

**The digestive mechanisms of an intertidal grazer,
the sea urchin *Parechinus angulosus*.**

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ABSTRACT

Echinoids are important grazers in the near-shore ecosystem and can significantly effect their ecology. The sea urchin *Parechinus angulosus* occurs inter- and subtidally along the southern African coast. Within this range it consumes an extremely wide variety of algae. Since algal cell walls have an almost species specific chemical composition, the question arises as to how it can digest the algae that it consumes.

In order to investigate the digestive mechanisms employed by *P. angulosus*, an ultrastructural study of the gut was undertaken to characterize the tissue and identify functional regions in the gut. Ten structural and storage polysaccharides commonly found in macroalgae were used as substrates to assay the digestive polysaccharidases of the sea urchin. The enteric bacteria of the sea urchin were isolated and tested separately for polysaccharidase activity using the same substrates.

The results shown that the gut of *Parechinus angulosus* is regionally specialized, with the foregut primarily responsible for the production of hydrolytic enzymes, while the hindgut is primarily absorptive. The occurrence of lamellar bodies, heterolysosomes, cytoplasmic blebs and paddle cilia among other characteristic features of the digestive epithelium are described and discussed.

Two levels of enzyme activity are apparent. Generally the urchin could hydrolyze the reserve polysaccharides, but only partially hydrolyze the structural polysaccharides, of red and green algae. *P. angulosus* was unable to digest alginic acid, the main structural polysaccharide of brown algae. Mixed cultures of bacteria utilized only the reserve polysaccharides of red and green algae. Significantly, the bacteria were able to hydrolyze alginic acid. Enteric bacteria also showed agarolytic activity.

Parechinus angulosus has the ability to digest red and green algae. No lysozyme activity was detected. The enteric bacteria can digest the same algal reserve polysaccharides and so may compete for carbon in the gut. However, in the case of brown algae, bacteria have a potentially important endosymbiotic role as agents of digestion. These results correspond with food preference studies which have shown that, although *P. angulosus* consumes the kelp *Ecklonia maxima*, in the western Cape, it is amongst its least preferred food species. The reasons for this are its unpalatability and the urchin's inability to digest brown algae. The digestibility of algal material can be an important factor in determining algal-herbivore interactions.

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CHAPTER 1

INTRODUCTION

The importance of echinoid herbivores

Intertidal and subtidal marine ecosystems rely primarily on herbivores, detritivores and filter-feeders for the distribution of energy through the system (Barnes and Hughes, 1982). It has been clearly demonstrated that variation in the factors limiting the herbivorous population of intertidal communities can have marked effects on the structure of that community (e.g Vadas, 1977; Lubchenco, 1978 and Harrold and Pearse, 1987; Lubchenco and Gaines, 1981). Such effects are now classically illustrated by the example of the effects of overgrazing by the sea urchin *Strongylocentrotus* spp. in kelp beds on the coast of Nova Scotia (Lawrence, 1975; Breen and Mann, 1976; Ebeling, Laur and Rowley, 1985 and Novaczek and McLachlan, 1986).

The last twenty years have seen extensive reviews and monographs on the role of echinoderms in intertidal and shallow water marine environments (Ferguson, 1969; Lawrence, 1975; Vadas, 1977; Lawrence and Sammarco, 1982; Harrold and Pearse, 1987 and Andrew 1989). The majority of these reviews (and papers cited in them) have repeatedly illustrated the importance of echinoids in structuring the community in which they occur. For example, in 1975, Lawrence wrote, "Sea urchins are often the determining factor with regard to the abundance and distribution of marine plants in shallow water marine environments" (Lawrence, 1975; p.213).

While this literature documents the mechanisms and effects of herbivory by echinoids, only Vadas (1977) has dealt with the

evolutionary implications of herbivory by sea urchins. He suggests that selective pressures, due to herbivory by sea urchins, may have resulted in the co-evolution of algae and urchins so that different reproductive strategies of algae have evolved in response to differential feeding preferences among urchins. Vadas suggests that large macrophytic algae generally adopt two broad types of strategies to permit the continued co-existence of this "predator-prey" interaction. These are either "escape strategies" which include short annual life cycles and high reproductive capacity (r-selection) or "defence strategies" which entail incorporation of calcium carbonate in tissues, low energetic value, toxic chemicals and reduced reproductive capacity (k-selection).

Andrew (1989) discussed the physiological basis of the ecological importance of sea urchins by comparing them with their herbivorous gastropod competitors. Comparing the capacity of sea urchins to withstand food limitation with that of ecologically similar gastropods, he showed that sea urchins are better able to tolerate starvation because:

- 1) they are capable of negative growth and can resorb tissues under conditions of severe food deprivation,
- 2) they possess a relatively simple body plan with little muscle and small investment in soft tissue, resulting in a low requirement for nutrients.

The consequence of these facts is that survivorship and food abundance are largely decoupled and so density-dependent mortality is significantly less prevalent in sea urchins than in herbivorous gastropods. This explains why, for example, high densities of sea urchins can persist for years, even in periods

of low food availability.

As De Ridder and Lawrence (1982) point out, the diets of echinoids are extremely varied. The diet of a single species may display spatial and temporal variation, dependent on the food available when and where feeding occurs (Lawrence, 1975). Some echinoids may be functionally or predominantly herbivorous, but this does not make them obligate herbivores (Lawrence, 1975).

The multifarious structure of algal cell walls.

The major classes of marine macroalgae are identified in terms of their photosynthetic pigmentation. While debate about the ecological implications of these pigments continues, they represent one of the unique adaptations of marine macrophytes to shallow water environments (Lobban, Harrison and Duncan, 1985). Variation and attenuation of light are not the only features of the benthic ecosystem to which these primary producers are adapted. Many examples of varied types of adaptations to minimize the effects of herbivory exist (e.g. Lubchenco and Cubitt, 1980; Slocum, 1980; Santelices, Correa and Avila, 1983; Johnson and Mann, 1986 and Tugwell and Branch, 1989). The pressure of herbivorous grazers as well as the physical demands of this environment have led to the evolution of relatively tough cell walls (Lobban *et al.*, 1985). The presence of sulphated polysaccharides, acid polysaccharides and extensive intracellular matrices are unique in marine algal cell walls and enable them to play a vital part in the regulation of the morphology, hydration and ionic regulation of the algal cell (Kloareg and Quatrano, 1988).

Until recently, the polysaccharides of only 190 of the approximately 250 000 known species of marine algae had been studied (Painter, 1983). From these studies alone it is clear that, whether co-evolved or evolved in response to specific selective pressures, marine algae produce polysaccharides of almost unparalleled diversity and complexity (Painter, 1983). These polysaccharides, generally of a structural function, are poorly understood and are usually still described in terms of their solubility characteristics and component units as opposed to their structural characteristics (McCandless, 1982). Detailed studies of the polysaccharides of relatively few species have been conducted, using X-ray diffraction and electron microscopic techniques (Percival and McDowell, 1982). Kloareg and Quatrano (1988) have produced an exhaustive review that summarizes our knowledge of the structure and function of algal cell walls. Painter (1983) suggests that the evolution of algal polysaccharides may have occurred in association with morphological evolution, with the result that it is possible to loosely group polysaccharide types with the major classes of algae. For example alginates and laminarin occur exclusively in the Phaeophyceae (Percival, 1979). Nevertheless, it is clear from the literature that the composition of cell walls is not only species specific, but may even vary between generations of the same species (Percival and McDowell, 1982).

The digestive mechanisms of generalist herbivores

The extreme variability of algal cell wall composition and the diet of sea urchins raises a question about the physiological mechanisms of herbivory. A generalist marine herbivore requires mechanisms to digest the variety of structural algal

polysaccharides described above. Differential ability to digest different algal polysaccharides could have profound ecological implications (Lubchenco and Gaines, 1981). Even if the structural material is not utilized as a food source itself, it still has to be degraded to allow access to the cell contents. There are three possible mechanisms that a herbivore can employ to degrade algal structural polysaccharides : 1) Mechanical, using mouth parts to break up macrophytic material in order to ingest it,

2) Biochemical, using an enzyme system to extracellularly break down ingested material,

3) Microbiological, by co-opting enteric bacteria to break down ingested structural material. These mechanisms need not be mutually exclusive and any combination of them may suffice.

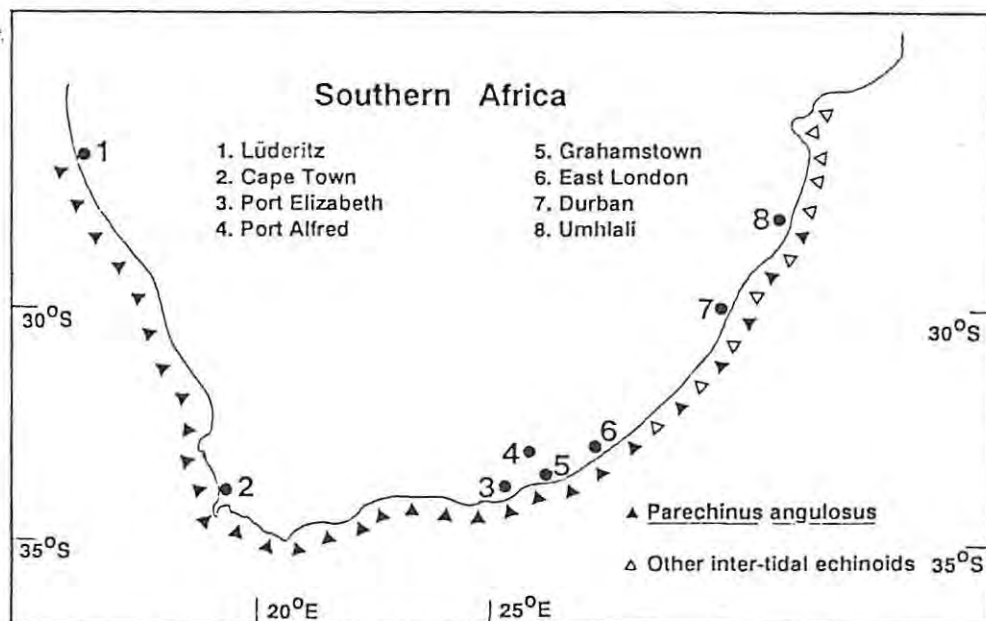
The purpose of this study is to investigate the digestive capability of the South African sea urchin, *Parechinus angulosus* (Leske), with the aim of identifying which mechanism(s) it employs in dealing with algal polysaccharides. It is accepted as a premise that mechanical breakdown of macrophytic material is achieved with remarkable efficiency by the Aristotle's lantern, however, since no masticatory organ exists, whole pieces of algal material are ingested. Emphasis is thus placed on the latter two mechanisms in this study.

The study animal

Parechinus angulosus is the only species of regular sea urchin that occurs intertidally (down to twenty metres depth) on the southern African coast from Luderitz in Namibia to East London in the eastern Cape (Day, 1974). North of East London, it occurs sympatrically with several other species up to the limit of its

distribution on the northern Natal coast (Stephenson, 1948; Day, 1974; Fig 1.1). Stephenson's analysis of the biogeographical distribution of the South African intertidal biota (Stephenson, 1944; Stephenson, 1948), identified three distinct components of the biota that occurred in the three biogeographical provinces along the coast. Stephenson grouped a separate 23 species as the "ubiquitous component," which occurs in all three provinces. One of these is *Parechinus angulosus*, although its highest recorded densities are in the kelp forests of the western and south-western Cape (Fricke, 1980).

Figure 1.1: Map of the distribution of *Parechinus angulosus*



P. angulosus is clearly an example of a typically generalist herbivore (Fricke, 1979; Anderson and Velimirov, 1982; Lawrence, 1982). Within its distributional range on the South African coast it consumes an undetermined range of algae, ingesting species from the Chlorophyceae, Phaeophyceae, and Rhodophyceae and is thus a good candidate for a study of the digestive physiology of herbivores.

Various aspects of the biology of *P. angulosus* have been studied, but these studies have generally been limited to populations on the western and south western Cape coast. Field studies on the biology of *P. angulosus* were carried out by Fricke (1979; 1980) who examined kelp grazing and aspects of the population structure. Respiration and ecological energetics were studied by Stuart and Field (1981) while a comparative study of two populations was conducted by Greenwood (1980), who examined growth and respiration and presented a tentative energy budget for *P. angulosus*. A food preference study was carried out with western Cape specimens using western Cape algae, by Anderson and Velimirov (1982) and feeding, defecation and absorption efficiency were examined by Buxton and Field (1983).

