteria, respectively, closely approximate one another. These studies were conducted with pure cultures of bacteria.

The purpose of the present study was two-fold. The first aspect was to examine a psycrophilic, marine bacterium, ANT-500

(a Vibrio sp.), and its ability to degrade chitin. This involved the effects of temperature, salinity, and pH on the chitinase system (crude preparation) as well as the limitations of the organism to produce the enzymes.

Second was the search for free chitinase in the marine environment. Fish digestive tracts and marine sediments were tested as probable sites of accumulation of chitinous material. The samples were assayed for chitinase activity under varying experimental conditions with the intent of obtaining data which could be applied to rates of chitin decomposition. Finally, an attempt was made to determine the origin of the enzyme activity found in fish stomachs using aquarium reared buffalo sculpin (Enophrys bison).

LITERATURE REVIEW

Chitin is a mucopolysaccharide composed of 2-acetamido-2-deoxy-D-glucopyranose (N-acetyl-D-glucosamine) units joined by β-1, 4-glucosidic bonds (37, 49). It has, therefore, the same basic structure as cellulose and like cellulose its function is structural. Chitin is the major carbohydrate constituent of most invertebrate "skeletons". Chitin is found in the cuticle of all arthropods and in some members of the molluscs, coelenerates and protozoa as well as in the walls of many fungi. Chitin has also been found in many of the phyla of the lesser invertebrates. Jeuniaux (23) presented an extensive review of the incidence of chitin in the animal kingdom.

Estimates of the amount of chitin produced per year in the world's oceans have appeared for a few species. Johnstone (25) reported the sub-class of crustaceans, the copepods, can produce several billion tons of chitin annually based on rate of synthesis, numbers of organisms and numbers (some producing 10 to 12 casts per year) of ecdyses, or molts. Jerde and Lasker (19) have estimated that the single, planktonic crustacean, Euphausia pacifica, produces approximately 1.9×10^{13} g dry weight of molt skeleton/year. The authors do not indicate the percent chitin content of the species; however, with a conservative estimate of 10% chitin per unit dry weight this species would contribute about 1.8×10^{6} metric T of

chitin per year. No large areas of chitin settlement have been reported; therefore, it must be assumed that the chitin is degraded or made available for other utilization.

Chitinase is the name given the enzyme capable of hydrolyzing chitin. Yet nearly all reports indicate that those living organisms capable of decomposing chitin possess at least two chitinase enzymes. The chitinolytic enzymes consist of a "chitinase" which is responsible for the hydrolysis of the large oligosaccharide moieties and of a chitobiase which is responsible for the hydrolysis of the two- and three-unit N-acetyl-D-glucosamine (NAG) chains, chitobiose and chitotriose, respectively.

Karrer and Hofman (28) helped establish that NAG was the sole building block of chitin when they utilized an enzyme from a snail gut to obtain 85% conversion of chitin to NAG. Jeauniaux (20) found that exocellular, or extracellular chitinases were produced by a chitonoclastic soil actinomycete and by a member of the Eubacteriales isolated from the snail gut. Reynolds (47) using the exocellular chitinase produced by a Streptomyces sp., reported on some of the properties of the enzyme including pH range, substrate concentration, enzyme induction and stability. The Streptomyces enzyme was found to have a pH optimum between 6.5 and 7.5, was induced only by chitin and was stable after treatment with 0.01% thiomersalate at pH 7 and stored at 4 C.

Monreal and Reese (40) found that large quantities of chitinase were produced by Serratia marcescens and Enterobacter liquifaciens, although a wide variety of bacteria and fungi tested also produced the enzymes. It was shown that the Serratia marcescens chitinase system was induced by chitin. A crude enzyme preparation had maximum activity at pH 6.4 and 50 C. Skujins et al. (60) employed an extensive purification procedure to obtain a purified chitinase fraction from a Streptomyces sp. Their enzyme was homogeneous and crystallizable with a molecular weight of ca. 29,000 and a requirement for calcium. Ohtakara (42) reported similar findings using the strain Streptomyces griseus. Attempts were unsuccessful at obtaining a purified chitobiase free of chitinase activity.

Prior to 1934 there were relatively few reports of isolated bacteria capable of hydrolyzing chitin. Benecke (6) was the first to describe a bacterium capable of using chitin as a food source. The bacterium was an aerobic, gram-negative, non-spore forming rod with peritrichous flagella. Benecke named the organism Bacillus chitinovorous. Folpmers (13) and Rammelberg (45) also isolated a chitinoclastic bacterium which appeared to be similar to, if not identical, to Benecke's organism. Several reports of chitin decomposition by actinomycetes and myxococci also appeared in the early literature (18, 24).

The first extensive search for chitinoclastic bacteria was

performed by Benton (7). She isolated over 250 pure cultures from lake water, sediments, and plankton, and from the intestines of frogs and bats as well as from insects and crayfish. The isolates were divided into 17 distinct groups. Even though twelve of the groups consisted of both gram-negative and monotrichous organisms, there were significant differences among the groups which led Benton to believe that they were different genera. The few phenetic characteristics used by Benecke to describe his isolates, preclude any valid taxonomic comparison between his isolate and Benton's organisms.

ZoBell and Rittenberg (65) described pure cultures of chitinoclastic bacteria isolated from marine environment sources, including sediments, water, tide pools, crustacean and squid. Genus and species names were not supplied; however, the authors indicated that some of the isolated organisms came from laboratory stock cultures which presumably were identified. All isolates were gram-negative and 27 of the 31 were actively motile. Some chitinoclasts were described which derive their complete carbon and nitrogen requirements from chitin. Other chitinoclasts were able to utilize the nitrogen from chitin but required an additional carbon or energy source. Still others could utilize chitin only in the presence of other carbon and nitrogen sources.

Kihara and Morooka (29) extensively studied six chitinoclastic isolates from marine sediments. They placed the isolates into three

genera, Agarbacterium, Pseudomonas and Beneckea.[1] Seki and Taga (51, 52, 53, 54, 55, 56, 57) reported on the occurrence of chitinoclasts in pelagic and neritic waters and in the intestines of whales as well as on the effects of various environmental parameters, such as, temperature, salinity, pH, and radiation on the distribution of chitinoclasts. Chitinoclastic bacteria have been implicated in many crustacean diseases of the shell and appendages (59). Timmis et al. (62) have successfully isolated chitinoclastic clostridia from marine sediments containing decaying chitinous material. Many of the species isolated do not belong to any described clostridial species.

The distribution of chitinoclastic bacteria in the marine environment has been examined for a few water masses. ZoBell and

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[1] Beneckea is a genus of "uncertain taxonomical position" in the 8th Edition of Bergey's Manual of Determinative Bacteriology (9). An extensive taxonomical study was performed by Baumann et al. (4) and many pre-existing Vibrio, Aeromonas, and Pseudomonas species were placed into the genus Beneckea. The confusion surrounding the controversy has decreased to a single taxonomic character, flagellation. It, therefore, must be resolved whether that single character should be weighted in such fashion as to alter all previous classification schemes. For the purposes of this thesis, the designations utilized by the cited authors will be used. The term "chitinoclast" will be employed wherever possible to prevent as much confusion as possible, since very similar, if not the same, bacteria are referred to as a Vibrio sp. by some workers and a Beneckea sp. by others.

In a recent report (17) the International Subcommittee on taxonomy of vibrios has suggested that the species V. parahaemolyticus and V. alginolyticus be restored to the genus Vibrio. The type species of the genus Beneckea, B. labra, no longer exists for comparison purposes.

Rittenberg (65) found the distribution of chitinoclasts in California waters to vary significantly. They speculated that the random distribution of particulate matter was the primary reason for their findings. The greatest concentration of chitinoclasts was found in the uppermost layer of sediment (10^3 cells/g). Based on examination of total numbers of bacteria, only 0.1 to 1.0% of the bacteria in the waters and sediments were chitinoclastic.

Veldkamp (63) reported that terrestial (soil) chitinoclasts influenced the numbers of total chitinoclasts found in pelagic waters. Actinomycetes predominated among the soil chitinoclasts. Lear (31) found that the concentration of chitinoclasts in the waters off California decreased with increasing depth. Few chitinoclasts were found below 1000 m in the water column. Bianchi (8) sampled the deep sediments of the Mediterranean Sea. Only 11% of the samples taken were found to contain chitinoclasts, however, one of the samples examined had 2.4×10^4 cells/g. Chan (11) working in the waters and sediments of Puget Sound reported slightly less than 10% of the bacterial biomass to be chitinoclastic. He found greater than 10^4 chitinoclasts/g in sediments from subtidal and intertidal sediments.

Seki and associates in a series of papers have concluded an extensive research on chitinoclasts in the marine environment. Seki and Taga (51, 57) also found that the concentrations of chitinoclasts decreased with increasing depth and that the concentrations were

affected by water temperature and, in general, increasing temperature yielded higher population densities. They also noted a direct relationship of specific chitinoclastic species to the presence of specific plankton species. Seki and Taga (52) tested the effects of pH, temperature and salinity on growth of the chitinoclasts. Ultraviolet light resistance was also examined to determine lethal effects of solar radiation in the upper water layer.

Hood and Meyers (16) reported that the distribution of chitinoclasts in a salt marsh was related to three factors: 1) organic matter, 2) chitin deposition, and 3) temperature. Highest concentrations of chitinoclasts were found in the sediments. Peak concentrations of chitinoclasts in the water column coincided with peak concentrations of chitin-producing organisms. Penaeid shrimp had large numbers of chitinoclasts associated with their exoskeleton and digestive tract. These investigators felt that the organisms in the digestive tract possibly served multi-purposes including producing enzyme (chitinase), elaborating growth factors and/or serving as a food source.

Many workers have reported on the close association of chitinoclasts with chitin-containing organisms. Seki and Taga (51, 57) found large numbers of chitinoclasts associated with copepods in the water column. Jones (26) had shown that the concentration of bacteria on the surface of a radiolarian species was a thousand fold greater than in the surrounding sea water. Buck and Barbaree (10) reported

similar results using a large copepod species. Kaneko and Colwell (27) examined the adsorption of Vibrio parahaemolyticus onto chitin and copepods. They found the efficiency of adsorption was dependent on pH, NaCl concentration, and on other ion concentrations in seawater, particularly Mg^{++}. Surprisingly, a pH range of 8.0 to 9.0, which is the pH of normal seawater, was not conducive to adsorption; in fact, seawater in this range tended to prevent adsorption. Maximal adsorption was found in the pH range 4.0-8.0.

Several workers have recorded findings of large numbers of chitinoclasts in the digestive tracts of fishes. Hood and Meyers (16) suggested that "indigenous" bacteria of the digestive tract relate to the fact that only certain bacterial types can grow in a restrictive environment; e.g., not that one will consistently encounter only one or two types of bacteria, rather that if bacteria are encountered they will probably be of one or two types. The controversy surrounding "indigenous" bacteria was a result of the findings of Liston (32) who found a direct relationship between the species of bacteria and the species of fish in which they were found, of Potter and Baker (44) who failed to find such a correlation, and of Margolis (36) who regarded the bacterial flora of the digestive tract to be a function of the food consumed. Sera et al. (58) suggested that fish with developed stomachs have specific species of Vibrio indigenous to the digestive tract. They found that the total number of bacteria in both the

stomachs and intestines was not influenced by the composition of the diet. Okutani (43) found that over 90% of the intestinal, pyloric cecal, and stomach flora of the Japanese Sea Bass consisted of chitinoclasts, with populations ranging from 2.0×10^2 cell/g to 5.5×10^9 cells/g. Seki and Taga (54) reported chitinoclastic populations in the intestines of squid (4.3×10^5 cells/g), octopi (1.5×10^5 cells/g) and swell fish (4.4×10^3 cells/g) and in the digestive tracts of whales (55). Aiso et al. (1) found that the "indigenous" flora of the jackmackerels' digestive tract were almost exclusively vibrios. Chan (11) reported up to 1×10^7 chitinoclasts/g in the intestines of fishes of Puget Sound.

Rates of chitin degradation in the marine environment have appeared in several papers. Seki and Taga (57) have estimated that it would take 40 to 70 days to decompose one gram of planktonic chitin at 25 C based on in vitro studies using pure cultures. They also found that the rate was greatly affected by hydrostatic pressure. The rate at 200 atm was only 40% of the rate at 1 atm. Earlier Seki and Taga (52) noted that the growth of chitinoclasts at 15 C was half that at 25 C. From this data and data obtained from The Oceans (61) they calculated the rate of chitin decomposition in temperate oceans (based on 15 C) to be 70 to 110 days. Seki (50) republished these basic findings increasing their estimates to 140 days at 15 C and adding 370 days at 5 C and 500 days below 5 C (with the stipulation that hydrostatic

pressure be neglected). They also hypothesized that "if barophilic, psychrophilic, chitinoclastic bacteria are present in the deeper parts of the ocean, chitin would be expected to decompose within 140 days at any part of the ocean." In Seki and Taga's (53) earlier studies they employed N. D. Ierusalimski's formula to conclude that in vitro studies involving chitin decomposition are not unlike the results one would expect from in situ studies of the sea.

Chan (11) reported 80-130 μg of chitin decomposed per hour by 10^{10} chitinoclasts at 22 C. Converted to 19-31 mg/day/10^{10} bacteria at 22 C, this compares favorably with Seki's (50) findings of 30 mg/day/10^{10} bacteria at 25 C. Liston et al. (34) showed the rate of chitin decomposition in Puget Sound sediment as 18.8 mg/day. Hood (15) demonstrated a rate of 87 mg/day/g chitin in in situ studies of a salt marsh environment.

The effect of hydrostatic pressure on chitinase was examined by Kim and ZoBell (30) using commercially prepared chitinase (obtained from an actinomycete grown at 1 atm). They showed that this chitinase was extremely barostable and no differences were noted in activity at 1, 200, 500, or 1000 atm when tested at 4 C and 22 C, respectively. The overall rate of chitin decomposition at 4 C was much less than at 22 C.

MATERIALS AND METHODS

Bacterium and Culture Maintenance

A psychrophilic, marine vibrio designated ANT-500 was used. The organism, which was isolated from the Antarctic convergence during the 1971 cruises of the R/V ELTANIN, has a maximum growth temperature of 13.5 C, an optimum at 7 C and a minimum below -2 C.

MINIMAL SEAWATER SALTS (MSWS)

Sodium chloride	24.0 g
Potassium chloride	0.7
Magnesium chloride (hydrated)	5.3
Magnesium sulfate (hydrated)	7.0
Distilled water	1.0 liter
pH adjusted to 7.5	

LIB-X MEDIUM

Bacto yeast extract	1.2 g
Trypticase	2.3
Sodium citrate	0.3
L-glutamic acid	0.3
Sodium nitrate	0.05
Ferrous sulfate	0.005
MSWS	1.0 liter
pH adjusted to 7.5	

LIB-X (2) medium was used to grow and maintain the culture. Minimal seawater salts consisted of the major cation and anion constituents of seawater. The pH adjustments were made with dibasic potassium phosphate.

Growth Curves

Temperature optima for growth were determined for ANT-500. Using a temperature gradient incubator (Scientific Industries Inc., Mineola, NY), the gradient was set to include temperatures from -2.5 C to 30 C. Fifteen ml of LIB-X broth was added to each L-shaped tube. The tubes were sterilized at 120 C under 15 psi pressure for ten min and then placed in the gradient incubator and allowed to equilibrate for approximately two h.

A 5%-inoculum of ANT-500 was grown in 500 ml of LIB-X broth at 5 C for 96 h. The cells were centrifuged at 27,000 x g for 5 min at 0 C and were then washed two times with fresh LIB-X broth. (Unless otherwise specified all procedures were conducted under aseptic techniques). The cells were resuspended in LIB-X to a concentration which would yield about 1×10^6 cells/ml when one ml of the suspension was added to the 15 ml volume in an L-shaped tube.

The growth was monitored every six to twelve h by OD readings in a colorimeter (Bausch and Lomb Spectronic 20) at 600 nm. Plate counts using the spread plate technique were performed at the start

and finish and at 48-h intervals during the incubation period. Growth was observed over a 7-day period.

Salinity tolerance was measured as above. Minimal seawater salts solution was modified to yield salt concentrations (‰) of 10, 15, 20, 25, 30, 37, 50, 60, and 70.

Chitin Preparation

Commercially-available squid, Loligo opalescens, was used as the source of chitin. The cuttlebones, or pens, were removed and soaked in 1% hydrochloric acid for 96 h. The acid solution was changed every 24 h. The pens were washed with distilled water and placed into 2% potassium hydroxide for an additional 96 h. The solution was heated to just below boiling after each 24-h period and then exchanged for a fresh 2%-KOH solution. Again, the pens were washed with distilled water and placed into 95% ethyl alcohol for 24 h. The pens were washed with distilled water and allowed to air dry. The dried pens were placed into a Waring blender and pulverized.

Five hundred ml of concentrated sulfuric acid (specific gravity, 1.84) was mixed in a 2800 ml Fernbach flask with an equal volume of distilled water and cooled to 2 C. The temperature was maintained by placing the flask in ice in a 5 C controlled environment incubator. The solution was continually mixed using a magnetic stirrer (Bellco). The pulverized chitin (50-75 g) was added to the solution and allowed to

dissolve (18-24 h). The chitin was reprecipitated in 15 liter of distilled water cooled to 5 C in a 50 liter carboy. The sulfuric acid-chitin solution was poured through a Buchner funnel using glass wool as the filter into the distilled water.

After the colloidal chitin had settled out of solution, most of the liquid was decanted off. The remaining solution was centrifuged in 250 ml Nalgene centrifuge bottles at 10,400 x g for 10 min at 0 C. The supernatant was poured off and the chitin was pooled. Potassium hydroxide was used to adjust the pH to 7.0 ±0.5. The adjusted solution was poured into 4.8 cm diam. viscose dialysis tubing containing two large marbles to facilitate mixing. The tubes were placed in running distilled water for one week to remove excess ions. The tubes were hung to air dry.

When the chitin within the tube had reached the consistency of very thick paste, it was removed and resuspended in MSWS, pH 7.5, to a concentration of 0.15 to 0.3 g/ml. The chitin was stored in 200 ml dilution bottles after autoclaving for thirty min at standard temperature and pressure.

Chitin Media

Two chitin-containing agar media were routinely used, seawater-chitin agar (SWCA) (11), and LIB-X-Chitin agar. LIB-X-chitin broth was identical to LIB-X agar without the agar.

LIB-X-CHITIN AGAR

Reprecipitated chitin	60. 0 g
Bacto agar	15. 0
LIB-X medium	1. 0 liter
pH adjusted to 7. 5	

SEAWATER-CHITIN AGAR

Trypticase	1. 0 g
Bacto yeast extract	0. 5
Reprecipitated chitin	60. 0
Sodium chloride	15. 0
Rila marine salts	20. 0
Bacto agar	15. 0
Distilled water	1. 0 liter
pH adjusted to 7. 5	

Chitinase Induction

Several substrates were added to MSWS medium containing (g/liter) trypticase, 1. 0; and yeast extract, 0. 5 per liter. The substrates (g/liter) N-acetyl-D-glucosamine, 1. 0 and 10. 0; cellulose, 10. 0; D-glucose, 10. 0; D-glucosamine, 10. 0, and lactose, 10. 0, were employed to determine if they could induce ANT-500 to produce chitinase. Controls included no additives and chitin in concentrations of 1. 0 g/liter and 10. 0 g/liter. A 50 ml volume of medium was placed

into a 250 ml Erlenmeyer flask and was incubated at 5 C for four days in a PsycroTherm (New Brunswick Scientific, Inc., New Brunswick, NJ). The cells were then centrifuged at 27,000 x g for 5 min at 0 C. One ml of the supernatant was used for the chitinase assay. Samples were performed in duplicate.

Chitinase Assay

Purified chitin was added to a solution suspected of containing chitinase. The pH of the assay solution was controlled using a buffer system, and toluene was added to prevent bacterial activity in the reaction solution. After a designated reaction period the mixture was sampled and the concentration of N-acetyl-D-glucosamine (NAG) was determined as an indirect measurement of the amount of enzyme present in the original solution.

Two dibasic sodium phosphate-citric acid buffers were prepared to yield pH 5.1 and pH 7.5. Buffer at pH 5.1 was used for samples from fish stomachs and intestines and supernatant from ANT-500 cultures. Buffer at pH 7.5 was used for samples from sediments as well as culture supernatant of ANT-500. A chitin suspension of about 5 mg/ml in MSWS was used. MSWS was also used to bring assay solutions up to volume.

The following schematic procedure (22) was used:

1. One (1.0) volume chitin suspension plus one (1.0) volume

citric acid-sodium phosphate buffer plus one tenth (0. 1) to two (2. 0) volumes of enzyme-containing solution up to four (4) volumes with MSWS. To this mixture was added toluene.

2. Incubate at desired temperature for desired time.
3. Centrifuge sample (or 5-ml subsample) at 27, 000 x g for 5 min at 0 C.
4. Utilize 0. 5 ml of supernatant in NAG assay.

Fifty ml and 250 ml Erlenmeyer flasks were used for examining fish stomachs and sediments. Ten ml of buffer, chitin solution, and MSWS were combined with 5 ml toluene and 5 ml sample (both the contents of the stomachs and the sediment were diluted with MSWS approximately 1:10) in 250 ml flasks. Two ml of buffer, chitin solution, and MSWS were combined with one ml toluene and one ml of sample in the 50 ml flasks. The latter concentrations of reagents and sample (one ml of supernatant) were used in the L-shaped tubes to examine the supernatant of ANT-500.

The fish stomach and sediment samples were incubated at 5 C or 10 C in a PsycroTherm as well as 20 C on a desk-top rotary shaker (Laboratory rotary Model G-2, New Brunswick Scientific Co., Inc., New Brunswick, NJ) for a designated period of time. ANT-500 supernatant samples were incubated in the gradient incubator at various temperatures, salinities, pH's and periods of time. Controls

included assays 1) without sample, 2) without chitin, and 3) without toluene; volumetric difference was compensated for by addition of MSWS.

Chitinase Assays under Hydrostatic Pressure

Contents of fish stomachs were prepared for examination in concentrations of sample and reagents used in the previous experiments for the 250 ml flask assay. The 10 ml portion of chitin solution was added last, just prior to pressurization. Five ml plastic syringes (Becton, Dickenson and Company, Rutherford, NJ) with detachable needles were used. Five ml of the test solution was drawn into the syringe with the needle portion removed. The needle portions had previously been clipped and sealed by crimping to prevent any air or fluid from leaking into or from the syringe. The sealed needle was placed on the syringe. The syringes were then put into pressure cylinders similar to those described by ZoBell and Oppenheimer (64). The pre-equilibrated cylinders filled with water were sealed and pumped up to pressure using a hydrostatic pressure pump (Autoclave Engineers, Inc., Erie, PA). (Total time from the loading of the syringe to pumping up to pressure was less than one min.) The cylinders were incubated in a refrigerated water bath. All experiments were performed in duplicate.

Pressures of 1000, 500, 300, 100 and 1 atmospheres were employed. Incubation temperature was 5 C and the cylinders were incubated for 24 h.

After the incubation period, pressure was released from the cylinder. The contents of the syringe was emptied into a 15 ml Corex centrifuge tube (Corning) and centrifuged at 27, 000 x g for 5 min at 0 C. A 0. 5 ml sample, in duplicate, was tested for NAG according to the NAG assay listed below. Appropriate controls were also run.

N-acetyl-D-glucosamine Assay

The assay procedure was the Reissig et al. (46) modification of the Morgan-Elson (39) method for the colorimetric determination of N-acetyl-hexosamines. The assay is not a direct measure of the quantity of chitinase but rather it is a measure of the end product of the enzyme system.

Reagents used were a borate buffer (1. 12 mole boric acid + 0. 56 mole potassium hydroxide, pH 9. 1) and the color reagent, p-dimethyl-amino benzaldehyde (DMAB), (10 g DMAB in 100 ml glacial acetic acid with 12. 5% v/v concentrated hydrochloric acid; diluted 9x with glacial acetic acid for experimental use.)

The assay was performed in a 13 x 100mm screw-capped test tube. The assay was as follows:

1. 0. 1 ml borate buffer was added to 0. 5 ml of sample,

standard (specific concentrations of N-acetyl-D-glucosamine--5, 10, 20, 30, 40, and 50 μg NAG/ml of MSWS) or blank control (MSWS). (The standard curve is shown in Fig. 1.)

2. Heat tubes in boiling water bath for exactly three (3) min. Cool in running water or ice bath.
3. Add three (3) ml of diluted DMAB solution, mix, incubate at 36-38 C for exactly 20 min. Cool in running water or ice bath.
4. Read tubes at 585 nm in Bausch and Lomb Spectronic 20 within 20-30 min.

Sampling Area

Most samples were taken in the Yaquina Bay estuarine area at Newport, Oregon aboard the R/V Paiute. The area of the bay and Yaquina River studied was that between buoy 8 near the Yaquina Bridge (U. S. Highway 101) and buoy 15 just off Coquille Point in the bay, a distance of approximately 2. 25 miles (3. 62 km) (Zone I, Fig. 2). In the river, the sampling area, Zone II, was that from halfway between buoy's 19 and 20, just opposite Parker Slough, to an area past buoy 23, just South of Oneatta Point, a distance of one mile (1. 61 km).

Sediment samples were obtained near buoys 9 and 15.

The remainder of the fish samples came from the Pacific Ocean

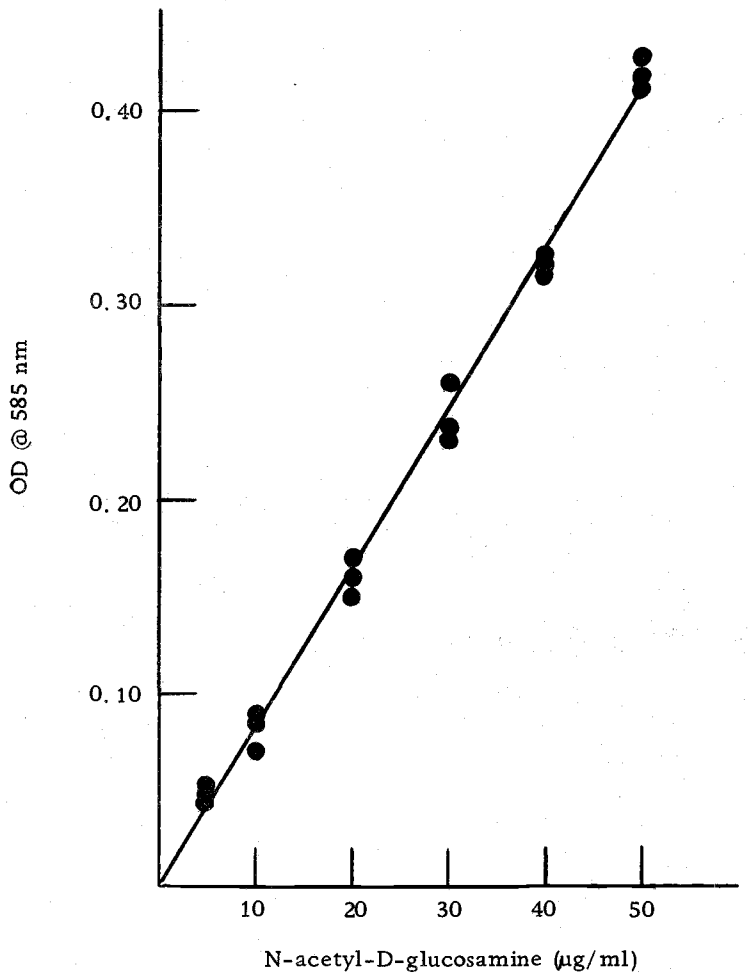

Figure 1. Standard curve of N-acetyl-D-glucosamine. Six concentrations of NAG were utilized to yield a standard curve for the conversion of OD readings at 585 nm to μg NAG produced. The assay was the Reissig et al. Modification of the Morgan-Elson colorimetric assay for N-acetyl-D-hexosamine. Correlation coefficient is 0.9974; m=0.00815; b = 0.00061.

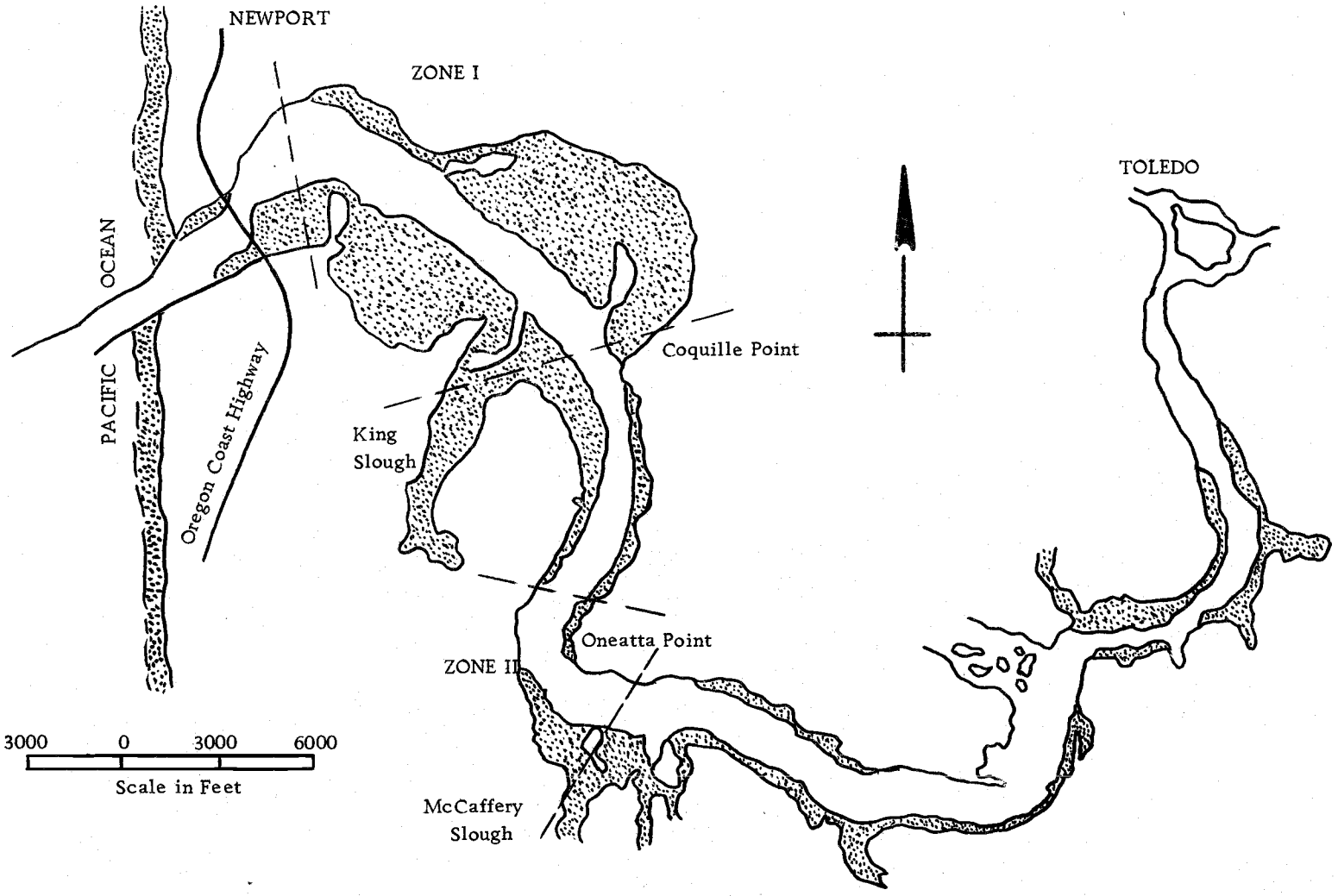

Figure 2. Location of study zones in Yaquina Bay, Oregon.

off the Oregon Coast on the continental shelf. The samples were taken on cruises of the R/V Cayuse by members of the biological oceanography contingent at Oregon State University.