This study was conducted using three approaches. These were:

- 1) Ultrastructural: The ultrastructure of the gut and surface mucosa were investigated in order to characterize the digestive epithelium.
- 2) Enzymological: Enzyme extracts of the gut were assayed for a range of digestive polysaccharidases.
- 3) Microbiological: The utilization of algal polysaccharides by bacteria isolated from the gut of sea urchins was investigated.

Since there appears to be a functional distinction between different regions of the gut (Hyman, 1955; De Ridder and Jangoux, 1982) the entire study was conducted separately for the hindgut and the foregut.

In this presentation, each of the above approaches is considered in a separate chapter, consisting of an introduction, methods section, results section and a preliminary discussion. The final chapter presents a synthesis of the discussion and the conclusions.

CHAPTER 2
ASPECTS OF THE HISTOLOGY OF THE GUT OF *Parechinus angulosus* AND A
MICROSCOPIC EXAMINATION OF THE GUT MUCOSA AND CONTENTS

Introduction

The histology of the gut of a wide variety of species in the phylum Echinodermata has been studied at the light microscope level, but only a few studies have examined the ultrastructure of these tissues (see De Ridder and Jangoux, 1982 for review). For echinoids, Holland and Lauritis (1968) studied the fine structure of the exocrine tissue of the gut of *Strongylocentrotus purpuratus* and Tokin and Filiminova (1977) studied the ultrastructure of the gut of *S. droebachiensis*. These two studies were in close agreement in their conclusion that the digestive epithelium was protein producing and responsible for the production of digestive enzymes.

Some degree of regional specialization is distinguishable histologically in the gut, although this is variable, (Tokin and Filiminova, 1977 and De Ridder and Jangoux, 1982). Generally the tissues are secretory in the foregut (or stomach) and absorptive in the hindgut (or intestine; De Ridder and Jangoux, 1982). Various cell types have been described in the gut of echinoids. Enterocytes occur throughout the gut, are flagellated and seem to have a variable function (De Ridder and Jangoux, 1982). Gastric exocrine cells are reported to occur mainly in the foregut (Holland and Lauritis, 1968 and Tokin and Filiminova, 1977) and are characterized by copious rough endoplasmic reticulum, numerous mitochondria and secretory vacuoles.

The results obtained in these studies correlate well with those for other marine herbivores. Bush (1988) studied the ultrastructure and function of the digestive tract of the limpet

Patella vulgata to reveal that gastric exocrine glands found in its gut were almost identical in structure to those of echinoid herbivores.

No scanning electron microscope studies of the gut mucosa or gut contents of echinoids have been published. Certain features of the gut are therefore undescribed. Furthermore, the surface of the gut and of the gut contents have not been examined for microorganisms.

For the purpose of characterizing the tissue of the gut of *Parechinus angulosus* in terms of function, an ultrastructural study was conducted on the foregut and the hindgut. Gut tissue was examined for the presence of glycogen and attempts were made to histochemically label lysosomes and immunolabel amylase. Scanning electron microscopic examination of the gut mucosa of the foregut and hindgut as well as the surface of the gut contents from these two regions was conducted.

Materials and Methods

Specimen collection:

All specimens were collected at low tide at Port Alfred, South Africa (33° 35'S, 26° 55'E; Fig. 1.1) and were either treated in the field or were transported directly to the laboratory where they were processed in the shortest time possible (less than 2 ours).

Ultrastructure:

1) Tissue processing for TEM: The foreguts and hindguts of fresh specimens were dissected out and processed separately. The gut tissue was cut into pieces of approximately 2mm³ and washed in

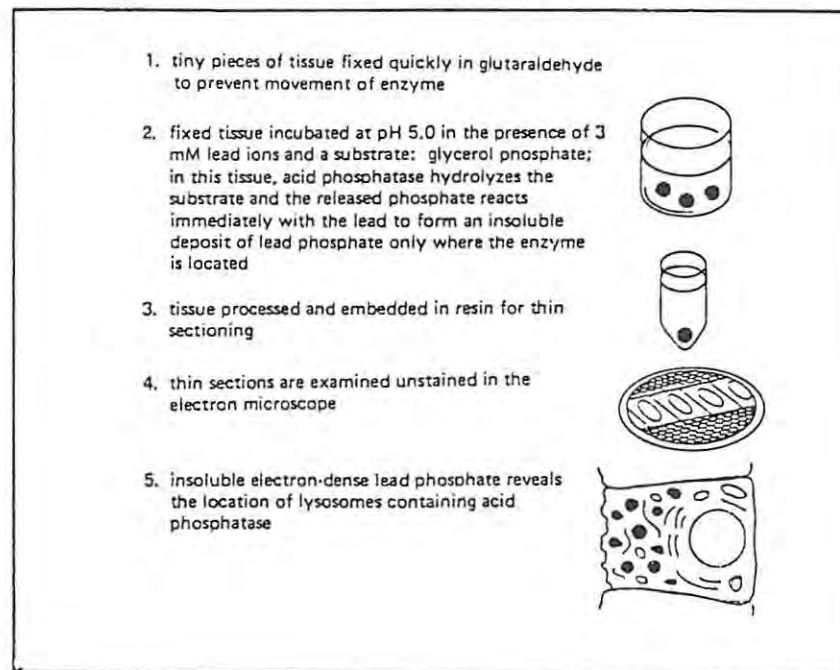
cold, 0.45 μm , filtered sea water. The tissue was then fixed in 0.1M sodium cacodylate buffered sea water (Hackney, McCrohan and Hawkins, 1983) for approximately 6 hours and post-fixed in 1% osmium tetroxide in 0.1M sodium cacodylate buffer. The tissue was then dehydrated on an ethanol series and transitionally infiltrated using propylene oxide in an Araldite/Epon resin mixture (Cross, 1987). The tissue was then imbedded in resin moulds and polymerization carried out at 60°C for 36 hours. The tissue was sectioned on an LKB UM111 Ultramicrotome using glass knives. The sections were mounted on copper grids and then routinely stained with uranyl acetate and lead citrate and viewed on a Jeol CX 100 11 transmission electron microscope.

2) Glycogen staining: Sections used to stain specifically for glycogen were obtained in the manner described above except that sections were mounted on gold grids. The method used was the Periodic Acid-Sodium Chlorite-Uranyl Acetate technique of Vye and Fischman (1971). Sections were reacted with 1% periodic acid for 30 minutes, rinsed in distilled water and incubated for 15 minutes in 2.5% sodium chlorite in 10% acetic acid solution in the dark. The sections were then rinsed and stained with uranyl acetate and viewed on a Jeol CX 100 11 TEM.

3) Lysosome labelling: Acid phosphatases are marker enzymes for lysosomes (Alberts, Bray, Lewis, Raff, Roberts and Watson, 1983). These enzymes were labelled in the digestive epithelium of the foregut and hindgut in order to ascertain whether the vacuoles present were lysosomal in nature. The method used was adapted from Alberts *et al.* (1983) and Bancroft and Hand (1987). The principle of the technique is that in an acid environment and in the presence of an excess of lead ions and a suitable organic

phosphate compound, the acid phosphatase enzyme will cleave the phosphate molecules from the organic compound. The phosphate molecule will then form a lead phosphate deposit at the site of the reaction. Provided that no other source of heavy metal is present local staining of the acid phosphatase on the tissue sections is visible with a transmission electron microscope as electron dense deposits (Fig. 2.1). The need to avoid additional sources of heavy metals precludes staining by conventional techniques in this procedure.

Figure 2.1: Diagram illustrating the lysosome staining technique. (After Alberts *et al.*, 1983).



Two problems with this technique should be pointed out. Firstly, the tissue is only fixed for 1 hour in glutaraldehyde (brief enough to maintain the cell structure and prevent the enzyme from diffusing without enzyme denaturization) with no post-fixation. This results in superficial fixation of the tissue as a whole and

reduces the quality of the tissue, while not affecting the outcome of the technique. Secondly, no staining other than the enzyme stain is used in this technique so that, when viewed on the electron microscope, almost no contrast is distinguishable within the tissue. This does not affect the outcome of the results, but it causes a further compromise in the quality. This problem can be partially overcome with the use of image enhancing equipment as described below.

Procedure: The foregut or hindgut from freshly dissected specimens were washed and cut into pieces of about 2mm³. This tissue was then fixed in 2.5% gluteraldehyde in filtered sea water for 1 hour. After fixation, the tissue was incubated at room temperature in the following medium for 2 hours.

10ml Acetate buffer (ph5)

20mg PbNO₂

32mg β-glycerophosphate

A control was run simultaneously by incubating identically prepared tissue in the same medium excluding the β-glycerophosphate. The tissue was then processed as for the ultrastructure described above, but without heavy metal staining. The images were then recorded on film using contrast enhancing Scanning Transmission Image Mode (STEM) on the microscope.

Immunocytochemical staining:

Specific labelling of hydrolytic enzymes in the tissue of the gut would establish, beyond doubt, the origin of the enzyme and its location in the tissue as well as relative concentrations of the enzyme in the foregut and hindgut. An attempt was therefore made to immunocytochemically label amylase in the digestive epithelium

of the foregut and hindgut. The method used here was an immunogold labelling technique, based on that of Polak and Van Noorden (1984). The method used human anti-alpha amylase raised in rabbit as the primary antibody and normal goat serum as the blocking agent (see Appendix 1 for staining protocol). The tissue was routinely obtained, fixed for 2hrs in 3% paraformaldehyde and 0.1% gluteraldehyde in filtered, cacodylate buffered sea water, dehydrated on an ethanol series and transitionally infiltrated using acetone in an Araldite/Epon resin mixture. The tissue blocks were polymerized at 37°C for approximately 10 days before being sectioned and stained.

Scanning electron microscopy:

In order to establish whether bacteria adhered to either the gut mucosa or the gut contents of the urchin, the surfaces of these were microscopically examined using SEM.

Specimens were injected with 2.5% gluteraldehyde in 0.45µm filtered, cacodylate buffered sea water, through the mouth and anus, in the field as soon as they were collected. They were then placed into glass containers where they were submerged in fixative and left overnight. This procedure was performed to prevent any artifactual movement of gut contents from the foregut to the hindgut which usually occurs due to handling. The fixed animals were then dissected and the foregut, and contents, treated separately from the hindgut, and contents. The tissues and gut contents were then post-fixed in 1% aqueous osmium tetroxide and dehydrated on an ethanol series. This material was then critical point dried. In the case of the gut contents, the amyl-acetate step was omitted to prevent the disintegration of the particles. The material was mounted on copper specimen

stubs, coated with 12nm of gold palladium on a sputter coater and examined on a Jeol JSM 840 Scanning Electron Microscope.

Results

Ultrastructure:

The results obtained here correspond well with those obtained for other echinoids. The gross histology of the gut consists of four layers (Plate 1) that are consistent throughout the gut. The outermost layer (coelomic edge) is a single layer of epithelium that appears to be ciliated in places and serves as a basement epithelium. Immediately inside (distal) this layer, and somewhat interspersed in it, is a layer of smooth muscle. A layer of connective tissue lies between the smooth muscle layer and the digestive epithelium. Bands of collagen fibers and vacuoles are visible in this layer. The thickness of of this connective tissue layer varies between about 0.2 μ m in the foregut and 0.5 μ m in the hindgut. This may be related to the fact that the hindgut is extremely distendable.

The digestive epithelium of the gut is the innermost layer (lumen edge) and is the most diverse in structure. The cells are relatively long, 150 to 250 μ m (depending on where they are sectioned), and between 0.3 and 2 μ m thick. The epithelium itself forms pits in the tissue (Plate 2a) and is made up of several cell types. In the foregut epithelium, at least three cell types are distinguishable. The most numerous cell type is the zymogen cell (*sensu* Tokin and Filiminova, 1977; Plates 2b-d) which is characterized by three features. Firstly, it possesses numerous apical vacuoles and villus like projections (Plate 2b). Secondly, it possesses well developed rough endoplasmic reticulum (Plate

2c) and thirdly, a high density of mitochondria (up to 100 per μm^2) which tend to concentrate at the base of the cells (Plate 2d). These cells are also flagellated (see below). The other cell types are goblet cells and mucous glandular cells which occur mainly in the oral region of the foregut (Plate 3). The goblet cells are characterized by their light cytoplasm while the mucous glandular cells are filled apically with secretory granules.