Fish Sampling and Preparation for Assay

All fish were captured by use of an otter trawl net. Only bottom trawls were made in the bay and river. The fish were sorted on board ship and placed in ice chests with ice for transport back to the Oregon State University, Dept. of Microbiology, Corvallis, Oregon, about 56 miles (90 km) on the same day. Live fish were maintained in large plastic bags with approximately twelve liters of bay water on ice in an ice chest for transport. Fish captured on board the R/V Cayuse were dressed after capture and the stomach and intestines were placed in plastic bags and frozen due to the extended nature of the ocean cruises.

Upon arrival, the live fish were transferred to either 80 or 200 liter aquarium filled with aged seawater. The fish were maintained on shrimp obtained during the cruises and on squid.

The fish packed on ice were dissected within 24 h of capture and the stomach or intestinal contents, or portion thereof, transferred to sterile 20 x 150 mm screw-capped test tubes. The samples were diluted 1:10 with MSWS and mixed vigorously in a vortex-type

test tube mixer. Chitinase assays were run. One ml of the mixture was also placed in an aluminum weighing boat to determine dry weight (100 C for 24 h) and wet weight. In some cases a portion of the stomach or intestine content was frozen to determine the effects of freezing on samples, in particular those from the ocean cruises.

Sediment Sampling and Preparation for Assay

Sediment samples were obtained by using an orange-peel grab sampler. The sediment was transferred to a sterile 16 oz. (473 ml) jar. The jars were placed on ice for transport back to the lab. All samples were examined within 24 h. About two cm^3 of sediment was diluted with 10 ml MSWS and mixed. Chitinase activity as well as wet and dry weight determinations was made.

Fish

Among the most abundant bottom fish obtained and, therefore, most studied was the buffalo sculpin, Enophrys bison. Other members of the sculpin family caught included the red Irish lord, Hemilepidotus hemilepidotus, and the Pacific staghorn, Leptocottus armatus. The other abundant group of fishes studied were the flat fishes--starry flounder, Platichthys stellatus; sandsole, Psettichthys melanostictus; and English sole, Parophrys vetulus. A general population study of the fishes of Yaquina Bay was conducted by Beardsley

(5) and is presented in abridged form listing only those which were listed as common or abundant (Table 1).

Other fishes from the bay examined were the Pacific snake-blenny, Lumperus sagitta; lincod, Ophiodon elongatus; shiner perch, Cymatogaster aggregata; Pacific tomcod, Microgadus proximus; Pacific herring, Clupea harengus; skate, Raja binoculata; speckled sanddab, Citharichthys stigmaeus; cabezon, Scorpaenichthys marmoratus, and kelp greenling, Hexagrammos decagrammus. The dungeness and red rock crabs were also examined, Cancer magister and C. productus, respectively. Fishes studied from the ocean cruises were the sablefish, Anoplopoma fimbria, and rockfish, Sebastodes sp.

Origin of Chitinase in Sculpin Stomachs

Juvenile buffalo sculpin were captured in an otter trawl and transported live back to the laboratory at Oregon State University. The fish were kept in a 60 gallon aquarium with aged seawater obtained from the bay. They were maintained on a diet of shrimp and squid. Experimentation was performed in a 15 C walk-in incubator.

Four one-gallon fish bowls were prepared with three liters of Rila marine salts mixture (Rila Products, Teaneck, NJ) and covered with aluminum foil. Two bowls were autoclaved under standard conditions. Chlormaphenicol (CAP; Sigma) was added to each of the sterile

Table 1. Common and abundant fishes of Yaquina Bay, Oregon.[a]

Species	Common Name	Number[b]
Clupea harengus	Herring	Common
Engraulis mordax	Anchovy	Common
Hypomesus pretiosus	Surf smelt	Abundant
Microgadus proximus	Tomcod	Common
Cymatogaster aggregata	Shiner perch	Abundant
Embiocata lateralis	Striped perch	Abundant
Sebastodes sp.	Rockfish	Abundant
Hexagrammos decagrammus	Kelp greenling	Common
Ophiodon elongatus	Lingcod	Common
Enophrys bison	Buffalo sculpin	Common
Leptocottus armatus	Staghorn sculpin	Abundant
Pholis ornata	Saddleback gunnel	Common
Lumpenus sagitta	Snakeblenny	Abundant
Atherinopsis californiensis	Jacksmelt	Common
Citharichthys stigmaeus	Speckled sanddab	Common
Parophrys vetulus	English sole	Abundant
Platichthys stellatus	Starry flounder	Abundant

[a]Excerpt from Table 15, pp. 84-85, Beardsley (5).

[b]Number = Common (50 individuals); Abundant (1000 individuals).

bowls to yield 100 ug CAP/ml final concentration. Six fish (weight range, 25-30 g/fish) were placed into each of the four bowls.

Commercial pelletized fish food (prepared by OreAqua Foods, Newport, OR) containing shrimp and crab shells was used to feed the fish and induce chitinase activity. A portion of the food was sterilized by autoclaving at 120 C and 15 psi pressure for 30 min. Sterile food was placed into both a bowl with and without CAP. Similarly, non-sterilized food was placed into a bowl with and without CAP.

The fish were sampled at time zero, 24, 72, and 144 h. Two and one-half ml of sterile MSWS was added to the stomach contents of each fish by pipet through the mouth of the fish. Gut samples were withdrawn using the same procedure; one ml was used for plate count determinations on SWCA. Another ml was used for the NAG assay.

Effects of N-acetyl-D-glucosamine and Stomach Fluid on Growth Rates

Stomach fluid was obtained from the pooled contents of twelve buffalo sculpin. The contents were centrifuged at 31,000 x g for 15 min. The supernatant was filter sterilized through a 0.45 μm membrane filter.

One hundred-ml portions of LIB-X medium were added to eight flasks. Ten ml of the sterile stomach fluid was added to each of four flasks; ten ml of LIB-X medium was added to the remaining flasks.

One-tenth g of NAG was added to two flasks with gut fluid and two flasks with LIB-X only.

Bacterial cultures used for the growth rate determination were isolated from the stomach of a buffalo sculpin. A pure culture of a chitinoclast and a mixed culture of all bacterial types isolated were grown in LIB-X medium at 10 C.

One set of flasks, 1) LIB-X, 2) LIB-X + NAG, 3) LIB-X + gut fluid, and 4) LIB-X + NAG + gut fluid, was inoculated with a 0.5% inoculum of pure culture and one set with mixed culture. The flasks were incubated at 15 C on desk-top rotary shakers at 125 rpm. Growth was followed by OD readings at 600 nm. Plate counts were performed at time zero and at various times during the incubation using LIB-X-chitin agar.

RESULTS

The marine vibrio ANT-500 has a maximum growth temperature of 13.5 C at salinities of 15‰, 25‰, 30‰ and 37‰. The maximum growth temperature decreased to 7.5 and 6.5 C at salinities of 50‰ and 60‰, respectively. There was no growth at either 10‰, or at 70‰. The minimum growth temperatures for ANT-500 varied with salinity and were -1.5 and -1.0 C at 15‰ and 60‰, respectively, whereas at all intermediate salinities the minimum growth temperature was -2.5 C or less. The optimum growth temperature also varied with the salinity (Fig. 3).

Extracellular chitinase from a five-day ANT-500 culture gave three peaks of activity over a temperature range -2.0 C to 42.0 C. Using a 24-h incubation, supernatant assayed at pH 5.5 showed a small peak at 7.0 C, a large peak at 17.0 C, and a broad peak at 29.5 C with a shoulder at 32.0-33.5 C (Fig. 4). Activity dropped off rapidly at 36.5 C, with little or no activity at 40.0 C and above. The same enzyme preparation assayed at pH 7.5 showed a peak at 19.0 C, a large peak at 23.0 C, and a smaller peak at 35.0 C (Fig. 5).

Screw-capped test tubes containing 5 ml Seawater-Chitin Agar (SWCA) were streaked with ANT-500 and incubated over the temperature range -1.0 C to 19.0 C to determine, qualitatively, at which temperatures chitinase was produced. Growth occurred in all tubes

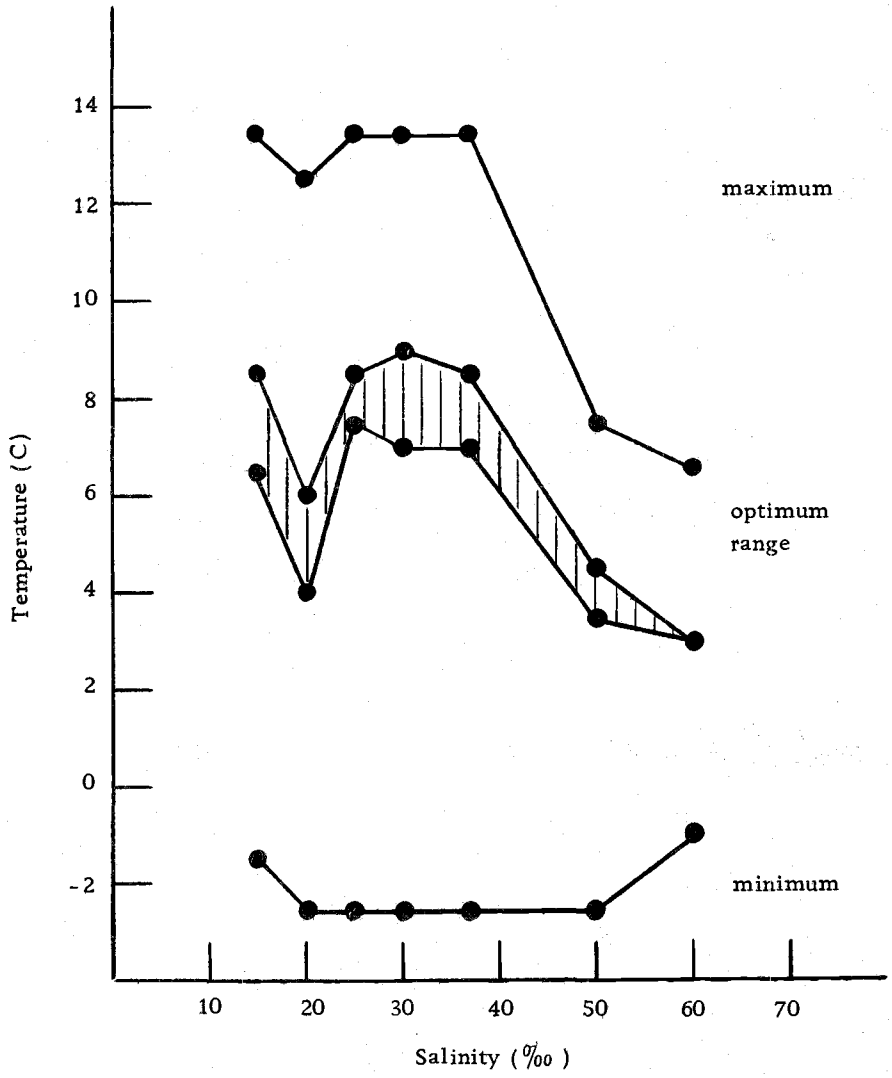

Figure 3. Temperature-salinity relationship of ANT-500. Maximum, minimum, and optimum temperatures for growth were determined at various salinities on a reciprocating temperature gradient incubator. Incubation time was 168 h; OD readings at 6-12 h intervals at 600 nm. IIIIII = optimum growth temperature range.

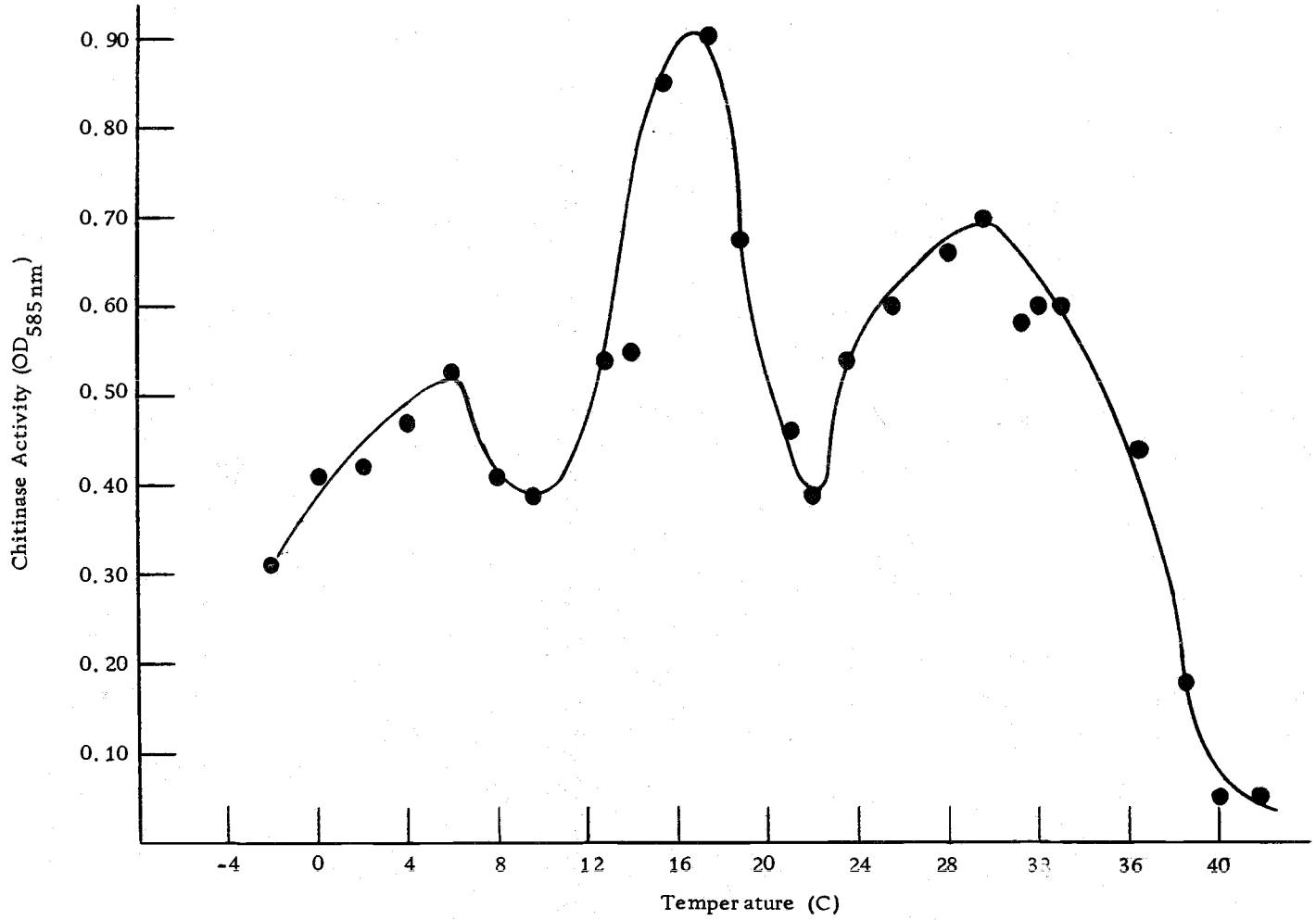

Figure 4. Chitinase activity of ANT-500 crude enzyme system at pH 5.5. Colorimetric determination ($OD_{580\,nm}$) of N-acetyl-D-glucosamine produced from one ml of culture supernatant. Incubation period was 24 h.

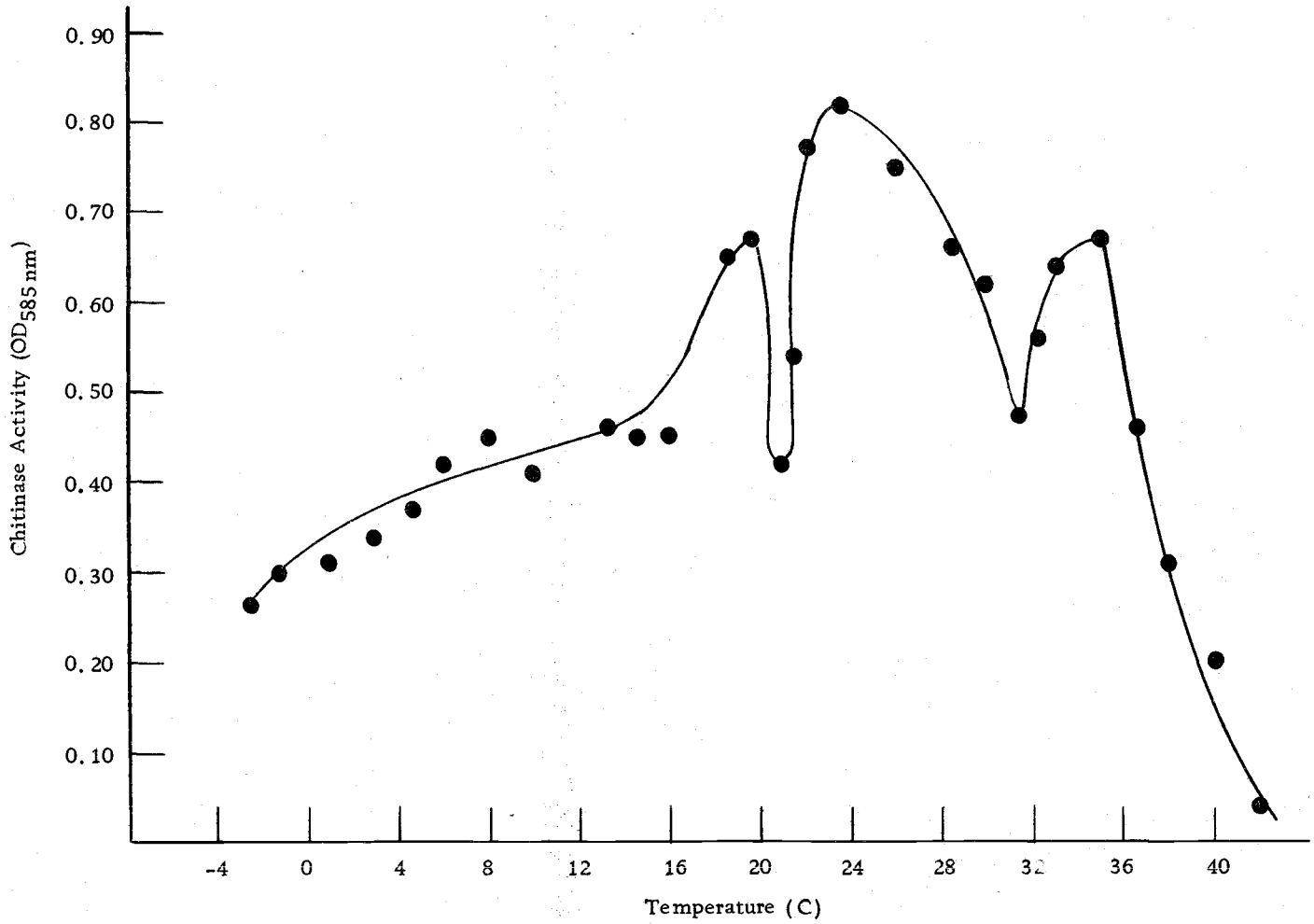

Figure 5. Chitinase activity of ANT-500 crude enzyme system at pH 7.5. Colorimetric determination ($OD_{585\,nm}$) of N-acetyl-D-glucosamine produced from one ml of culture supernatant. Incubation period was 24 h.

from -1.0 C to 13.5 C, inclusive. No hydrolysis (appearance of clear ring around a colony) was apparent at 1.5 C or below. Hydrolysis did occur in all remaining tubes which showed growth (Table 2).

ANT-500 has an inducible chitinase system. Chitin was the only substrate tested which would induce enzyme production. Cellulose, D-glucose, D-glucosamine, and D-lactose showed no inducible properties (Table 3). N-acetyl-D-glucosamine (NAG) results could not be interpreted, due to the high residual NAG present in the control.

Chitinase assays were performed on ANT-500 supernatant which had been filtered through sterile 0.45 μm membrane filters (Millipore type HA). The supernatant was assayed in the presence and absence of toluene to determine its effect on the assay procedure. No differences were observed (Table 4).

Enzyme kinetic values were determined on a crude enzyme fraction of culture supernatant. Plotting values of 1/chitin concentration vs. 1/units of NAG produced (Fig. 6) showed that some aspect of the enzyme system was applicable to Mechaelis-Menton kinetics. Linear regression on the data points gave a 0.9964 correlation coefficient.

No chitinase activity was detected in samples obtained from sediments in Yaquina Bay. To determine if the sediment caused a masking or inhibiting effect, crude enzyme from ANT-500 was added to additional sediment samples. Assays indicated there was no adverse

Table 2. Whole cell growth and chitin hydrolysis by ANT-500.

	Temperature (C)													
	-1.0	0	1.5	3.0	4.5	6.0	7.2	8.5	10.0	11.0	12.0	13.5	14.5	15.5
Growth[a]	+	+	+	+	+	+	+	+	+	+	+	+	-	-
Hydrolysis	-	-	-	+	+	+	+	+	+	+	+	+	-	-

[a] positive = +, negative = -.

Table 3. Substrate for induction of chitinase in ANT-500.

Inducer	$OD_{585\ nm}$[a]	
	Test	Control (NOCH)[c]
Nothing[b]	0.01	0.01
1% NAG	∞	∞
0.1% NAG	1.00	0.95
1% cellulose	0.01	0.01
1% glucose	0.01	0.01
1% glucosamine	0.03	0.03
1% lactose	0.01	0.01
1 ml chitin	0.10	0.01
0.1 ml chitin	0.05	0.01

[a]Chitinase activity of one ml supernatant of ANT-500 culture incubated 5 days at 5 C in presence of inducer.

[b]Basal medium = Minimal Seawater Salts + 0.1% peptone + 0.05% yeast extract + inducer.

[c]No chitin added to reaction mixture.

Table 4. Effect of toluene on chitinase activity of membrane filtered supernatant of ANT-500.

	$OD_{585\ nm}$
Supernatant	0. 23
Supernatant + Toluene	0. 23
Chitin control	0. 01
Chitin control + Toluene	0. 01
Blank	0. 00
Blank + Toluene	0. 00

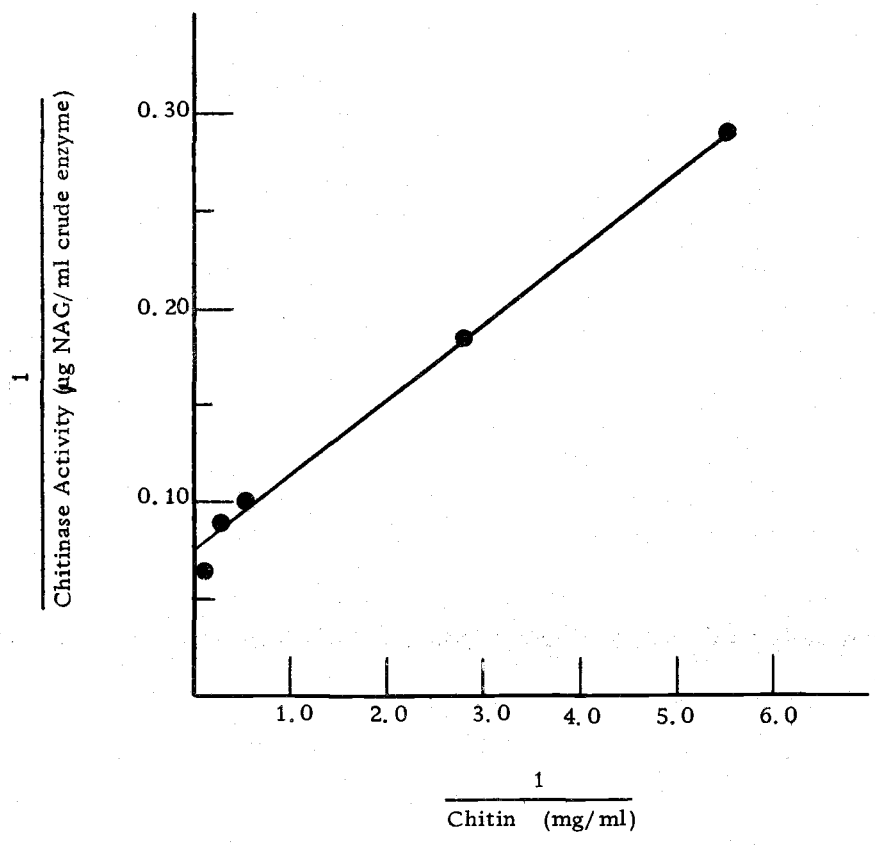

Figure 6. Effect of chitin concentration on enzyme activity. Reciprocal of µg N-acetyl-D-glucosamine produced per ml crude enzyme per unit time v. the reciprocal of chitin concentration (mg/ml). Correlation coefficient is 0.9964; m = 0.367; b = 0.0732; V_{max} = 13.65 µg NAG/ml enzyme·t; K_m = 0.50 mg/ml chitin. Incubation time was 24 h.

effect on the ability of the enzyme to breakdown chitin (Table 5). Low levels of chitinase activity were found in sediments from offshore (Table 6).

Only two species of fish were routinely obtained in large numbers from the otter trawls. Starry flounder (Platichthys stellatus) and buffalo sculpin (Enophrys bison) were the most abundant species captured; therefore, most interest was directed toward them. However, many other fish species did occur and were assayed when possible.

The stomach contents from rockfish (Sebastodes spp.) showed very high chitinase activity (Table 7) ranging as high as 1070 μg NAG produced per hour per gram dry weight of gut was obtained at 20 C. This compared with a low of 255 μg NAG/g. h at 20 C with an average of four samples of 650 μg/g. h. The range at 5 C was 196-771 μg NAG/g. h with an average of 490 μg NAG/g. h.

Sablefish (Anoplopoma fimbria) had slightly lower activities (Table 7). At 20 C the range was 321-547 μg NAG/g. h with a four sample average of 420 μg NAG/g. h and at 5 C, range 132-328; average, 230 μg NAG/g. h.

Due to their small size, the stomach of speckled sanddabs (Citharichthys stigmaeus) were pooled--2 to 4 stomachs per sample (Table 7). Activities ranged from 14. 9 μg NAG/g. h at 5 C up to 69. 7 μg NAG/g. h at 20 C.

Table 5. Chitinase activity in seawater and sediment from Yaquina Bay, Oregon.

Assay Mixture	Seawater	Sediment	Control[a]
1 ml chitin + 1 ml sample + 1 ml buffer + 1 ml ASW	0. 0	0. 0	0. 0
1 ml supernatant + 1 ml sample + 1 ml buffer + 1 ml ASW	0. 007	0. 006	0. 01
1 ml chitin + 1 ml enzyme + 1 ml sample + 1 ml ASW	0. 29	0. 29	0. 30
1 ml sample + 1 ml buffer + 2 ml ASW	0. 0	0. 0	0. 0

[a]For controls, one ml of ASW was substituted for one ml of sample.

Table 6. Chitinase activity in sediments off-shore from Yaquina Bay, Oregon.

Station[a]	Depth in Meters	Chitinase activity[b] µg NAG/g dry wt sediment	
		Sample	Control[c]
D	600	230	10
C	550	267	22

[a]Station designations used by crew of Yaquina cruise No. 47503-E.

[b]Activities resulting from 20 h incubation.

[c]No chitin added to reaction mixture.

Table 7. Chitinase activity in stomach contents of Anoplopoma fimbria, Sebastodes sp., Citharichthys stigmaeus, and Cancer magister.

Fish	Date Sampled	# Sampled	Assay Temperature	Range of Activity μg NAG/g·h	Average
Anoplopoma fimbria (Sablefish)	March 25, 1975	4	5 C	132-328	230
		4[a]	20 C	321-547	420
Sebastodes sp. (Rockfish)	March 25, 1975	4	5 C	196-771	496
		4	20 C	255-1060	650
Citharichthys stigmaeus (Speckled sanddab)	October 29, 1974	2[b]	10 C	51.1	-
	November 20, 1974	3[b]	5 C	14.9-38.2	26.2
		3	10 C	22.0-44.2	32.4
		3	15 C	27.7-57.0	44.8
		3	20 C	40.5-69.7	55.1
Cancer magister (crab)	October 29, 1974	2	10 C	146.3-158.5	152.4
	December 5, 1974	4	10 C	119.3-761.1	392.9
	February 25, 1975	1	5 C	159.4	-
		1	20 C	229.8	-

[a] = sample assayed at two temperatures indicated.

[b] number sampled were pooled prior to assay.

Crabs (Cancer magister) were the only non-fish sampled and assayed (Table 7). Activities of 146, 158, 164 and 119 μg NAG/g· h were obtained at 10 C in October and December, 1974. One crab assayed in February, 1975 gave 159 and 230 μg NAG/g· h at 5 C and 20 C, respectively.

Other species of fish were captured occasionally in small numbers, usually one or two per sampling trip. These data are shown in Table 8.

Effects of hydrostatic pressure were measured on chitinase activity in stomachs from skate (Raja binoculata), herring (Clupea harengus) and starry flounder (Platichthys stellatus). The levels of NAG produced (μg NAG/g dry weight of gut) over a 24 hour period were neither greatly enhanced nor inhibited at pressures from one atm to 1000 atm (Table 9). Average values of 420, 491, 422, 416, and 485 μg NAG/g were obtained from skate stomachs at 1, 100, 300, 500, and 1000 atm, respectively, at 5 C and 813 μg NAG/g at one atm and 20 C. When the same gut contents were assayed under anaerobic conditions, 493 μg NAG were produced at one atm and 5 C.

The herring and starry flounder gut contents gave a different pattern of NAG production under pressure. The chitinase activity in the herring was 186 μg NAG/g in 20 h at one atm and 5 C. At increased pressures, more NAG was produced (400, 387 and 347 μg NAG at 100, 300 and 500 atm at 5 C, respectively) than at one atm.

Table 8. Chitinase activity in stomach contents of several fishes from Yaquina Bay, Oregon.

Fish	Date	# Sampled	Assay Temperature	Range of Activity µg NAG/g·h	Average
Perch	October 29, 1974	2	10 C	9.7-57.2	33.4
	June 17, 1975	8[b]	5 C	-	20.6
		8[a]	20 C	-	28.9
	October 8, 1975	1	10 C	-	0
		1	20 C	-	0
Lingcod	October 29, 1974	1	10 C	-	28.0
	December 5, 1974	1	10 C	-	398.2
		1	20 C	-	637.4
	October 15, 1975	1	10 C	-	28.5
		1	20 C	-	57.0
Blackcod	December 10, 1974	1[c]	5 C	-	7.7
		1	20 C	-	12.8
Tomcod	June 17, 1975	1	5 C	-	26.0
		1	20 C	-	76.7
Herring	October 29, 1974	1	10 C	-	56.4

Table 8. (Continued)

Fish	Date	# Sampled	Assay Temperature	Range of Activity μg NAG/g·h	Average
Herring (cont'd)	June 4, 1975	1	5 C	-	9.3
		1	20 C	-	21.4
Snakeblenny	June 17, 1975	1	5 C	-	23.2
		1	20 C	-	33.6
Staghorn sculpin	June 17, 1975	6[c]	5 C	-	39.1
		6	20 C	-	113.0
	October 8, 1975	2	10 C	0-0	0
		2	20 C	0-0	0
English sole	December 5, 1974	1	10 C	-	50.7
	December 5, 1974	1	10 C	-	5.4
		1	20 C	-	14.1
Sand sole	December 5, 1974	1	10 C	-	47.8
	December 10, 1974	1[c]	5 C	-	19.9
		1	20 C	-	39.8

Table 8. (Continued)

Fish	Date	# Sampled	Assay Temperature	Range of Activity μg NAG/g·h	Average
Sand sole (cont'd)	March 11, 1975	1	5 C	-	692.0
		1	20 C	-	1051.0
Kelp greenling	October 9, 1975	1	10 C	-	0
		1	20 C	-	0
	October 15, 1975	1	10 C	-	459.8
		1	20 C	-	395.7
Cabezon	October 15, 1975	2	10 C	69.4 - 179.3	124.3
		2	20 C	108.5 - 228.5	168.5

a { = sample assayed at two temperatures indicated.

b Number sampled were pooled prior to assay.

c Intestine contents, not stomach.