In the hindgut, the main cell type is the storage cell although some zymogen cells do occur. Apically the storage cells display "cytoplasmic blebs" (*sensu* Bush, 1988; Plate 4A). Some storage cells possess villi and are flagellated (Plate 4B). The cytoplasm of the storage cells is generally more electron translucent than the zymogen cells and they possess considerably less rough endoplasmic reticulum and fewer mitochondria. There are two other distinguishing features of storage cells that require attention. Firstly, the cells contain varying numbers of "lamellar bodies," (*sensu* Tokin and Filiminova, 1977; Plate 5A) which are thought to be the remains of undigested algal plastids that are released into the lumen by apical lysis of the cells (Plate 5B). Secondly, heterolysosomes (*sensu* Tokin and Filiminova, 1977; Plate 4B and Plate 6) are apparent in this tissue. These are relatively large (1 μm diameter) lysosomes that contain a number of electron dense particles (see below).

Lysosome staining:

The results obtained from the lysosome staining procedure indicate that acid phosphatase activity is localized and occurs apically in the digestive epithelium of the foregut. Discrete electron dense deposits have a semi-circular to circular shape

(Plate 7) and appear to be membrane bound, although the membranes themselves are unstained. These deposits did not occur in the control sections. The deposits have a diameter of about 0.5 to 1µm and are not numerous or small enough to account for all the vacuoles found in the zymogen cell apex. These deposits are clearly distinguishable from the lead contaminants that occur in both the control and the experimental sections which are much smaller, scattered inclusions in the apical regions of the cell (Plate 7). Phosphatase activity was not detected in the hindgut.

The presence of glycogen in the gut epithelium:

Glycogen was detected in both the hindgut and foregut although no pattern in its distribution was discernible. In the foregut glycogen was detected in the basement membrane as well as in the digestive epithelium (Plate 8A). Glycogen in the hindgut (Plate 8B) was more densely packed and was found in the digestive epithelium in greater abundance than in the foregut. In Plate 8A, lipid-like bodies are visible. This suggests, but does not confirm, the occurrence of lipid storage in the foregut.

Immunocytochemical staining:

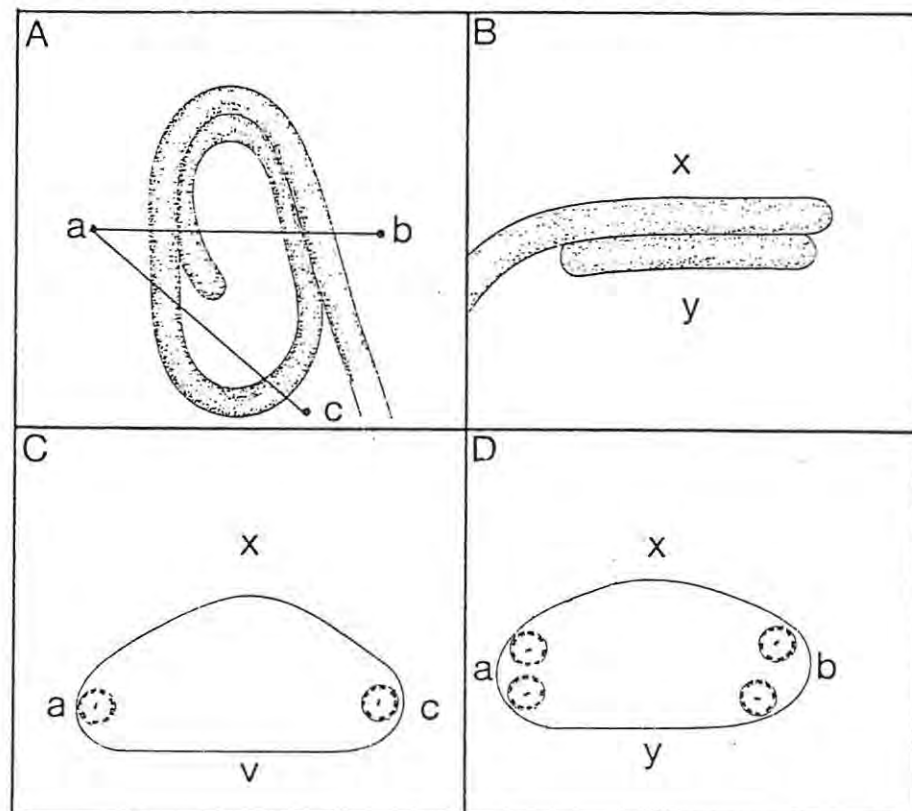
No result was obtained from the attempt to immunolabel sites of amylase production. The staining resulted in non-specific labelling, probably due to unsuitability of the primary anti-body and/or blocking agent. Ideally, the antigen should be isolated from the tissue to be labelled and an anti-body specific to that antigen generated.

Paddle cilia of the zymogen cells:

Scanning electron microscopy of the gut mucosa revealed the presence of "paddle cilia" (*sensu* Matera and Davis, 1982). Plates

9A and 9B show the surface of the gut epithelium at low magnification. The density of cilia is greater in the foregut than in the hindgut in accordance with TEM findings which show a greater number of flagellated zymogen cells than in the hindgut. The structure of these cilia is identical in the foregut and hindgut. They originate apically from the cell and are surrounded by folds of the cell membrane (Plates 10A and 10B) which can be seen in both the SEM and TEM micrographs. Plate 10B shows that these cilia are kinocilia since they possess the typical striated rootlette in the apex of the cell.

Figure 2.2: Theoretical representation of the structure of a paddle cilium head and section obtained from it.



It is from the terminal ends (or heads) of the cilia that the name paddle cilia (or disco-cilia) is derived. The cilia have swollen heads (Plates 11A and 11B), which are variable in

appearance. This variation may be due to differential swelling or shrinking of the heads. Figures 2.2A-D are theoretical representations of the head of a paddle cilium without the covering membrane. They are derived from the micrograph Plate 11b.

If the cilium head were sectioned through the indicated planes in Figure 2.2A, then two or four axonemes should be visible within a single membrane (as represented in Figures 2.2C and 2.2D). Two sections through the heads of these cilia corresponding exactly to the predictions in Figure 2 were obtained (Plates 12A and 12B). It is noteworthy that the appearance of the membrane surrounding the head is different in these two micrographs. In Plate 12A the surrounding membrane is swollen which would correspond to a spherically shaped head as in Plate 11A. Plate 12B shows the head with one convex side and one concave side which could correspond to a head structure as in Plate 11B. Which of these (if either) is artifactual is a matter of conjecture. However, the cupuliform structure of the cilium head in Plate 12B (cf. De Villiers and Hodgeson, 1987) is appropriate for a structure with a paddling function. Furthermore, the biconvex shape in Plate 12B could be explained as a swollen form of the head in Plate 12B. No microorganisms were detected adhering to the gut mucosa.

The surface of the gut contents:

The gut contents in the foregut are often covered in a layer of mucous although this is not always the case (Plate 13A and B). While the mucous could obscure microorganisms that are attached to the food particles, they were not detected on pieces free of mucous. In the hindgut, bacteria were clearly visible adhering to

the gut contents (Plates 14A-C). A wide variety of strains was apparent including rods, cocci, filamentous strains, and capsulated bacteria. Whether these bacteria were present in the foregut (i.e ingested with the food) or were resident in the gut is difficult to ascertain from these results. No bacterial film surrounding the gut contents was observed although they did occur relatively densely in certain cases. The greater density of bacteria in the hindgut may be due to bacterial growth in the gut resulting in easier detection.

In summary, the foregut and the hindgut differ distinctly in a number of respects. In the foregut, protein producing (exocrine) cells are prevalent and acid phosphatase activity was detected here only. Paddle cilia, with a likely circulatory function, occurred more abundantly in the foregut than in the hindgut. Glycogen was detected in both regions but in greater abundance in the hindgut. Storage cells were concentrated in the hindgut and displayed some unique features such as lamellar bodies and heterolysosomes. Gut microorganisms were not detected adhering to the gut mucosa while a variety of strains were observed on the gut contents of the hindgut only.

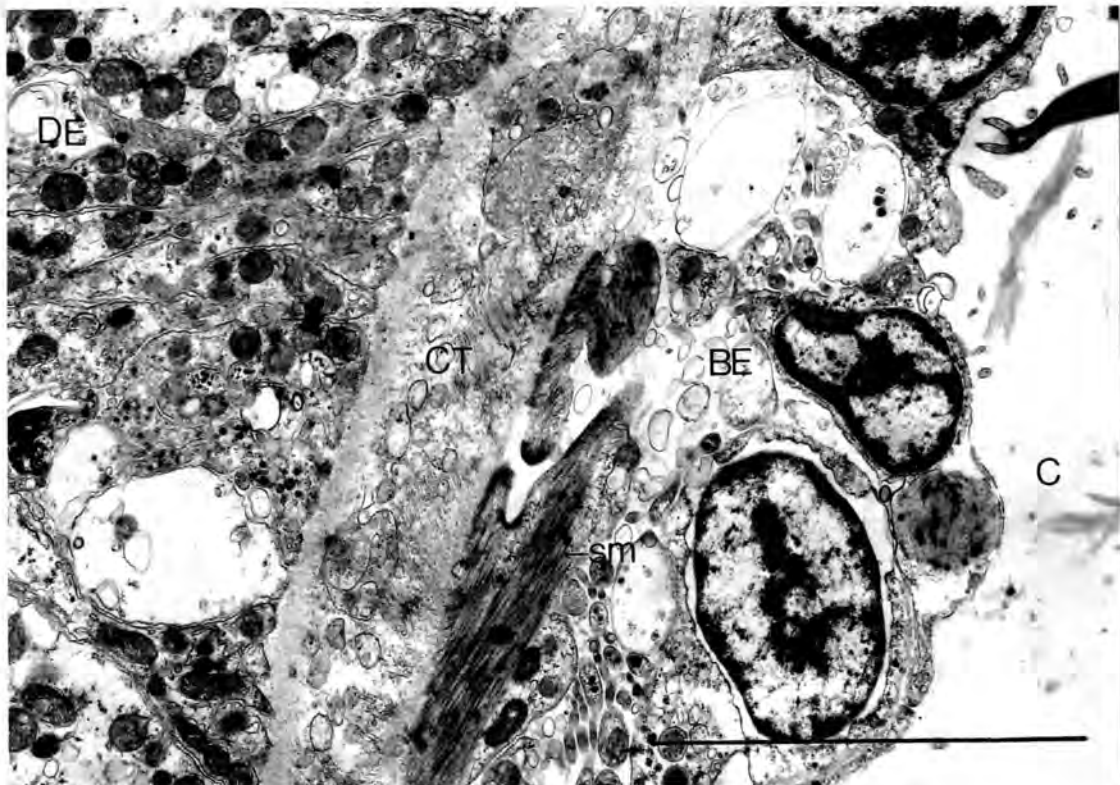


Plate 1: Four tissue layers in the gut. C = coelom, sm = smooth muscle, CT = connective tissue, DE = digestive epithelium. SCALE BAR = 0.5 μ m

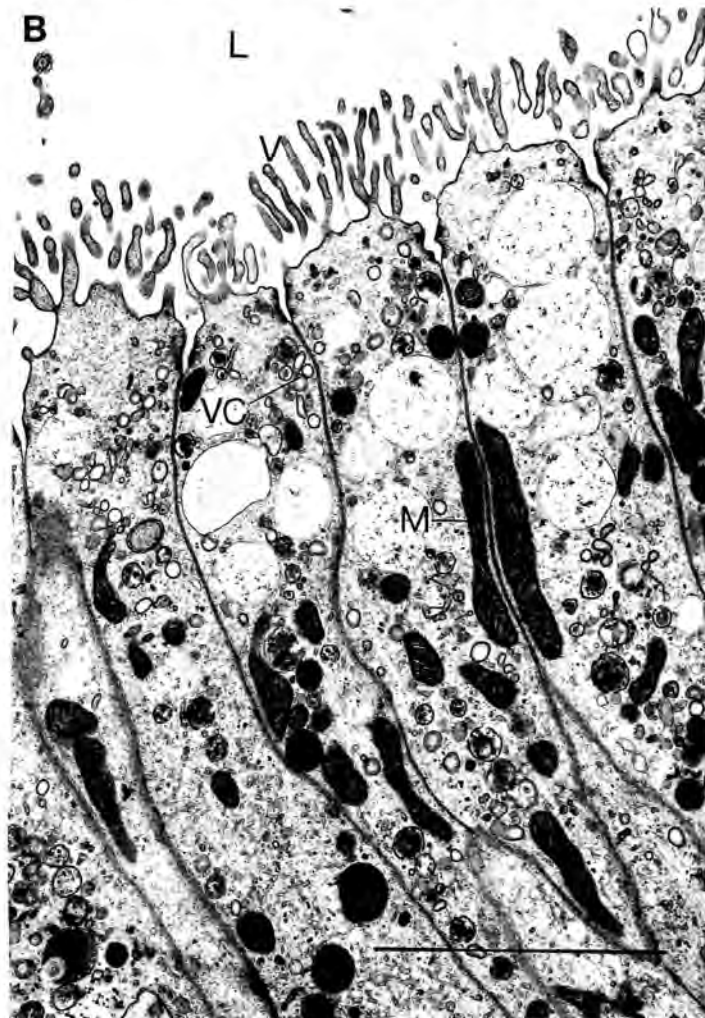
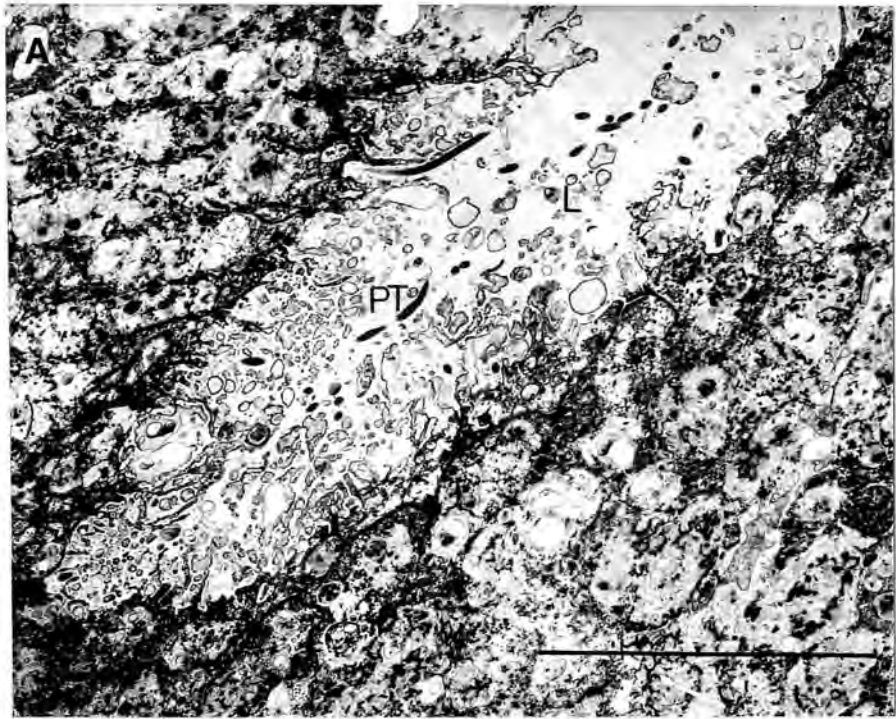


Plate 2: (A) Pit in the digestive epithelium. PT = pit, L = Lumen. SCALE BAR = 1 μ m. **(B)** Micrograph demonstrating some feature of a typical zymogen cell. VC = secretory vacuoles, M = mitochondrion, L = lumen. SCALE BAR = 0.5 μ m.

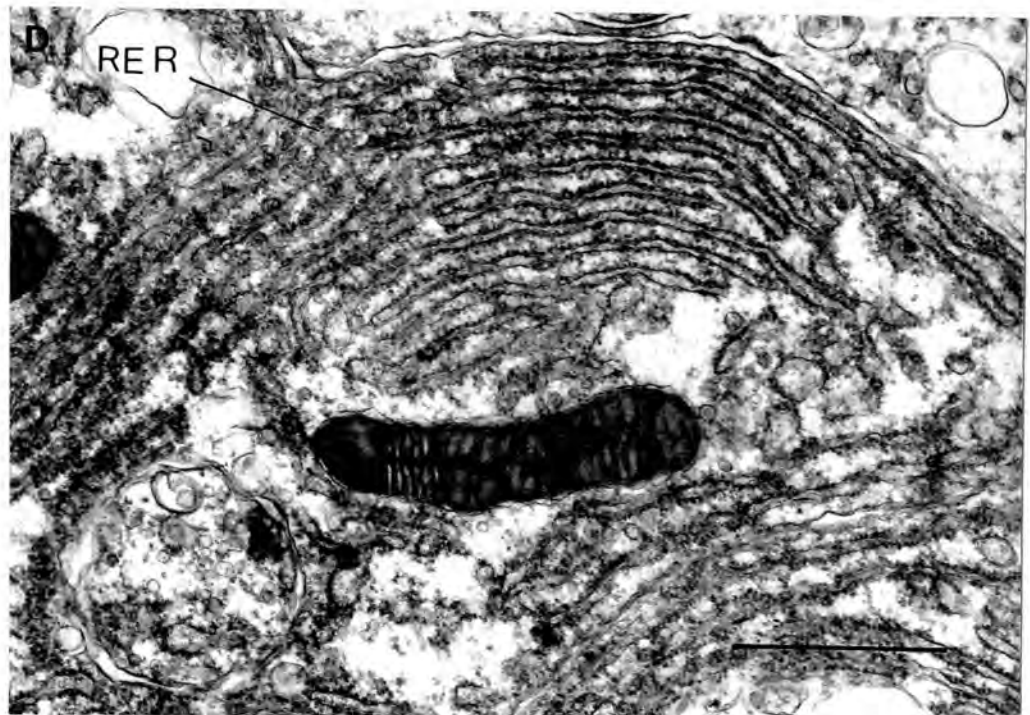
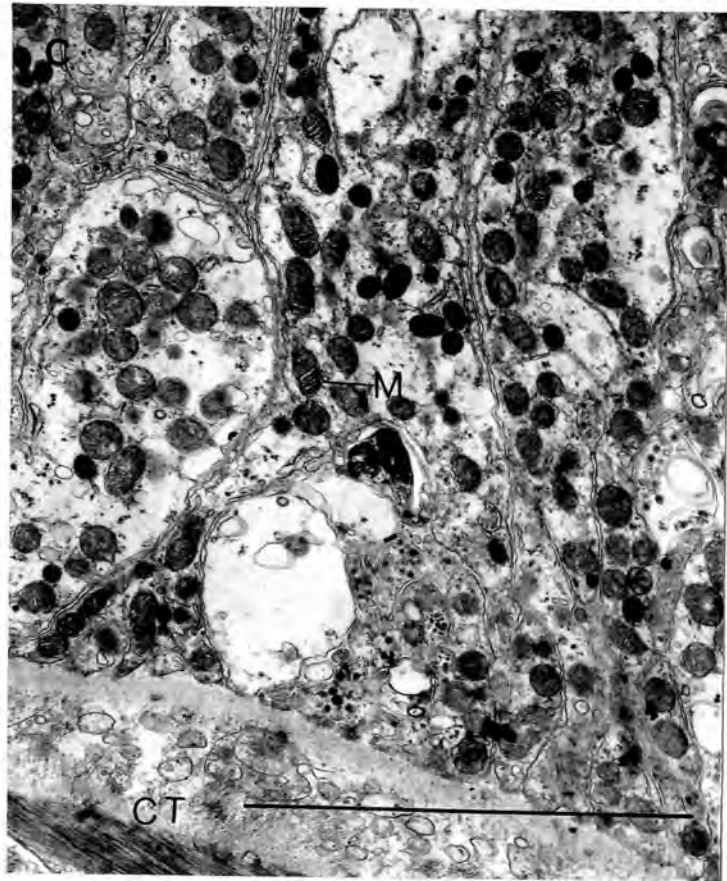


Plate 2 continued: (C) Mitochondria at the base of a zymogen cell. CT = connective tissue, M = mitochondria. SCALE BAR = 0.5 μ m. **(D)** Well developed Rough Endoplasmic Reticulum (RER) of the zymogen cells. SCALE BAR = 0.1 μ m.

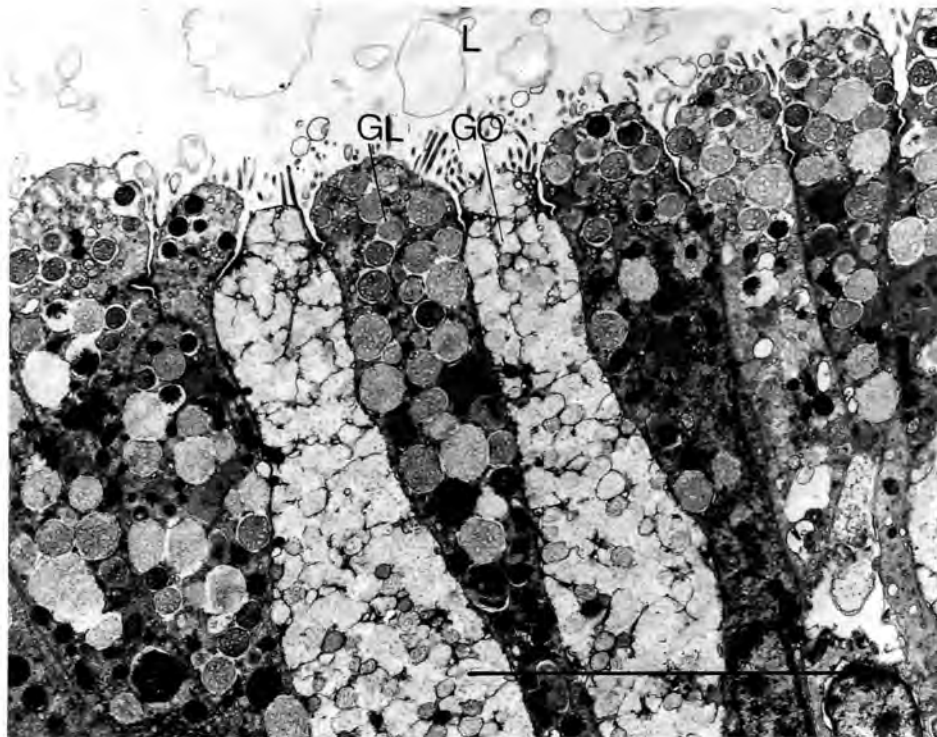


Plate 3: Cell types of the digestive epithelium. GO= Goblet cell, GL= mucous glandular cell, L= lumen. SCALE BAR= 1 μ m.

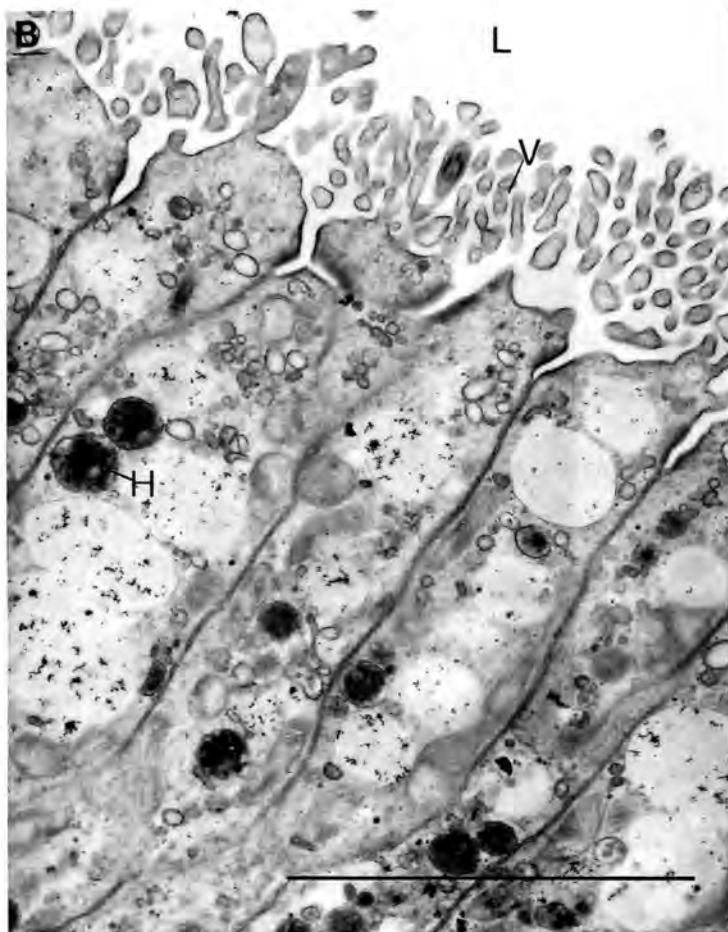


Plate 4: (A) Storage cell of the hindgut. B = Bleb, L=lumen. SCALE BAR = 0.5 μ m. (B) Alternative cell type in the hindgut V=villi, L=Lumen, H = Heterolysosome. SCALE BAR = 0.5 μ m.