Table 9. Effect of hydrostatic pressure on chitinase activity in stomachs of Raja binoculata, Clupea harengus, and Platichthys stellatus.

	Chitinase Activity (µg NAG produced/g dry wt gut)					
Pressure (atm)		1	100	300	500	1000
Temperature (C)	20	5	5	5	5	5
Species of Fish						
Raja binoculata (Skate)	836	413 (599)[a]	547	402	454	536
Raja binoculata (Skate)	798	426 (388)[a]	436	442	377	431
Clupea harengus (Herring)	427	186	400	387	347	-
Platichthys stellatus (Starry Flounder)	196	174	262	229	196	-

[a] anaerobic assay.

It is noted that, unlike the skate, the amount produced by herring and starry flounder decreased with increasing pressure. The same was true of gut activity from the starry flounder. At one atm and 5 C, 174 μg NAG were produced, while at 100, 300 and 500 atm and 5 C 262, 229 and 196 μg NAG were produced, respectively. This compares with 196 μg NAG produced at 20 C and one atm from the same starry flounder gut sample.

The contents of buffalo sculpin (Enophrys bison) intestines gave low levels of chitinase activity, 15.4 and 18.2 μg NAG/g· h at 10 C (Table 10). The activity doubled to 33.2 μg NAG/g· h at 20 C. Similarly, in the absence of visual chitinous animals in the stomach, the levels of activity were low--14.1 μg NAG/g· h at 5 C and 59.2 μg NAG/g· h at 20 C--or no detectable activity, as was the case of two stomachs assayed on October 15, 1975 and October 22, 1975 at both 5 C and 20 C.

The stomach contents of sculpin collected in February and March, 1975 were predominantly shrimps and crabs. The levels of chitinase activity were highest during these two collection trips with maximum activities of 365 and 632 μg NAG/g· h at 5 C and 20 C, respectively. Lower chitinase activities were found in those stomachs containing vegetation and/or fishes in addition to chitinous animals. Ranges of activity at 20 C were 123-163 μg NAG/g· h. A level of 111 μg NAG/g· h at 5 C was obtained for three pooled stomachs on

Table 10. Chitinase activity in stomach contents of Enophrys bison (Buffalo sculpin).

Date Sampled	# Sampled	Chitin[a]	Assay Temperature	Range of Activity µg NAG/g·h	Average
October 29, 1974	1[b]	+	10 C	51.1	51.1
	1[c]	?	10 C	18.2	18.2
December 5, 1974	3	++	10 C	107-249	157
December 5, 1974	1[c]	?	10 C	15.4	15.4
	1[c]	?	20 C	33.2	33.2
February 25, 1975	4	++	5 C	193-365	282
	3	++	20 C	440-632	558
March 11, 1975	2	++	5 C	123-192	157
	2	++	20 C	251-385	318
March 11, 1975	1	-	5 C	14.1	14.1
	1	-	20 C	59.2	59.2
June 17, 1975	3[d]	+	5 C	111	111
	3[d]	+	20 C	123	123
October 15, 1975	1	-	10 C	0	0
	1	-	20 C	0	0

Table 10. (Continued)

Date Sampled	# Sampled	Chitin[a]	Assay Temperature	Range of Activity µg NAG/g·h	Average
October 22, 1975	3	+	10 C	95-143	112
	3	+	20 C	144-163	155
October 22, 1975	1	-	10 C	0	0
	1	-	20 C	0	0

[a] Chitin = refers to presence or absence of chitinous animals in stomach contents.
++ = predominantly chitinous animals in stomach
+ = some chitinous animals plus vegetation and/or fishes
- = no chitinous animals present
? = due to digested state of intestine contents, positive identification of chitinous animals not possible by visual means.

b { = Samples assayed at two temperatures indicated.

[c] Intestinal contents, not stomach.

[d] Number sampled were pooled prior to assay.

June 17, 1975.

Lack of chitinous animals in the stomach of starry flounder showed results similar to those found for sculpin (Table 11). Many guts had no chitinase activity and even when activity was present, values were low (9.7 and 14.5 μg NAG/g· h at 10 C and 20 C, respectively).

Guts containing predominantly shrimp and crabs (March 11, 1975) gave maximum values of 402 and 690 μg NAG/g· h at 5 C and 20 C, respectively. Guts containing lesser amounts of chitinous material showed an overall activity range of 23.3-98.7 μg NAG/g· h with an average of 44.8 μg NAG/g· h at 10 C. At 20 C the range was 45.5-85.0 μg NAG/g· h with an average 61.5 μg NAG/g· h.

Time sequence studies of chitinase activity were carried out using guts from sculpin, starry flounder, and sand sole. Time increments of one, two, three, and four hours were used in this study. Activity in μg NAG produced/g dry weight of gut was plotted against time and linear regression analysis was applied to the data. Slopes (μg NAG/g· h) and correlation coefficients were determined. Samples were collected during March, 1975.

Slopes of 133.5 and 151.7 μg NAG/g· h were obtained from the two sculpin stomachs at 5 C, based on correlation coefficients of 0.976 and 0.930, respectively (Figs. 7 and 8). At 20 C the slopes were 255.6 and 292.1 μg NAG/g· h. The correlation coefficients were

Table 11. Chitinase activity in stomach contents of Platichthys stellatus (Starry flounder).

Date	# Sampled	Chitin[a]	Assay Temperature	Activity Range µg NAG/g·h	Average µg NAG/g·h
October 29, 1974	1	+	10 C	98.7	-
November 20, 1974	3	+	5 C	17.1-39.2	26.2
	3[b]	+	10 C	26.4-45.8	33.6
	3	+	15 C	35.6-53.9	43.7
	3	+	20 C	45.5-85.0	61.5
November 20, 1974	1	-	5 C	6.0	-
	1	-	10 C	9.7	-
	1	-	15 C	12.7	-
	1	-	20 C	14.5	-
December 5, 1974	4	+	10 C	23.3-58.7	39.7
March 11, 1975	2	++	5 C	344-402	373
	2	++	20 C	495-690	592
June 4, 1975	1	-	5 C	8.7	-
	1	-	20 C	9.8	-
October 8, 1975	4	-	10 C	0.0-9.4	4.3
	4	-	20 C	0.0-11.9	5.9

Table 11. (Continued)

Date	# Sampled	Chitin[a]	Assay Temperature	Activity Range µg NAG/g· h	Average µg NAG/g· h
October 15, 1975	3	-	10 C	0	0
	3	-	20 C	0	0
October 22, 1975	4	-	10 C	0	0
	4	-	20 C	0	0

[a] Chitin refers to presence or absence of chitinous animals in stomach contents.
++ = predominantly chitinous animals in stomach
+ = some chitinous animals plus vegetation and/or fishes
- = no chitinous animals present

[b] Sample assayed at temperatures indicated.

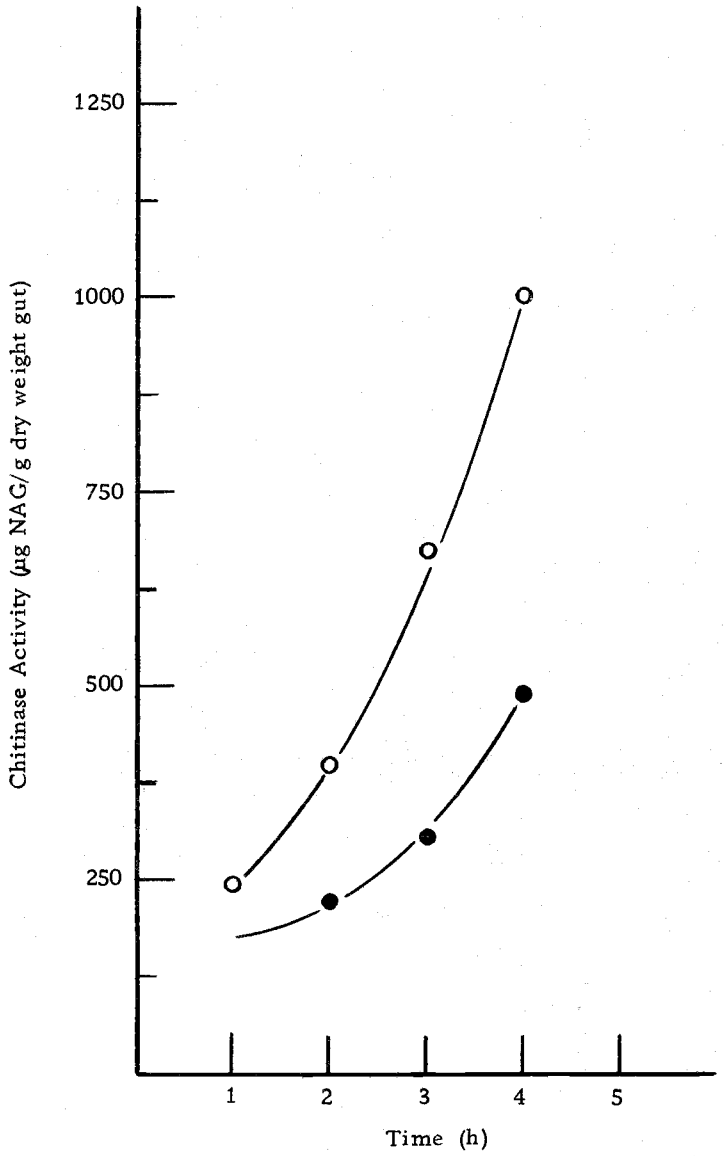

Figure 7. Chitin degradation vs. time by stomach contents of Enophrys bison. Chitinase activity was determined on gut contents of buffalo sculpin at t= 1, 2, 3, 4 h at 5 C (●) and 20 C (○). At 5 C, correlation coefficient was 0.9763; m = 133.5 µg NAG/g dry wt gut·h and at 20 C, c.c. = 0.9881; m = 255.6 µg NAG/g·h.

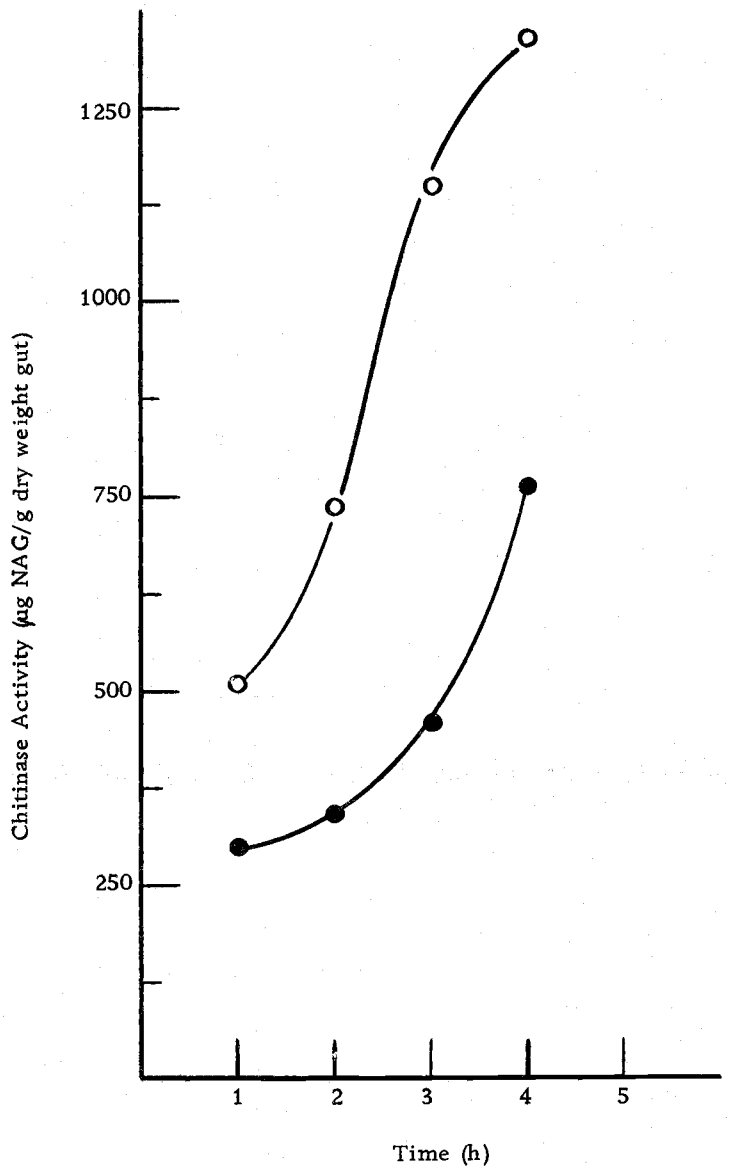

Figure 8. Chitin degradation vs. time by stomach contents of Enophrys bison. Chitinase activity was determined on gut contents of buffalo sculpin at t = 1, 2, 3, 4 h at 5 C (●) and 20 C (○). At 5 C, correlation coefficient was 0.9296; m = 151.7 µg NAG/g dry wt·h and at 20 C, c.c = 0.9895; m = 292.1 µg NAG/g·h.

0. 988 and 0. 990, respectively. Slopes of 222. 5 and 131. 1 μg NAG/g· h--correlation coefficients, 0. 937 and 0. 997, respectively--were obtained from the two starry flounder guts at 5 C (Figs. 9 and 10). At 20 C the slopes were 265. 3 and 228. 0 μg NAG/g· h with correlation coefficients of 0. 993 and 0. 975, respectively. The sand sole gut gave a slope of 221. 1 μg NAG/g· h at 5 C and 175. 9 μg NAG/g· h at 20 C. Correlation coefficients were 0. 985 at 5 C and 0. 999 at 20 C (Fig. 11).

Whereas the above guts contained predominantly chitinous animals, time sequence studies were also conducted on guts containing vegetation as well as chitinous material. Sculpin gut samples were taken on October 22, 1975. Slopes of 51. 8, 52. 7, and 96. 6 μg NAG/g· h were obtained at 10 C with correlation coefficients of 0. 961, 0. 961 and 0. 999, respectively (Figs. 12, 13, 14). At 20 C the slopes were 97. 0, 105. 5 and 103. 0 μg NAG/g· h. Correlation coefficients were 0. 999, 0. 990 and 0. 997, respectively.

Plate counts of gut contents from buffalo sculpin and starry flounder are shown in Table 12. The total counts ranged from 1. 6 x 10^7 to 1. 3 x 10^{10} bacteria/g dry wt gut in the flounder and 1. 3 x 10^7 to 3. 9 x 10^{10} bacteria/g dry wt gut in the sculpin. The percent of chitinoclasts varied with the contents of the gut. Stomachs with no chitinous material had low percentages of chitinoclasts (0 to 11%), while those with predominantly chitinous contents had from 90 to 95%.