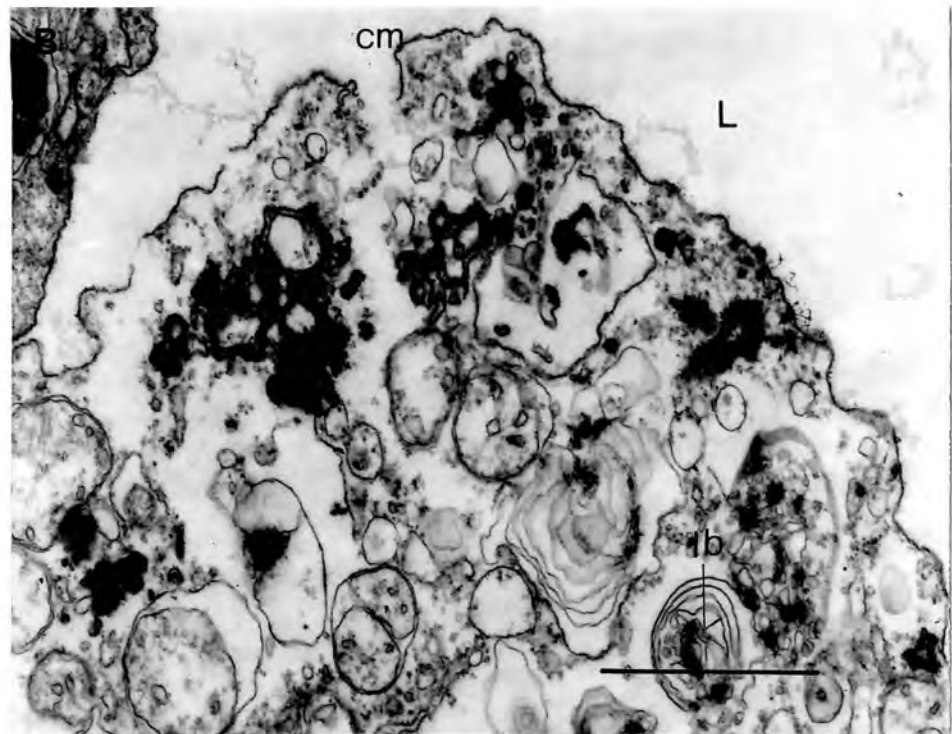
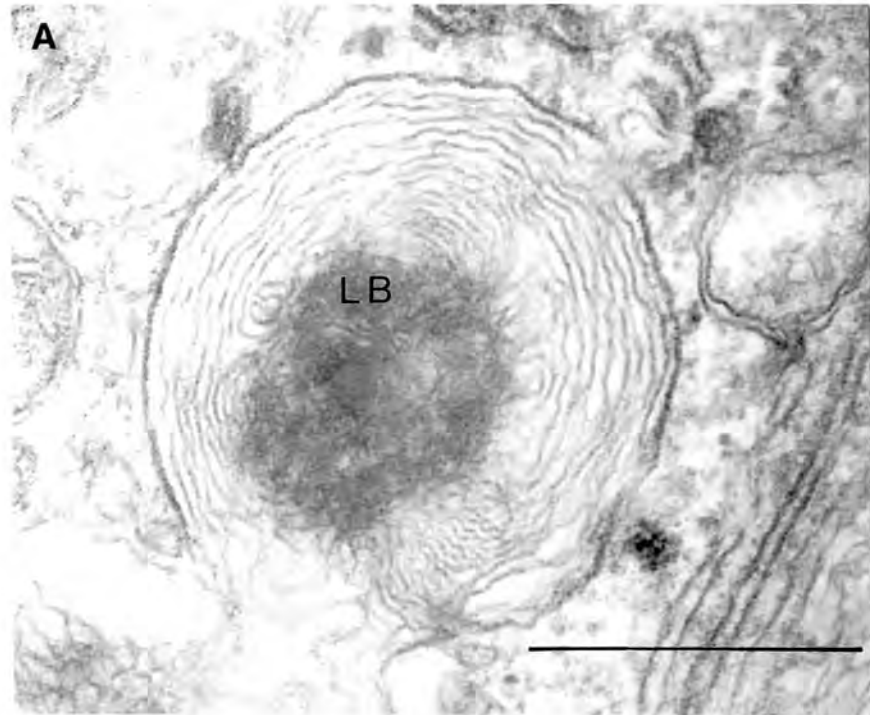


Plate 5: (A) Lamellar body (LB). SCALE BAR = 0.5 μ m. **(B)**: Apical lysis of the storage cells in the hindgut. L = Lumen. cm = cell membrane, lb = lamellar body. SCALE BAR = 0.1 μ m.

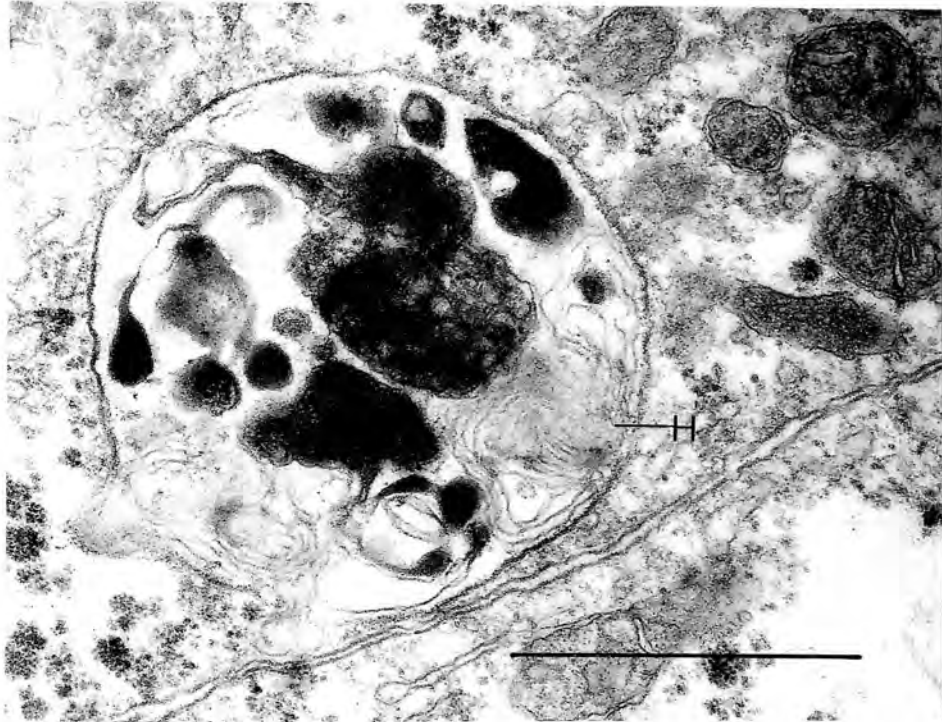


Plate 6: Typical heterolysosome in the hindgut storage cells.
SCALE BAR = 0.1 μ m.

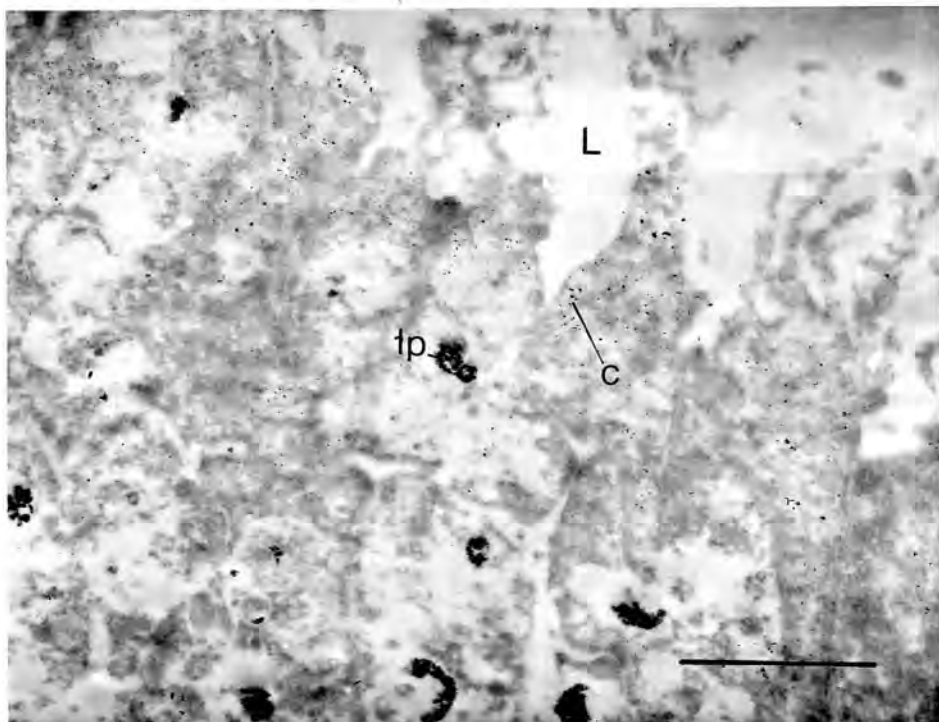


Plate 7: Result of the lysosome staining technique. lp = lead phosphate deposit, c = lead contaminant, L = Lumen.
SCALE BAR = 0.5 μ m.

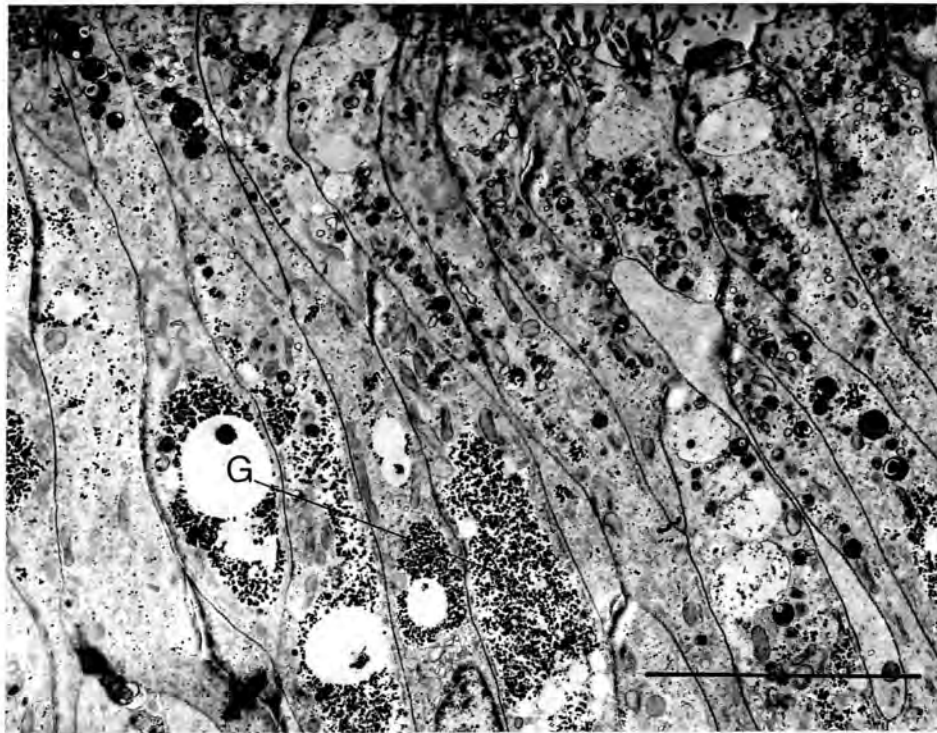
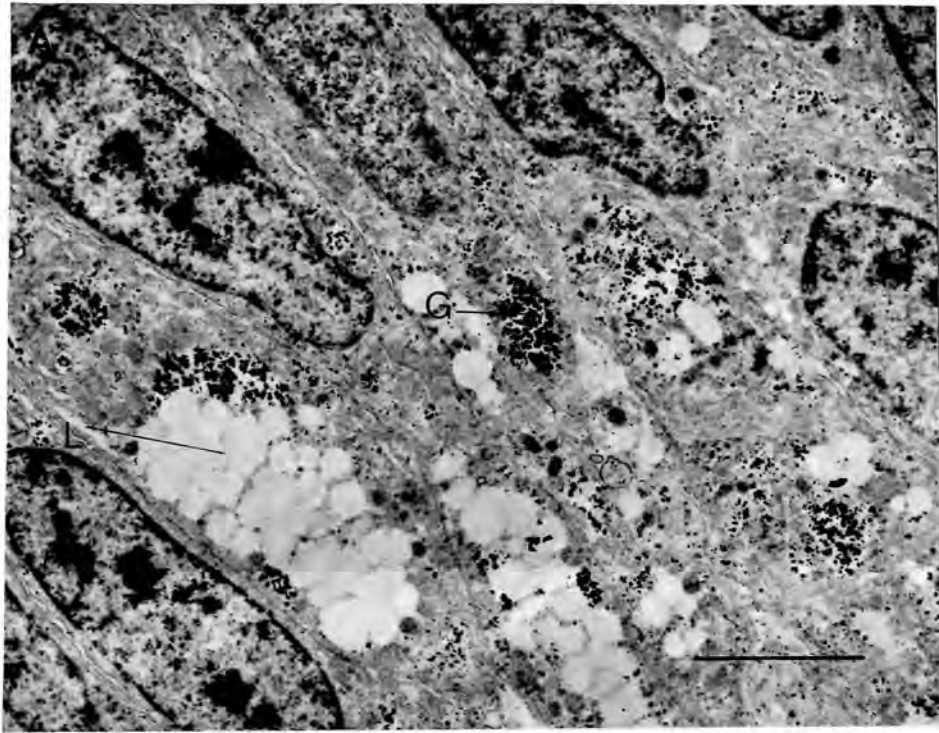


Plate 8: Reserve substances in the epithelium- glycogen staining technique. **(A):** Foregut. G = Glycogen, L = lipid like deposit. SCALE BAR = 0.3 μ m. **(B):** Hindgut. G = Glycogen. SCALE BAR = 1 μ m.

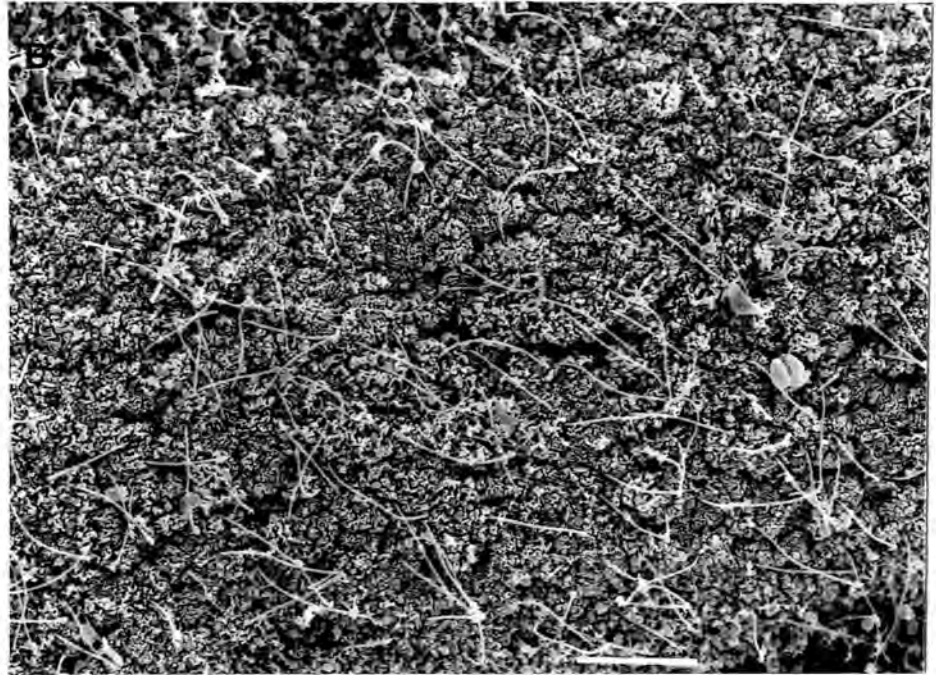
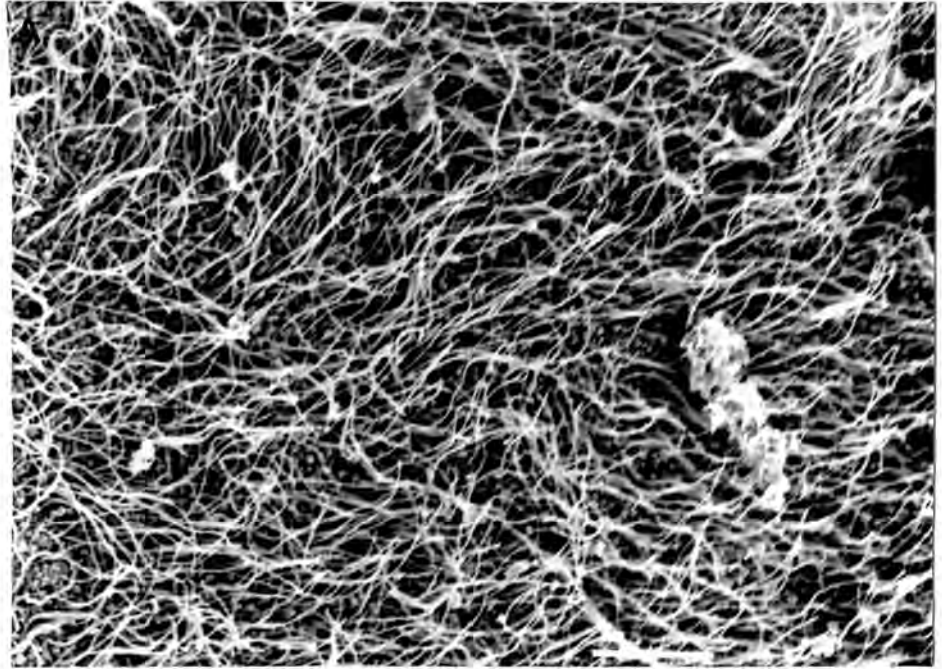


Plate 9: The surface of the gut mucosa. **(A):**Foregut. SCALE BAR = 10 μ m. **(B):** Hindgut. SCALE BAR = 10 μ m.

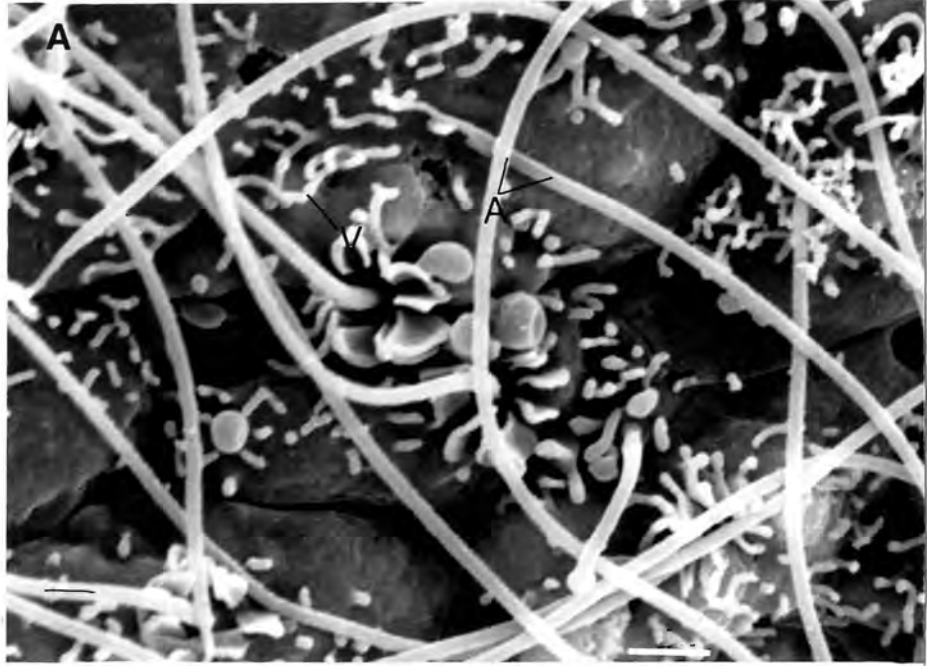


PLate 10: The origin of the paddle cilia. **(A):** SEM of the paddle cilium origin at the apex of a cell. A= axoneme, V = villus. SCALE BAR = 1 μ m. **(B):** TEM section through ciliary origin. C= cilium, L = Lumen, V= villi, sr = striated rootlet. SCALE BAR = 0.5 μ m

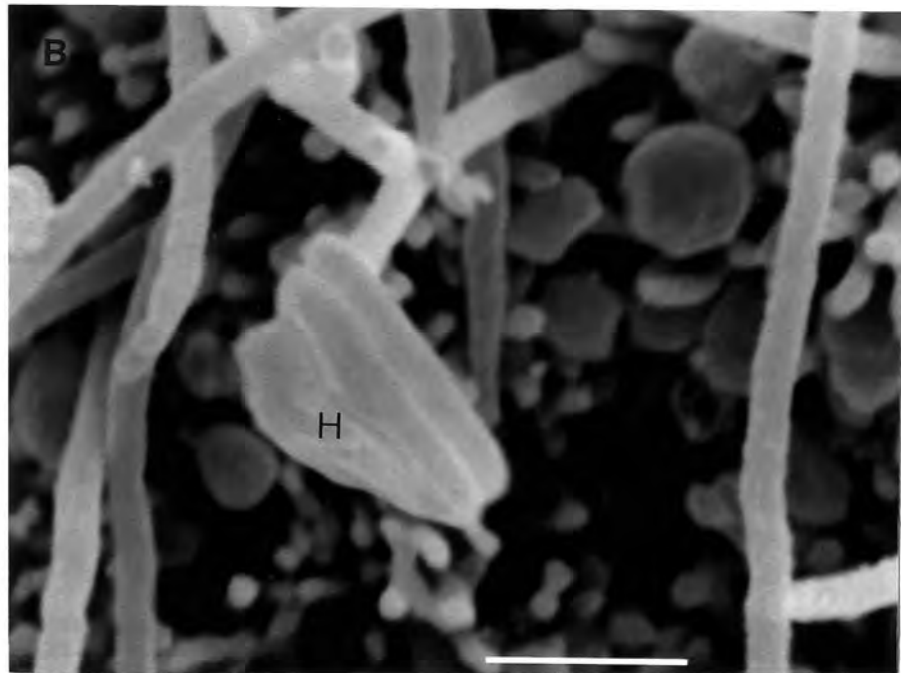
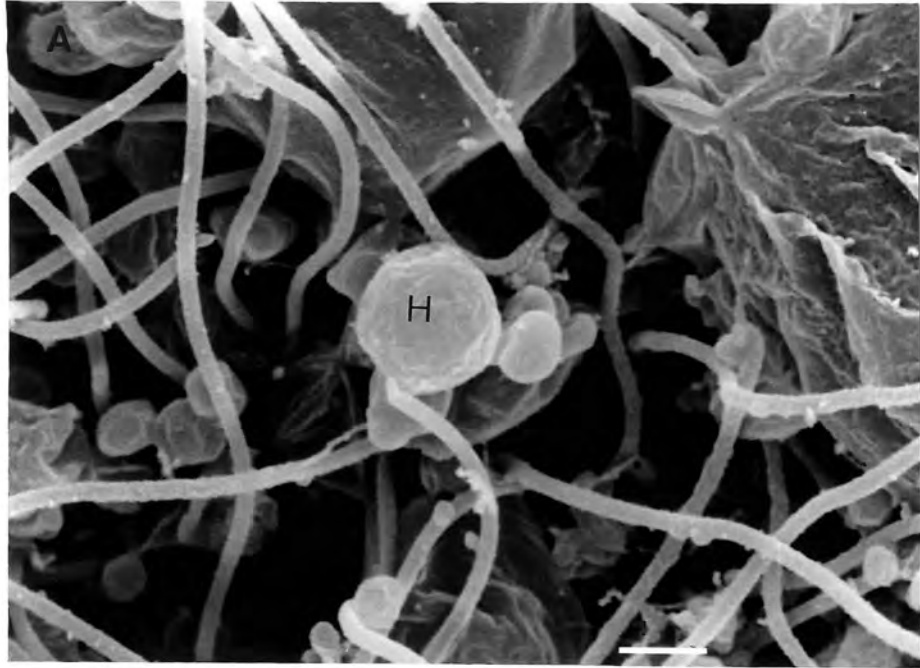


Plate 11: The terminal ends of paddle cilia. **(A):** Spherical head. H = head. SCALE BAR = 1 μ m **(B):** Cup-shaped head. H = head. SCALE BAR = 1 μ m.