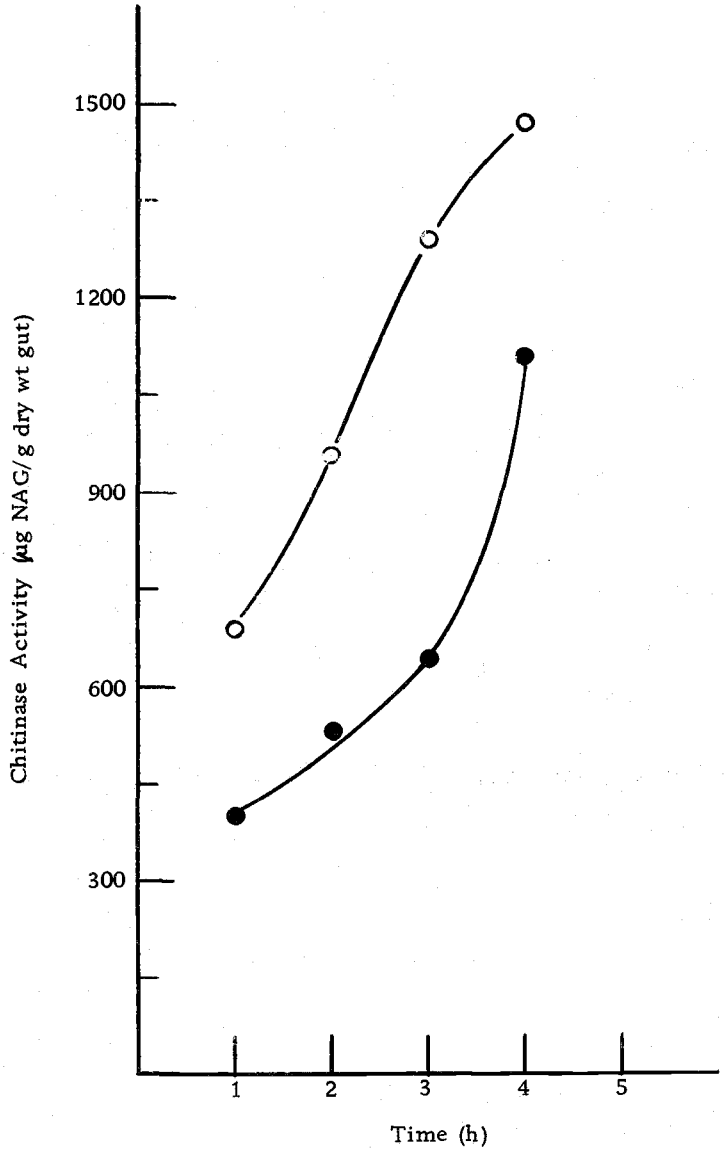

Figure 9. Chitin degradation vs. time by stomach contents of Platichthys stellatus. Chitinase activity was determined on gut contents of starry flounder at t = 1, 2, 3, 4 h at 5 C (●) and 20 C (○). At 5 C, correlation coefficient was 0.9371; m = 222.5 µg NAG/g dry wt gut·h and at 20 C, c.c. = 0.9932; m = 265.3 µg NAG/g·h.

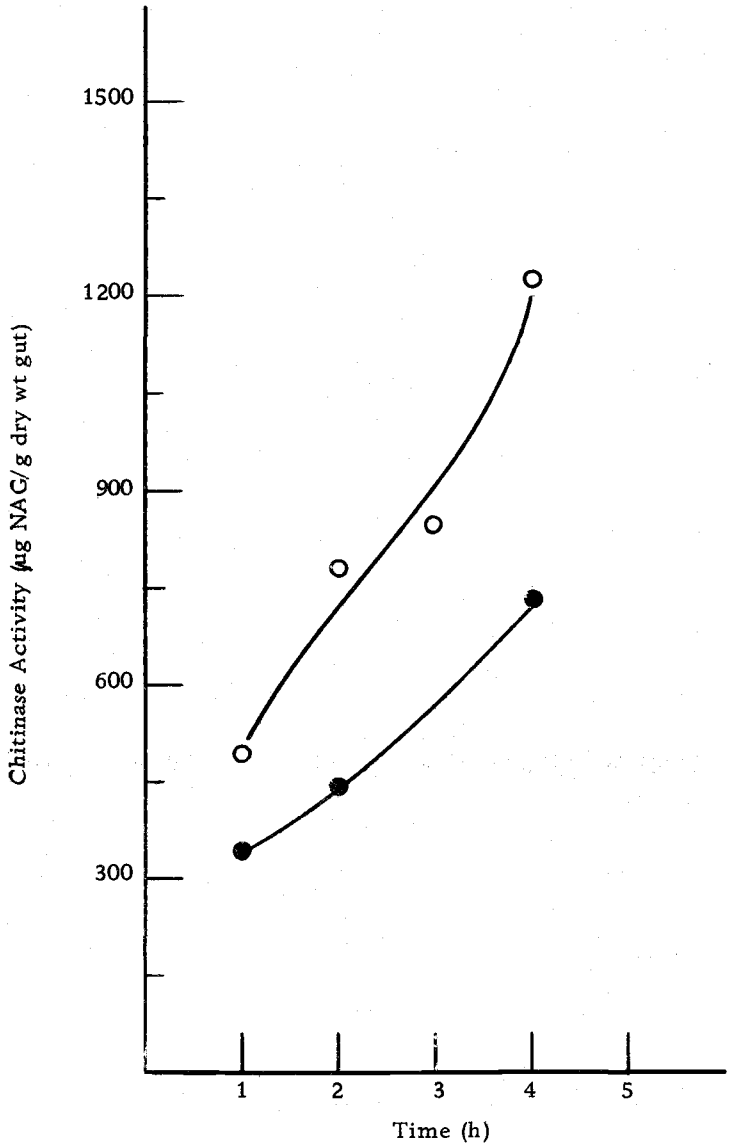

Figure 10. Chitin degradation vs. time by stomach contents of Platichthys stellatus. Chitinase activity was determined on gut contents of starry flounder at t = 1, 2, 3, 4 h at 5 C (●) and 20 C (○). At 5 C, correlation coefficient was 0.9968; m = 131.1 µg NAG/g dry wt gut· h and at 20 C, c.c. = 0.9747; m = 228.0 µg NAG/g· h.

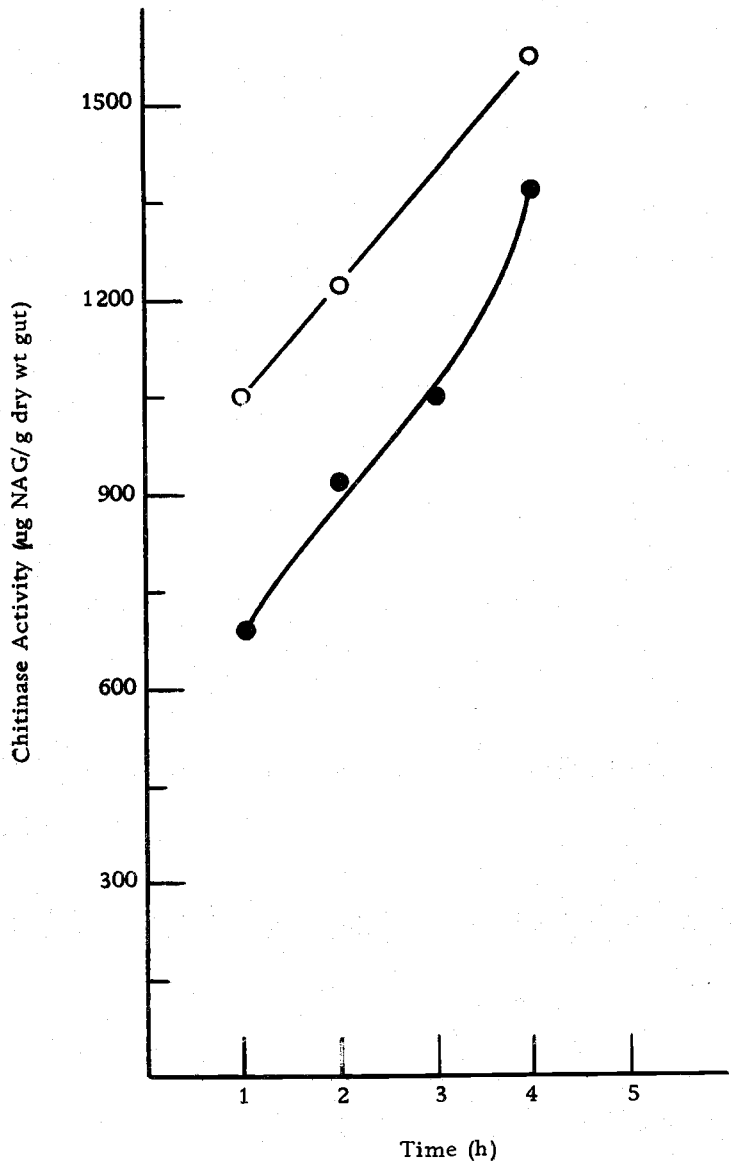

Figure 11. Chitin degradation vs. time by stomach contents of Psettichthys melanostictus. Chitinase activity was determined on gut contents of sand sole at t = 1, 2, 3, 4 h at 5 C (●) and 20 C (○). At 5 C, correlation coefficient was 0.9852; m = 221.1 µg NAG/g dry wt gut·h and at 20 C, c.c. = 0.9998; m = 175.9 µg NAG/g.h.

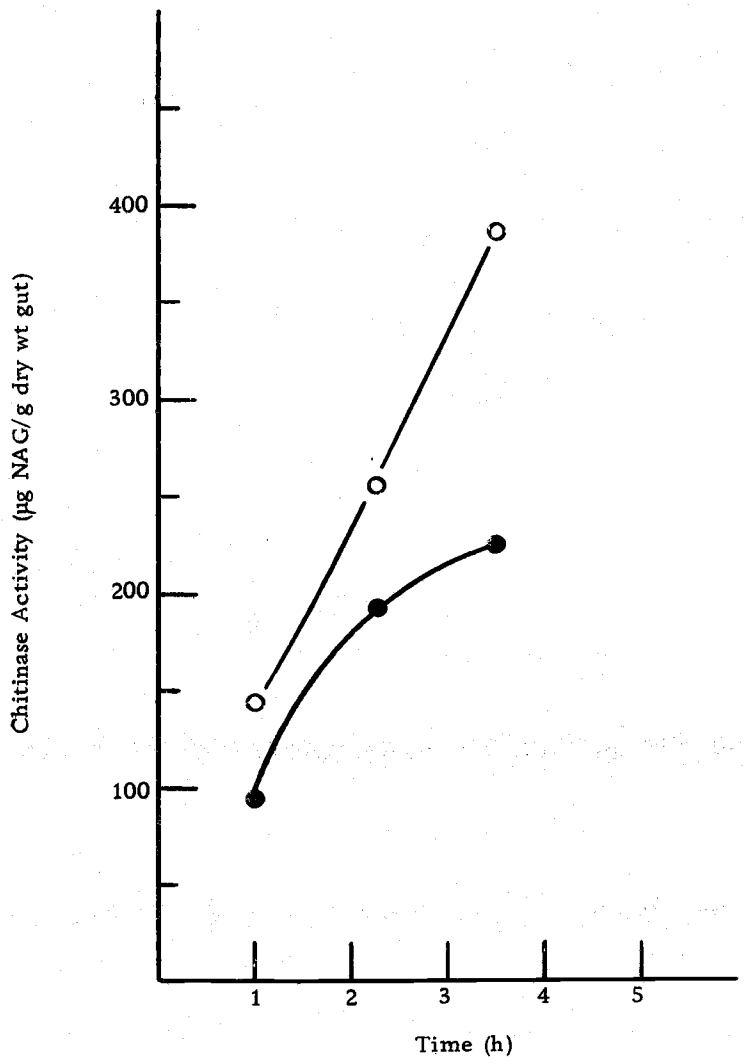

Figure 12. Chitin degradation vs. time by stomach contents of Enophrys bison. Chitinase activity was determined on gut contents of buffalo sculpin at t = 1, 2 1/4, 3 1/2 h at 10 C (●) and 20 C (○). At 10 C, correlation coefficient was 0.9608; m = 51.8 µg NAG/g dry wt gut· h and at 20 C, c.c. = 0.9992; m = 97.0 µg NAG/g· h.

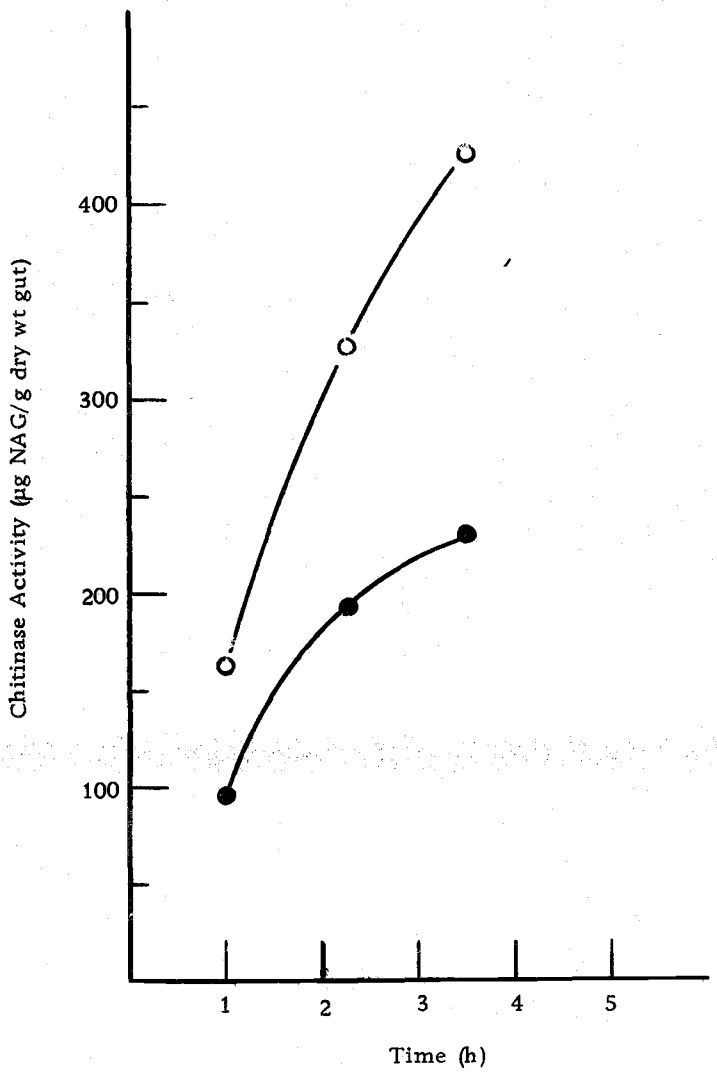

Figure 13. Chitin degradation vs. time by stomach contents of Enophrys bison. Chitinase activity was determined on gut contents of buffalo sculpin at t = 1, 2 1/4, 3 1/2 h at 10 C (●) and 20 C (O). At 10 C, correlation coefficient was 0. 9608; m = 52. 7 µg NAG/g dry wt gut· h and at 30 C, c. c. = 0. 9897; m = 105. 5 µg NAG/g· h.

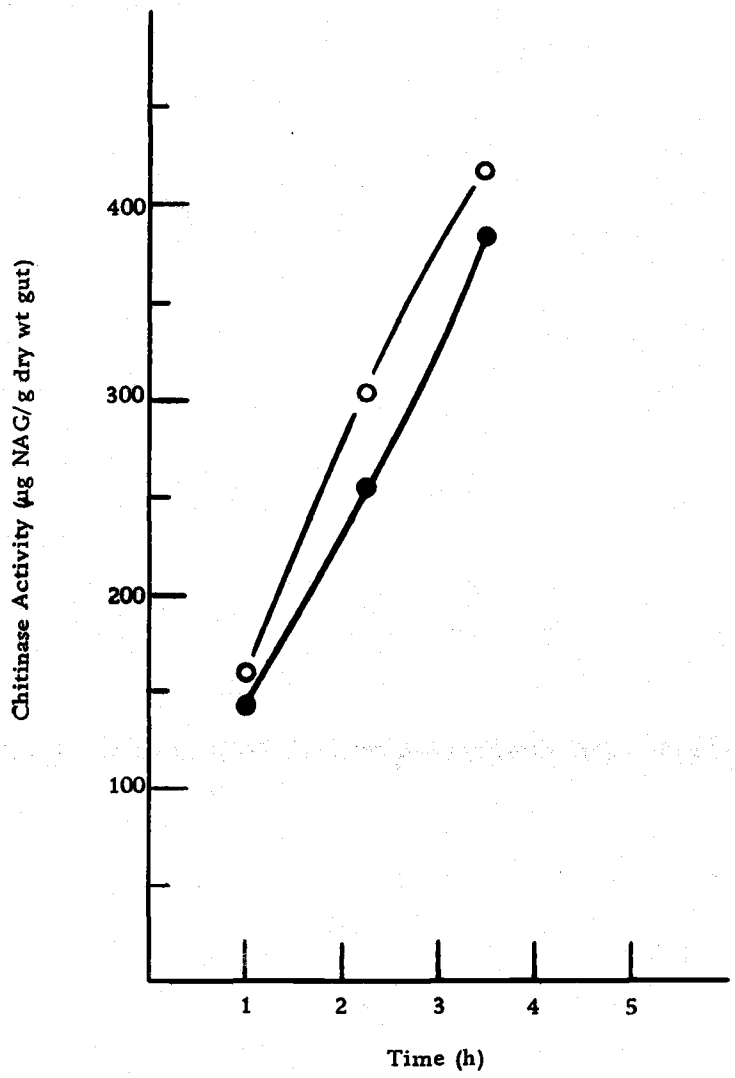

Figure 14. Chitin degradation vs. time by stomach contents of Enophrys bison. Chitinase activity was determined on gut contents of buffalo sculpin at 1, 2 1/4, 3 1/2 h at 10 C (●) and 20 C (○). At 10 C, correlation coefficient was 0.9992; m = 96.6 µg NAG/g dry wt gut·h and at 20 C, c.c. = 0.9974; m = 103.0 µg NAG/g·h.