A



B



Plate 12: Sections through ciliary heads. **(A):** Section showing two axonemes A = axoneme. SCALE BAR = 0.5 μ m **(B):** Section showing four axonemes. SCALE BAR = 1 μ m.

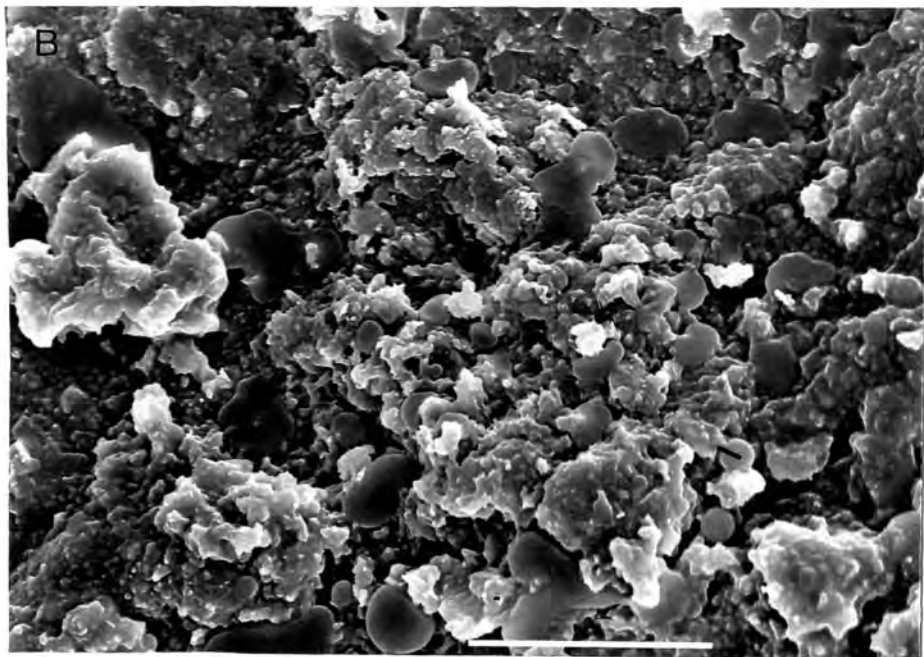
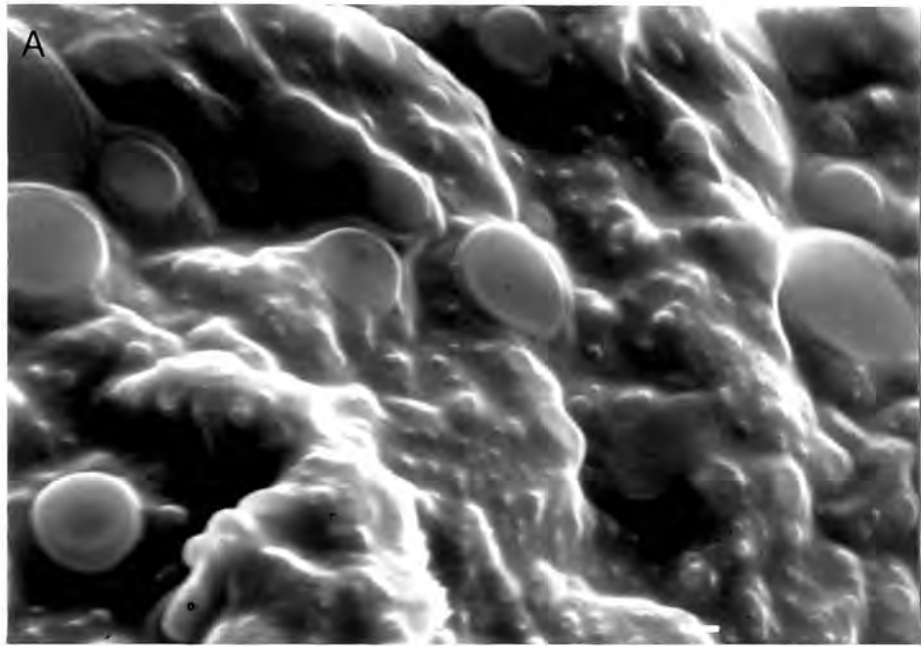


Plate 13: SEM micrographs of foregut contents surface. SCALE BAR = $1\mu\text{m}$ **(A):** Mucous-laden particle. **(B):** Mucous free. SCALE BAR = $10\mu\text{m}$.

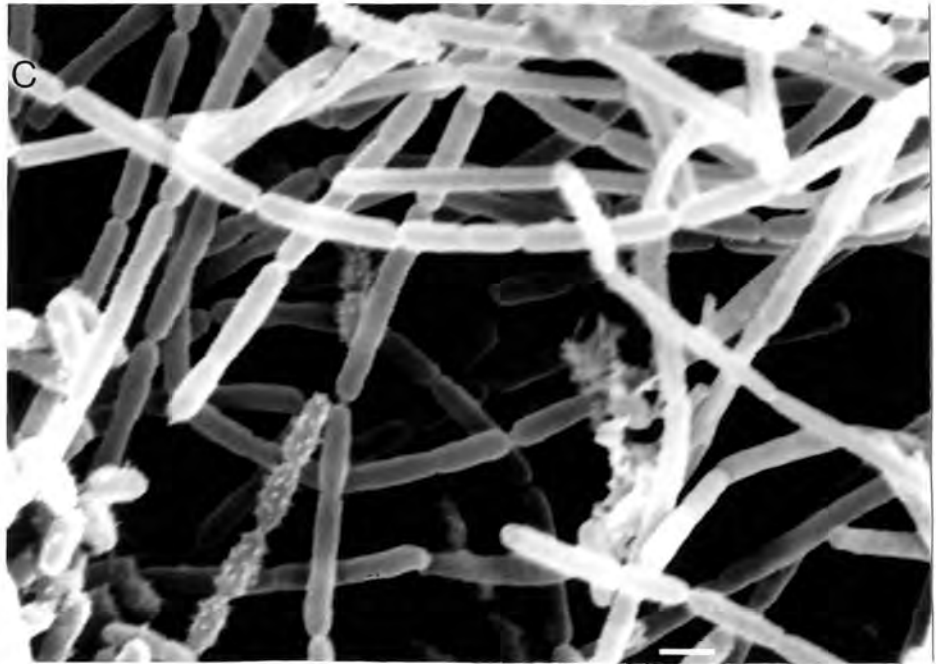
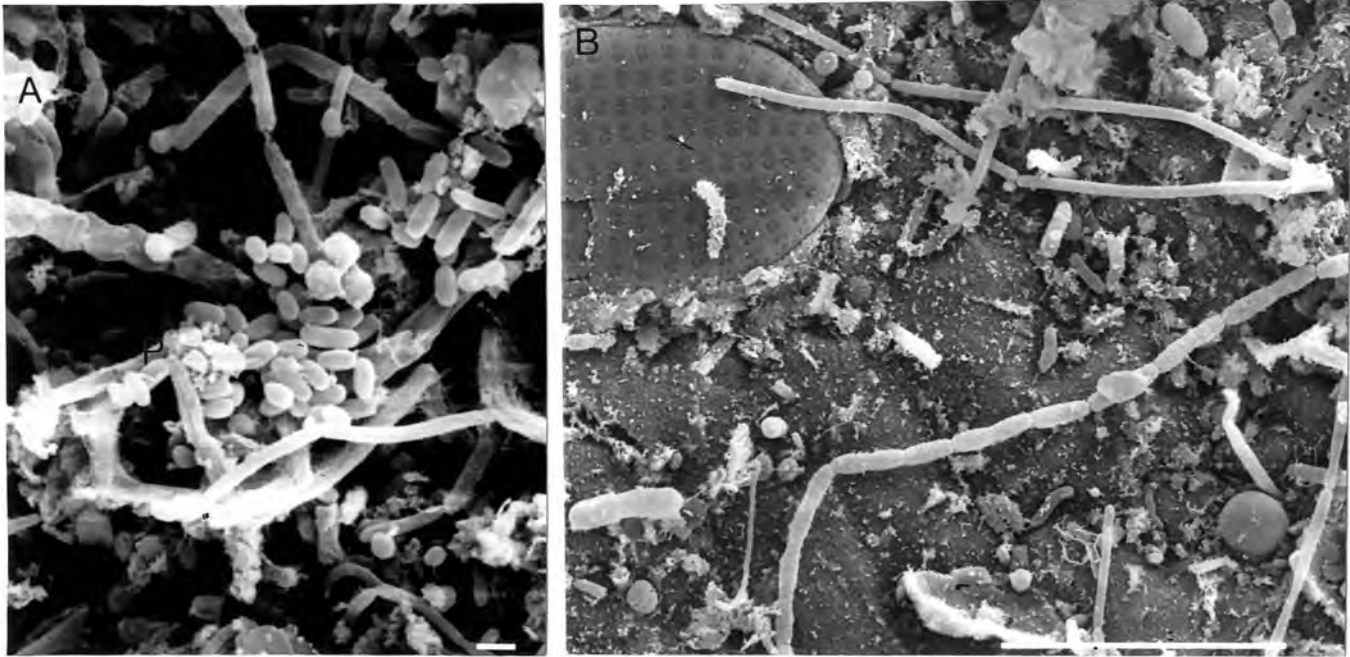


PLate 14: SEM micrographs of hindgut contents showing bacterial variety. **(A)** SCALE BAR = 1 μ m. **(B)** SCALE BAR = 10 μ m. **(C)** SCALE BAR = 1 μ m.

Discussion

Histology:

The basic histology of the gut of *P. angulosus* conforms closely to that described for other echinoid species (Fuji, 1961; Holland and Lauritis, 1968 and Tokin and Filiminova, 1977). While the cell length and width of the digestive epithelium falls within the range reported for other species, some cytological differences are apparent in the digestive epithelium. In *Strongylocentrotus purpuratus* (Holland and Lauritis, 1968) the rough endoplasmic reticulum was located basally in the zymogen cells while in *P. angulosus*, it is located mainly in the sub-nuclear region. Also, the extent of the rough endoplasmic reticulum was greater in *S. purpuratus* than in *P. angulosus*, although cell size and RNA concentration are affected by the nutritional status of the animal (Klinger, Watts and Forcucci, 1988). The location of the greatest concentration of mitochondria in the extreme basal regions of the cells was in agreement. The exocrine cells were generally limited to the foregut which is consistent with the results obtained for other species (see De Ridder and Jangoux, 1982).

The storage cells that occur predominantly in the hindgut possess features similar to those described for *S. droebachiensis* (Tokin and Filiminova, 1977). Of particular interest are the lamellar bodies that occur apically in these cells (plate 5). These are reputedly the remains of algal plastids that occur in vacuoles in the cells (Tokin and Filiminova, 1977) and are extruded into the lumen of the gut. In this study, these bodies were detected only in the hindgut whereas Tokin and Filiminova (1977) also observed them in the storage cells that occur in the foregut (or stomach)

of *S.droebachiensis*. The possibility that the lamellar bodies are artifacts cannot be dismissed on the basis of observation only. Their precise origin, function and significance can only be established experimentally. However, these observations support Tokin and Filiminova's contention that they are not artifactual as the organelles and cells in the immediate vicinity of the lamellar bodies were all well fixed and appeared normal.

Cytoplasmic blebs:

The same must be said for the cytoplasmic blebs (plate 4a) that occur at the apical extremity of the storage cells. Their occurrence was not reported by Tokin and Filiminova (1977) for *S.droebachiensis* although they are evident in the micrographs in their results. This feature and its significance were discussed by Bush (1988) for the limpet *Patella vulgata* who suggests that these blebs have a secretory function. His description of the blebs corresponds exactly with the results obtained here.

It is important to note that while some evidence of the disruption of the apical cell membrane was apparent on the surface of the gut in the SEM micrographs, features resembling blebs were not observed. This may imply that blebs are artifactual features caused during tissue processing for TEM or that they do not survive tissue processing for SEM.