Table 12. Total plate counts and percent chitinoclasts in stomachs of Enophrys bison (Buffalo sculpin) and Platichthys stellatus (Starry flounder).

Fish	Chitin[a]	Incubation Temperature	Total Count (bacteria/g dry wt gut)	% Chitinoclasts[b]	Activity (μg NAG/g·h)
Sculpin	++	5 C	7.2×10^7	93	245.3
Sculpin	++	5 C	1.7×10^9	90	364.9
Sculpin	++	5 C	4.3×10^7	95	324.3
Starry flounder	-	10 C	1.6×10^7	6.0	0
Starry flounder	-	10 C	1.3×10^{10}	11.0	0
Starry flounder	-	10 C	1.2×10^{10}	0.0	0
Sculpin	+	10 C	3.2×10^7	72	95.1
Sculpin	+	10 C	1.3×10^7	83	96.9
Sculpin	+	10 C	3.9×10^{10}	81	142.8

[a] Chitin refers to presence or absence of chitinous animals in stomach contents.
++ = predominantly chitinous animals in stomach
+ = some chitinous animals plus vegetation and/or fishes
- = no chitinous animals present

[b] Percent of colonies showing visual lysis of chitin in Seawater Chitin Agar after seven days.

Stomachs with chitinous material and vegetation had intermediate chitinoclast percentages (72 to 83%).

Sculpin maintained in aquaria containing chloramphenicol (CAP) had no detectable level of bacteria nor chitinase activities in their stomachs (Table 13). After 24 h, there was no chitinase activity in the fish from either bowl without CAP. Total counts were slightly higher in the fish fed the non-sterilized food than those fed the sterile food. Percent chitinoclasts were also slightly higher in the non-sterile food fish. At 72 h chitinase activity was present in the guts of fish in seawater not containing CAP. Slightly more activity was found after 144 h from the non-CAP fish. Plate counts and percent chitinoclasts also increased with time. (Chloramphenicol had no effect on the chitinase assay in controls run with bacterial enzyme from ANT-500.)

The growth rates of either a pure culture of a chitin digester or a mixed culture obtained from sculpin guts were unaffected by the additives of filter sterilized sculpin stomach fluid (Table 14). Both cultures, however, yielded greater bacterial numbers in the LIB-X with gut fluid than in LIB-X only (Table 15). N-acetyl-D-glucosamine (0.1%) was added to cultures growing in both LIB-X and LIB-X + gut fluid. The effect of NAG was to cause a cessation of growth of the pure culture in either LIB-X or LIB-X + gut fluid after attaining OD readings of 0.34 and 0.41, respectively (Figs. 15 and 16). Similarly

Table 13. Effect of chloramphenicol on the chitinase activity in the gut of Enophrys bison.

Chloramphenicol	Food	Chitinase Activity $OD_{585\,nm}$			Plate Count bacteria/ml			% Chitinoclasts		
		24 h	72 h	144 h	24	72	144	24	72	144
+		0.01	0.01	0.01	0	0	0	0	0	0
	sterile	0.01	0.01	0.005	0	0	0	0	0	0
		0.01	0.02	0.01	0	0	0	0	0	0
+		0.01	0.00	0.01	0	0	0	0	0	0
	non-sterile	0.01	0.00	0.01	0	0	0	0	0	0
		0.01	0.05	0.01	0	0	0	0	0	0
-		0.01	0.155	0.10	1.4×10^4	1.0×10^4	2.5×10^7	57	80	85
	sterile	0.01	0.06	0.08	1.7×10^3	1.5×10^6	3.7×10^7	59	67	89
		0.01	0.05	0.08	-	-	-	-	-	-
-		0.01	0.095	0.11	6.6×10^5	2.3×10^6	1.2×10^8	64	87	97
	non-sterile	0.01	0.095	0.12	6.0×10^4	3.1×10^7	6.9×10^7	67	96	96
		0.01	0.14	0.125	9.0×10^4	-	-	56	-	-

Table 14. Growth rates of pure and mixed cultures grown in the LIB-X medium and in LIB-X medium + gut fluid.

Culture	Medium	Growth Rate generations/h	Generation Time h/generation
Pure	LIB-X	0. 307	3. 25
	LIB-X + gut fluid	0. 342	2. 92
Mixed	LIB-X	0. 306	3. 26
	LIB-X + gut fluid	0. 289	3. 46

Table 15. Total plate counts and percent chitinoclasts from medium with and without gut fluid and N-acetyl-D-glucosamine.

Culture	Medium	Total Counts				Percent Chitinoclasts			
		0	15	24	50	0	15	24	50
Pure	LIB-X	2.1×10^{6}	-	4.5×10^{11}	-	100	-	100	-
	LIB-X + gut fluid	2.3×10^{6}	-	1.8×10^{13}	-	100	-	100	-
	LIB-X + NAG	2.0×10^{6}	4.0×10^{9}	-	8.2×10^{8}	100	100	-	100
	LIB-X + gut fluid + NAG	2.1×10^{6}	7.9×10^{9}	-	5.6×10^{9}	100	100	-	100
Mixed	LIB-X	1.6×10^{6}	-	3.9×10^{11}	-	80	-	92	-
	LIB-X + gut fluid	1.5×10^{6}	-	7.0×10^{12}	-	86	-	94	-
	LIB-X + NAG	1.7×10^{6}	1.4×10^{9}	-	4.4×10^{9}	82	93	-	41
	LIB-X + gut fluid + NAG	1.5×10^{6}	1.8×10^{9}	-	6.2×10^{9}	78	89	-	48

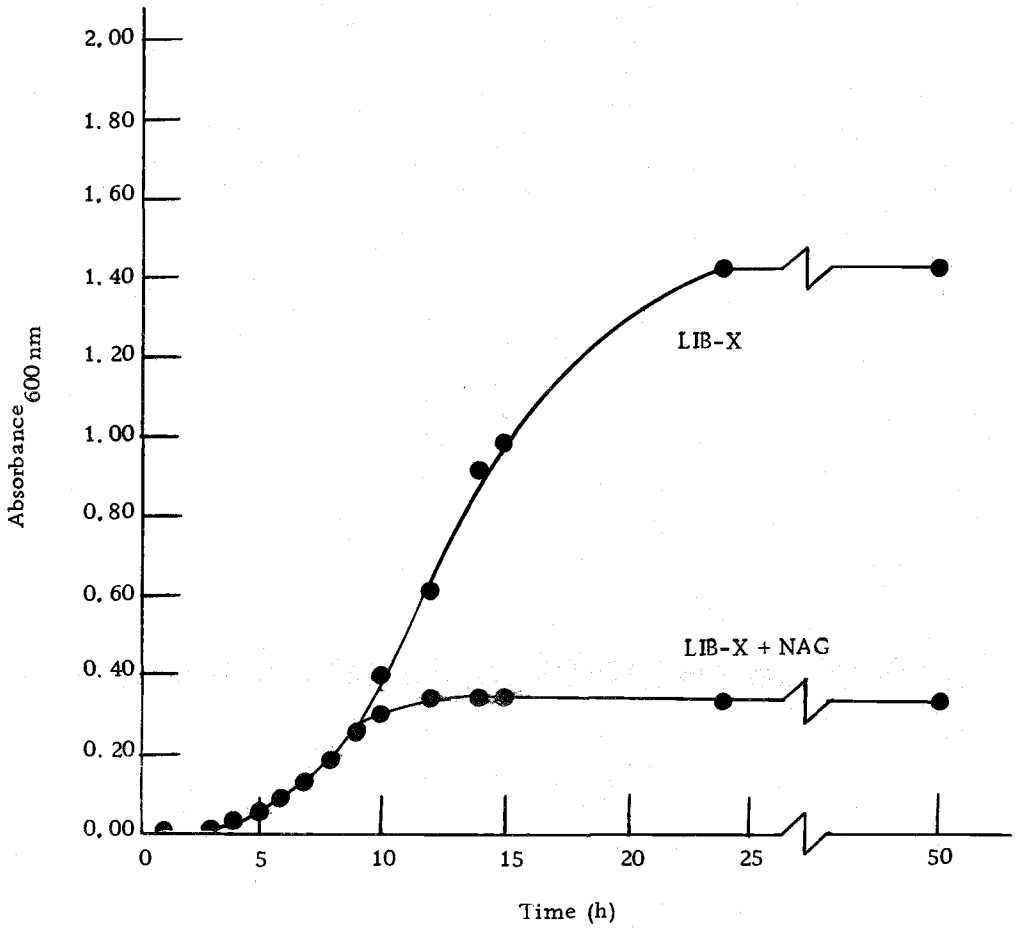

Figure 15. Growth curve in LIB-X of a pure culture isolated from the stomach of Enophrys bison. Optical density (OD) was read at 600 nm. One-tenth percent N-acetyl-D-glucosamine (NAG) was added to LIB-X in one set of culture flasks. The bacterium was chitinoclastic.

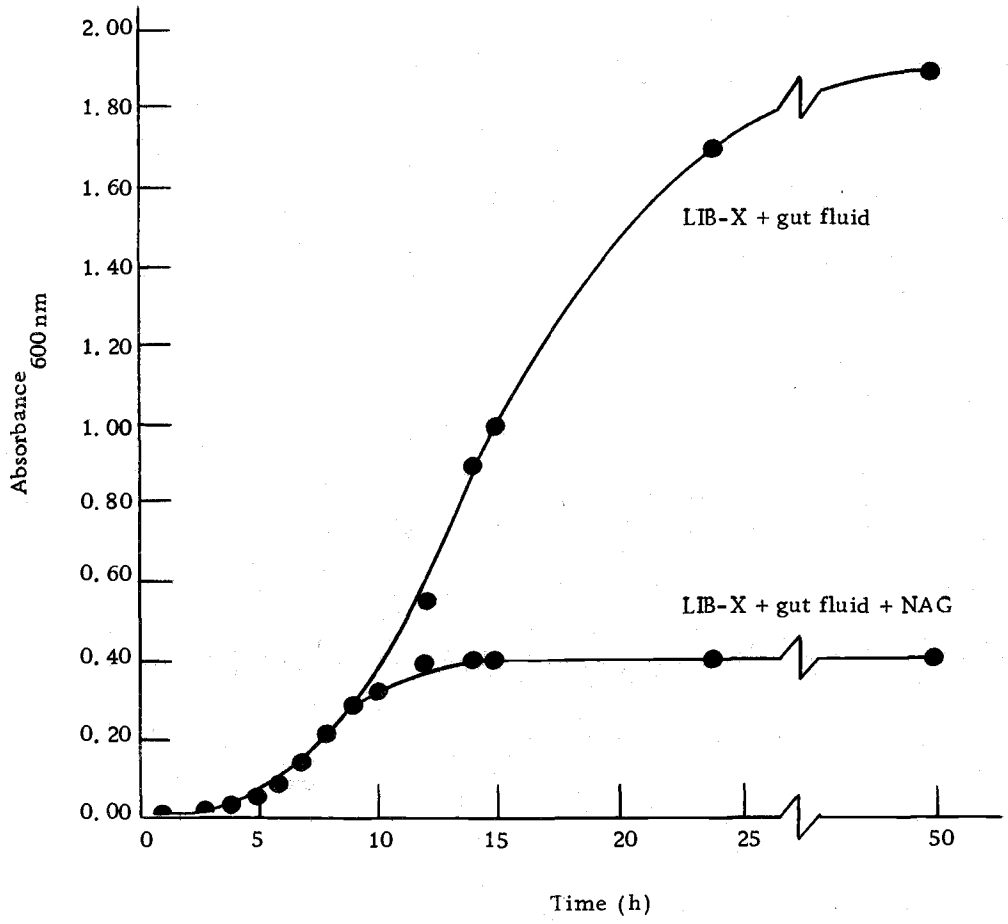

Figure 16. Growth curve in LIB-X + gut fluid of a pure culture isolated from the stomach of Enophrys bison. Optical density (OD) was read at 600 nm. One-tenth percent N-acetyl-D-glucosamine was added to LIB-X in one set of culture flasks. The bacterium was chitinoclastic. The gut fluid was collected from twelve pooled stomach contents of Enophrys bison and filter sterilized.

the mixed culture showed a period of no growth between the fourteenth and fifteenth hour of incubation and only reduced growth after the fifteenth hour (Figs. 17 and 18). Plate counts indicated a decrease in the number of chitinoclasts in the mixed culture after the fifteenth hour.

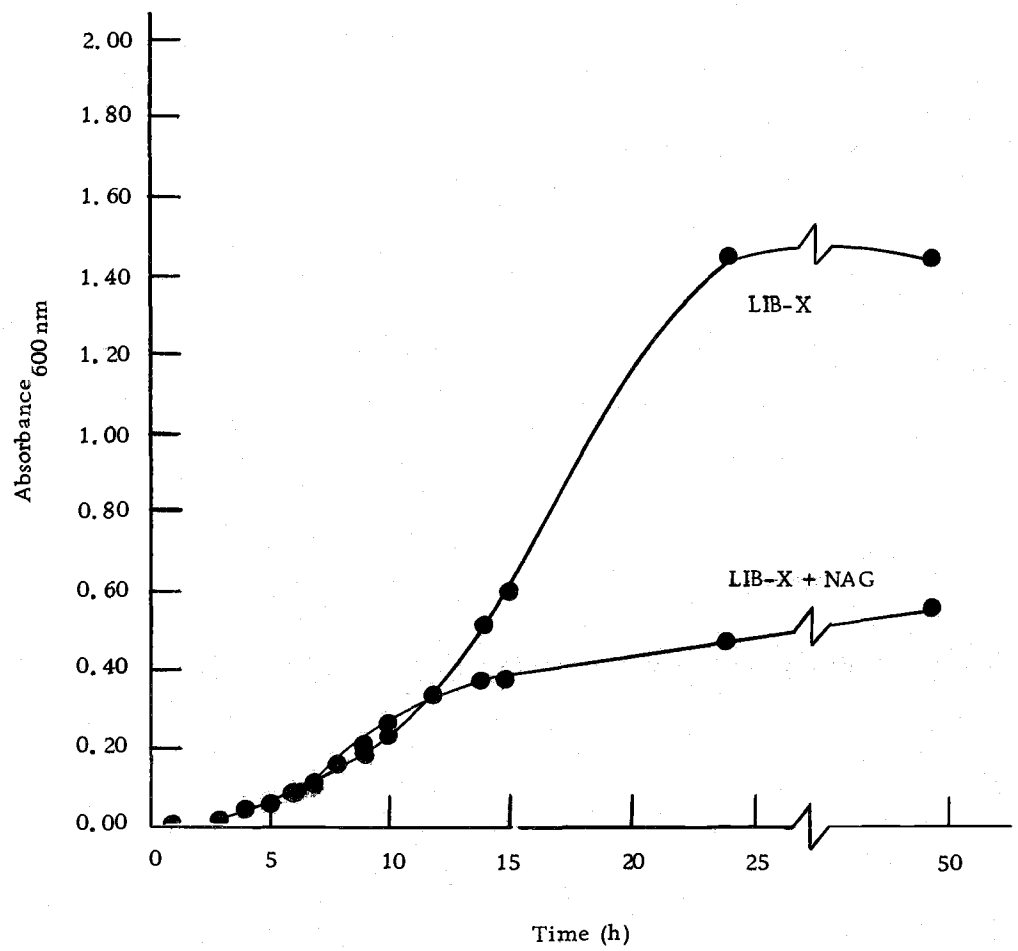

Figure 17. Growth curve in LIB-X of a mixed culture isolated from the stomach of Enophrys bison. Optical density (OD) was read at 600 nm. One-tenth percent N-acetyl-D-glucosamine (NAG) was added to LIB-X in one set of culture flasks. The mixed culture contained both chitinoclastic and non-chitinoclastic bacteria.

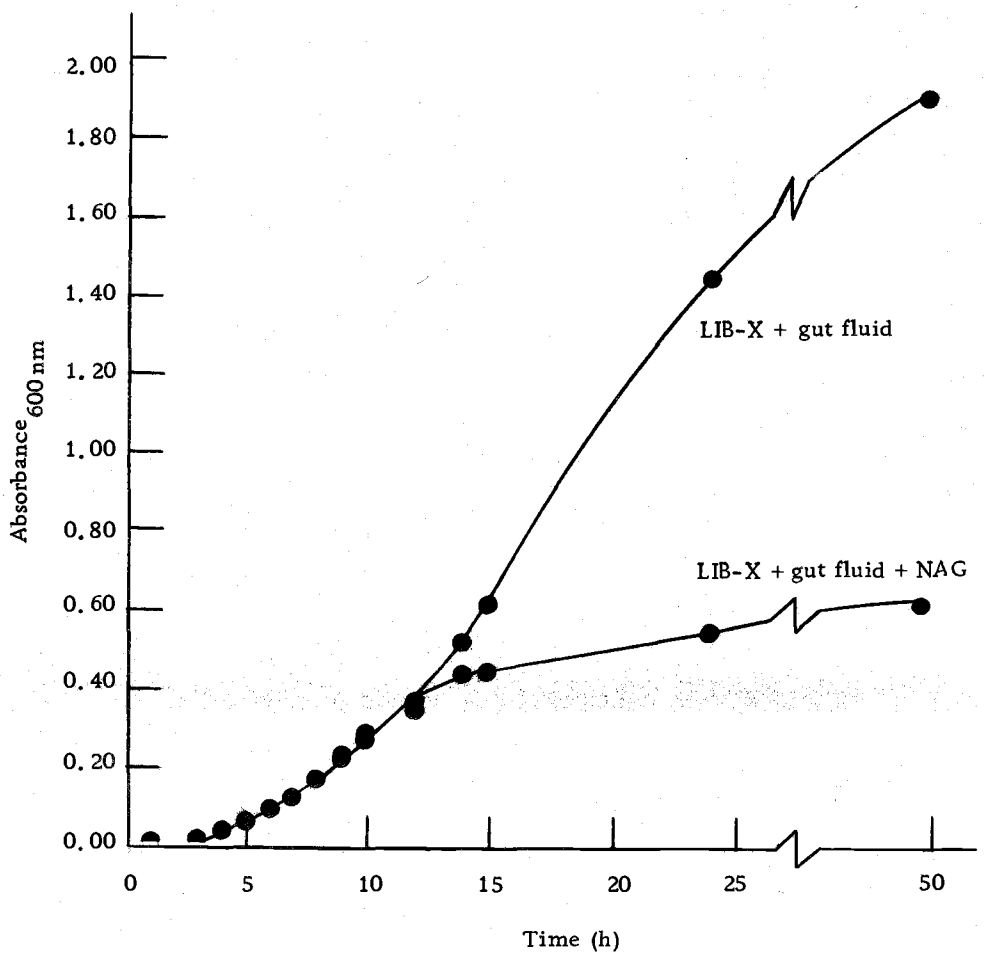

Figure 18. Growth curve in LIB-X + gut fluid of a mixed culture isolated from the stomach of Enophrys bison. Optical density (OD) was read at 600 nm. One-tenth percent N-acetyl-D-glucosamine (NAG) was added to LIB-X in one set of culture flasks. The mixed culture contained both chitinoclastic and non-chitinoclastic bacteria. The gut fluid was collected from twelve pooled stomach contents of Enophrys bison and filter sterilized.

DISCUSSION

The marine vibrio ANT-500 is a typical psychrophile according to the definition by Morita (41). The growth range is from a maximum of 13.5 C to a minimum of -2.5 C or less. Although the bacterium can grow at -2.5 C, it cannot elaborate chitinase at this temperature. The minimum temperature for enzyme production is 1.5 C. The loss of protein synthesis below this temperature could be a physical inhibition or possibly an inability of the organism to recognize the presence of the inducer. The latter could result from conformational changes in the cell surface proteins which detect the presence of chitin. Chitin is the only compound tested which could elicite the production of chitinase. It is likely that short oligosaccharide chains of β-1,4-linked N-acetyl-D-glucosamine would also induce enzyme production.

Enzyme kinetic data indicate that at least one reaction of the chitinase system does follow Mechaelis-Menton kinetics. This aspect appears to be the rate limiting step. It is not possible from the data accumulated to determine what step this could be. The situation is more complicated than many enzyme-substrate reactions since the substrate (chitin) is particulate. A further complication is that ANT-500 probably has three separate enzymes in its chitinase system, most likely two chitinases and one chitobiase. This conclusion was

indicated from the three separate peaks of activity shown at both pH 5. 5 and 7. 5. Therefore, several possibilities exist to explain the apparent Mechaelis-Menton kinetics. The step involved could include the action of either chitinase on the particulate chitin. Another possibility could be the action of either chitinase on the short oligosaccharide chains resulting from the initial action on the particulate chitin. The short oligosaccharide chains, in this case, would be in a soluable form. Finally, the step could be the action of the chitobiase on chitobiose and chitotriose.

All species of fish examined exhibited chitinase activity in their gut contents. Based on the findings of Beardsley (5) and on personal findings, the most accessible and most abundant fishes in Yaquina Bay were the buffalo sculpin and the starry flounder. Thus, the greatest effort was directed toward studying stomach contents of these two species.

Chitinase activity varied from one species to another and among individual members of the same species. Activities ranged from no detectable to 1070 μg NAG produced/g dry wt gut· h among all the fishes sampled. The most important factor related to the amount of chitinase activity was the nature of gut contents. When the gut contained only chitinous animals, e. g. crab, shrimp, copepod, etc., the activities were highest while if no chitinous material was found, the activities were very low or undetectable. This was further

supported when the guts contained combinations of chitinous organisms and vegetation and/or fish. The levels of chitinase activity were found to be intermediate to the two extremes. These findings indirectly support the hypothesis that the chitinase enzymes in the fish are bacterial in origin. Further evidence can be inferred based on the percent chitinoclasts isolated from the guts containing the varying amounts of chitinous material. Stomachs with mainly chitinous material had from 90-95 percent chitinoclasts; mixed gut contents had from 72-83 percent chitnoclasts, and guts that lacked chitnous material harbored 0-11 percent chitnoclasts.

Hydrostatic pressure up to 1000 atm had little detrimental effect on chitinase activity in fish stomachs. The skate (Raja binoculata) is a deep water benthic animal of the continental slope which migrates to estuarine environments to deposit its egg case. Therefore, one would expect the chitinase associated with its stomach contents to withstand high pressure since the chitinase must degrade the chitinous organisms in the skate's diet. Virtually no differences in activity were obtained at pressures ranging from one atm to 1000 atm at 5 C. Typically, a near doubling of activity could be noted at 20 C and one atm compared to the 5 C and one atm activity.

In contrast to the skate which normally inhabits the deeper benthic regions, both herring and starry flounder are generally found in the upper waters even though the flounder is also benthic.

Therefore, one would not expect the chitinase of these fishes to be as resistant to pressure, especially if one assumes that the fish can secrete its own chitinase. This was not the case, however, although increasing pressure did decrease the activity slightly in both fish. In the herring the activities resulting from assays at 100, 300, and 500 atm were twice as great as the activity at one atm. In the flounder the activities at elevated pressure also were greater than the activity at one atm, but not double.

From the preliminary data on the effect of anaerobic incubation, a lack of oxygen had no effect on the chitinase activity in the stomach contents of buffalo sculpin assayed. The average activities under aerobic and anaerobic conditions at 5 C and one atm were 420 and 493 μg NAG produced, respectively.

Rates for the degradation of chitin by chitinase preparations from fish guts were determined by measuring activity at various time intervals. Again the complications of a particulate substrate and a multiple enzyme system were encountered. Also the lack of precision of determining low levels of chitinase activity during short time intervals after zero time and general logistics of the necessary experimental design prevented the attaining of more instantaneous degradation rates. These problems are apparent when one compares the slope (rate) of any one rate curve with the value obtained at one hour. In each case the μg NAG produced during the first hour was greater

than the projected rate obtained from the linear regression. (In most cases controls without chitin added showed no residual NAG in the stomach contents.) Some curves at 5 C gave low correlation coefficients which points to the difficulty encountered when reading low activity results.

Sterile stomach fluid from a pooled sample of buffalo sculpin had no effect on the growth rates of bacteria isolated from the gut of another buffalo sculpin. There was, however, an effect on the population attained in late log-early stationary phase. The gut fluid medium yielded a greater population than did the LIB-X medium alone. This may have been the result of greater nutrient concentration in the LIB-X + gut fluid medium or the result of some other growth factor unique to the gut fluid.

The presence of 0.1% NAG in the culture media (LIB-X and LIB-X + gut fluid) exhibited a property similar to the glucose effect (35). The cells in either medium, after inoculation, continued growing until they reached early log phase and then, as with the glucose effect, the cells of the pure culture of chitinoclast were repressed and showed no further growth even after 50 h incubation. As previously mentioned, the mixed culture also showed repression during the same phase of the growth curve. The slow growth which followed the repression was by bacteria other than chitinoclasts as indicated by the decrease in percent chitinoclasts with time.

This seems to present a paradox, for why would an organism produce an enzyme to degrade a substrate if the organism could not utilize the degradation product of the enzyme reaction? This might be answered in the symbiotic relationship which exists within the gut of a fish. The stomach is well adapted for the adsorption of low molecular weight carbohydrates and keeps the concentration of NAG lower than the concentration necessary for repression of growth of the chitinoclast. This is likely since little or no residual NAG was detected in controls assayed without the addition of chitin. The apparent inability of the chitinoclasts to utilize NAG may be the result of an evolutionary loss of metabolic or transport enzymes necessary for its utilization. The possibility that other bacteria such as non-chitinoclastic organisms might utilize the NAG is unlikely. This may be surmised from the slow growth rate(s) observed after repression in the mixed culture and also the fact that gut samples with chitinous material had over 90% chitinoclasts. Chan (11) observed similar results.

Chloramphenicol (CAP) acts to prevent the formation of the ribosomal-messenger complex during protein synthesis in procaryotic organisms (14). The antibiotic is bacteriostatic. It was effective in deleting the bacterial flora from the digestive tracts of the buffalo sculpin placed in it. The CAP experiments show that in the case of the buffalo sculpin the enzyme is bacterial in origin and thus

substantiate the previous indirect evidence indicating a bacterial origin.

Bacterial origin of chitinase in other fish might also be predicted, specifically in those species tested in which no chitinous organisms in the gut were found, resulting in no chitinase activity. This is quite likely since fish are not known to possess inducible digestive enzymes. Barrington (3) stated that the production of cellulase and chitinase had not been a recognized property of vertebrates, for vertebrates "rely upon a symbiotic flora within the alimentary canal to enable them to deal with a specialized diet". Barrington found it doubtful that a fish could adapt its enzyme equipment in response to a diet change (i. e., not inducible). He listed the principle digestive enzymes as proteases (pepsin, trypsin, erepsin), carbohydrases (amylase, maltase), and lipases and esterases. Okutani (43) has reported a chitinase produced by the digestive glands of the Japanese sea bass. However, the experimental design does not completely eliminate the possibility that the chitinase was bacterial in origin because the extracellular nature of the bacterial chitinase would allow the bacteria to be washed from the stomach mucosa without removing the chitinase. The comparison of properties between the bacterial and the "fish" chitinase is also questionable since the assays were performed at pH 5. 1 for the "fish" chitinase and at pH 7. 5 for the bacterial chitinase. Also different fish samples were

used for the "fish" chitinase assay than for the bacterial chitinase assay. Based on the results of assaying ANT-500 at pH 5. 5 and pH 7. 5, different temperature optima can exist for the same enzyme. Also since the indigenous bacterial flora of fish is thought to be a group of organisms (32, 58) any of which could predominate in the same fish species, it is possible the bacterial chitinase Okutani studied came from one organism and the "fish" chitinase from another organism. Under properly controlled experiments it is likely that the Japanese sea bass chitinase would also be bacterial in origin.

A possible cycle for the degradation of chitin, therefore, can be proposed. A chitinoclastic bacterium can enter the fish gut adsorbed to the substrate (chitinous organism) or it may be maintained in the stomach or other part of the digestive tract until the substrate has entered the gut, much like the relationship which exists in the rumen. The stomach serves as an initial degradation center for the chitin, providing a suitable environment for the rapid proliferation of the chitinoclast. The bacteria produce the enzymes to degrade the chitin and the NAG is utilized by the fish. In turn the fish provides growth factors and a controlled environment for the bacterium. (The bacterium may also serve as a direct source of nutrition for the fish, but this is not pertinent to the chitin cycle.)

Small chitin particles onto which are adsorbed the chitinoclast are then excreted. The fecal pellets sink to the sediment layers

where they are fed upon by detritus-feeding organisms. (This could account for the lack of enzyme activity found in the sediment samples from Yaquina Bay.) The final stages of the chitin degradation could then take place in the digestive tract of the detritus feeder where again the bacterium has a suitable controlled enviornment in which to decompose the small chitin particles. The fate of the bacterium again could be to serve as a source of nutrition for the detritus feeder or to be excreted. Once excreted the bacterium would be free to adsorb to a chitinous organism and begin its cycle again.

Liston (32) reported that the predominate organisms in the gut contents of skate and lemon sole belong to a group of organisms designated "gut group vibrios". He suggests that growth conditions within the fish gut are such as to allow these vibrios to predominate, much like the coliform organisms are the major bacteria in the mammalian gut. He indicated, therefore, that a simple mechanical transfer of organisms associated with the food is not sufficient to produce the results he obtained. The diets of the lemon sole and skate as well as other flatfish (38) are fairly mixed including fish, worms, and crustaceans and would not support a theory that the bacterial flora is predominantly one group of organisms if mechanical transfer was solely involved. Results from the present study showed organisms from starry flounder and buffalo sculpin, which had mixed gut contents, had populations of chitinoclasts comprising 72 to 83% of the

total population.

Additional reports (12, 33) on the commensal bacteria associated with a variety of fishes and invertebrates from the North and South Pacific Ocean show one or two "groups" of bacteria are predominant, not only between fish of the same species, but also among fish of different species. These findings tend to support the theory that the gut organisms are maintained or stored in the gut until substrate enters the gut. This is important from an environmental standpoint for if only mechanical transfer were involved the chances of an active chitinoclast predominating in the gut is reduced. Also if stress conditions such as elevated water temperatues prevented the growth or maintenance of the "gut group vibrios", other chitinoclastic organisms, possibly the pathogenic strains Vibrio parahaemolyticus or V. anguillarum could be selected for and thus present a potential health hazard.

The presence of low levels of chitinase activity in sediments taken from off shore can also be explained by the proposal, if one takes into account the relative population densities of fishes (to serve as initial degradation centers) and of detritus feeders (to serve as final degradation centers) in the estuarine and the off-shore environment. It is more likely for detritus to settle onto off-shore sediment and not be taken up by a detritus feeder than for it to settle onto estuarine sediment and not be taken up. It would become necessary,

therefore, for part of the final degradation to take place in or on the sediment.

The necessity of an efficient cycle such as the one proposed can be understood when one recognizes the great quantity of chitin produced annually (19, 25). Since the decomposition of chitin in the stomach of the buffalo sculpin is bacterial, one could estimate the amount of chitin which could be degraded by a population of the fish in a year. If one were to assume favorable conditions for bacterial and fish growth and metabolism and an infinite supply of substrate (chitin), one could make such an estimate. Assume a population of 1×10^5 sculpin in the Yaquina Bay estuarine area with an average dry wt-gut content of 50 g and base this on a rate of chitinase activity of 300 μg NAG produced/g dry wt gut· h. This single species-population would be responsible for the production of ca. 14 metric T of NAG/yr. This is equivalent to the decomposition of ca. 16 metric T of chitin. It is not difficult, therefore, to see the important role that a single bacterial enzyme can play in the recycling of organic matter.

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