Reserve substances:

Storage of glycogen and possibly lipids in the digestive epithelium is common to all echinoids investigated (Fuji, 1961; Doezema and Phillips, 1970; Tokin and Filiminova, 1977). Results obtained here indicate that glycogen is ubiquitously distributed

in the gut although it was found more extensively in the hindgut. This is consistent with the results obtained by Doezeema and Phillips (1970) who showed that more glycogen occurred in hindgut (second circuit) of the gut than in the foregut (first circuit) of *Strongylocentrotus purpuratus*. The results obtained here agree closely with those obtained for *S.droebachiensis* (Tokin and Filiminova, 1977). The distribution of glycogen and lipid was extremely variable in the sub- and peri-nuclear zone and in both the zymogen and storage cells. Tokin and Filiminova (1977) suggest that the non-uniform distribution of glycogen in the gut is probably due to asynchronous functional states of the cells in the gut.

Acid Phosphatase activity:

Acid phosphatase activity in the foregut indicates the occurrence of hydrolytic activity in the digestive epithelium. Hydrolytic activity was localized in what appear to be membrane bound organelles found apically the digestive epithelium of the foregut only. This information must be treated cautiously since the design of the experiment does not ensure complete infiltration of the substrate and lead ions.

Fuji (1961) demonstrated that alkaline phosphatase activity occurs in the border of the digestive epithelium of *Strongylocentrotus purpuratus*. In that instance he was able to show that it occurred along the whole length of the gut and that more activity was detected in the foregut than in the hindgut. From his results, Fuji (1961) infers that the gut has an absorptive function presumably along the entire length, although more so in the foregut. The results obtained here do not necessarily imply this since the destination of the organelles

that contain the hydrolytic activity is not known. Whether the organelles that contain the activity are heterolysosomes which are responsible for the intracellular digestion of absorbed substances as in *S. droebachiensis* (Tokin and Filiminova, 1977) or whether the contents are destined for extracellular digestion is uncertain.

Paddle cilia:

The ciliation of the digestive epithelium in echinoids (and other echinoderms) is well documented (De Ridder and Jangoux, 1982) but no authors have described the presence of paddle or disco-cilia in the digestive epithelium. Paddle cilia have been described for other phyla where they have generally been assigned a sensory function (Matera and Davis, 1982). Oldfield (1975, cited in Matera and Davis, 1982) described similar structures on the pedicellaria of the urchin *Psammechinus miliaris*. No record of this type of structure exists for the gut of any invertebrate. Matera and Davis (1982) presented experimental evidence to show that in the gastropod *Pleurobranchia californica* these structures are not artifactual. The results presented here seem to concur. Matera and Davis (1982) also showed that the head of the cilium is able to dilate and suggest that this is a functional aspect of their sensory ability.

The significant difference between paddle cilia described elsewhere and those in the gut of *P. angulosus* is that each cell possesses one cilium only, as opposed to tufts of cilia that occur, for example, in *P. californica* (Matera and Davis, 1982) and the pulmonate limpet *Siphonaria capensis* (De Villiers and

Hodgeson, 1987). Also, where the paddle cilia occur in the *P. californica* the tissue tends to be well innervated whereas in the gut of *Parechinus angulosus* it is not inordinately innervated. It seems unlikely that the cilia have a sensory function in the gut.

Enterocytes of echinoids do possess cilia that are known to produce currents in the esophagus (De Ridder and Jangoux, 1982) as they do on the gill of *Siphonaria capensis* (De Villiers and Hodgeson, 1987). Considering the structure of the ciliary heads it is likely that the paddle cilia help to create currents to aid the passage of food in the gut of *Parechinus angulosus*. Cilia were also observed on the basement membrane of the digestive tract. They are probably responsible for creating currents in the coelomic fluid as are cilia of the coelomic epithelium (Endean, 1966). These cilia were not as numerous as the cilia in the digestive epithelium and were not examined with SEM in this study.

The differential occurrence of the paddle-cilia in the gut of *Parechinus angulosus* is difficult to explain in terms of their circulatory function since one would expect them to occur abundantly in the hindgut as well as the foregut. That they occur more abundantly in the foregut is a reflection of the fact that only certain cell types are ciliated. It appears that only the zymogen or exocrine cells are ciliated and that, while the storage cells do possess a brush border, they are generally not ciliated.

The structural heterogeneity of the digestive epithelium:

There is a strong similarity in the structure of the columnar cells described for the anterior section of the gut of the herbivorous gastropod *Patella vulgata* (Bush, 1988) and the gastric enterocytes of urchins, both here and in other echinoids (Holland and Lauritis, 1968 and Tokin and Filiminova, 1977). The limpet cells encompass the features that are described for the exocrine and storage cells in the echinoids. They are ciliated, have a brush border, possess "residual bodies" that appear to be lamellar bodies, display blebs, have well developed rough endoplasmic reticulum, a high density of mitochondria and contain reserve substances as described for *P. angulosus* below. The limpet cells differ only in that they are smaller and that the relative location of the features in the cells is different from that described in the echinoids.

Bush (1988) suggest that several of the cell types found in the limpet gut are possibly the same types at different stages, with the loss of particular features (such as ciliation) occurring with age. This implies that the cells could be functionally dynamic or even absorptive and secretory simultaneously. Tokin and Filiminova (1977) discuss the functional heterogeneity of the digestive epithelium of *Strongylocentrotus droebachiensis*. In the foregut (stomach) intracellular digestion the synthesis of reserve products occurs simultaneously in some cells resulting in cell structural heterogeneity.

Also, all the cells of the digestive epithelium possess villi except those with blebs. Villi are also present in the exocrine zymogen cells suggesting a possible absorptive function. In their review De Ridder and Jangoux (1982) refer to the

"enterocytes" in the gut of echinoids as having variable functions at different stages along the gut. It is therefore suggested that the cells of the digestive epithelium are variations of the same type and that functional specialization of the cells is facultative, resulting in a loose functional specialization in different regions of the gut. This is supported by enzymological studies conducted in the gut (see chapter 3) and the differential occurrence of paddle cilia in the foregut and the hindgut.

In summary, the foregut of *Parechinus angulosus* is predominantly secretory and responsible for the production of hydrolytic digestive enzymes whereas the hindgut is predominantly absorptive and responsible for intracellular digestion. These functions are not entirely mutually exclusive to either region of the gut. The synthesis of reserve substances occurs in both regions of the gut but predominantly in the hindgut. Paddle cilia occur along the entire length of the gut with a greater concentration in the foregut and have a likely circulatory function.

CHAPTER 3:

THE DIGESTIVE POLYSACCHARIDASES OF *Parechinus angulosus*

Introduction

A number of studies have been conducted on the polysaccharidases of freshwater and marine invertebrate herbivores. While some of these were directed at a range of polysaccharidases in several phyla (Huaug and Giese, 1958; Kristensen, 1972; Moldotsov, Vafina, Kim, Sundukova, Artyukov and Blinov, 1974; Elyakova, Shirokova, Oshitok, and Uvarova, 1974; Elyakova, Shevchenko and Avaeva, 1981; Yamaguchi, Araki, Aoki, Tseng and Kitamikado, 1989) others were surveys for specific polysaccharidases such as laminarinases (Sova, Elyakova and Vaskovsky, 1970 and Piavaux, 1977); alginases (Favorov and Vaskovsky, 1971); cellulases and chitinases (Elyakova, 1972) and β -N-Acetylglucosaminidases (Moldotsov and Vafina, 1972). Specific studies mainly concentrate on three phyla: Crustacea (e.g Telford, 1970; Stuart, Head and Mann, 1985 and Musgrove, 1988); Mollusca (e.g. Alexander, Cutler and Yellowless, 1979; Gianfreda, Imperato, Palescandolo and Scardi, 1979; Kesler, 1983; Mayasich and Smucker, 1986; Suzuki, Watanabe, Tanaka and Ohnishi, 1986; Hughes, 1986 and Barlocher, Arsuffi and Newell, 1989a); and most commonly on Echinodermata (see Lawrence 1982 for review and also Hultin and Wanntrop, 1966; Barnier, Sheehan and Williams, 1975; Scheibling, 1980; Propp, Ryabushko, Zhuchikhina and Propp, 1983; Klinger, 1984; Claereboudt and Jangoux, 1985; Klinger and Diehl, 1985 and Feral, 1989).

A minority of polysaccharidase studies conducted have tested a wide range of naturally occurring polysaccharides for breakdown by a single species. In his review, Lawrence (1982) points out

that even with the data available at that time, knowledge of digestion in echinoderms remained scanty and suggested that further digestive studies were needed with a wider array of substrates.

Three basic methods are used to assay polysaccharidases. The suitability of each of these methods depends largely on the properties of the polysaccharide concerned and whether the researchers are targeting enzymes that lyse specific bonds. Viscous polysaccharides such as alginates and some celluloses, for example, have been used in viscosimetric assays (Epply and Lasker, 1959; Hultin and Wanntrop, 1966). Artificial chromogenic substrates can be used assay specific enzymes such as β -glucosidases and β -galactosidases (Klinger, 1984). The most common assay for polysaccharidase activity is end-product analysis, which involves the measurement of a change in the levels of reducing sugars, in an incubation of enzyme and substrate (e.g Claereboudt and Jangoux, 1985 and Yamaguchi *et al.*, 1989). Generally, the Bernfield (3,5- dinitrosalicylic acid method) or the Nelson-Somogyi method for the determination of reducing sugars is used. In this study, the Nelson-Somogyi method was used to assay polysaccharidase activity.

The Nelson-Somogyi method:

The Nelson-Somogyi method for the determination of reducing sugars (Nelson, 1944; Somogyi, 1952), although widely used to assay polysaccharidases, has several limitations. These are:

- 1) Only reducing sugar levels are determined and lysis of long chain polysaccharides to oligosaccharides (of variable length) and disaccharides can be detected but not quantified.

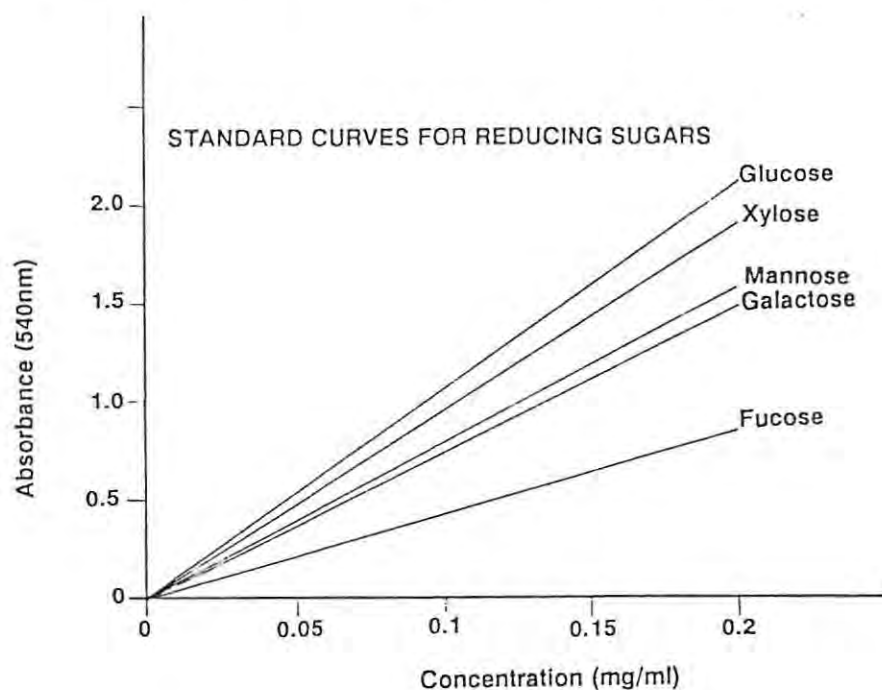
- 2) Comparisons between this method and that of Bernfield (3,5-dinitrosalicylic acid method) have revealed serious discrepancies in results (Breuil and Saddler, 1985 and Fielding, Harris, Lucas and Cook, 1986). Fielding *et al.* (1986) concluded that the Bernfield method results in higher reducing sugar values, while the Nelson-Somogyi method was significantly more sensitive. The implication is that these methods are unreliable when determination of absolute values of reducing sugars levels or enzyme activity are desired, as in the case of studies of enzyme rates and energy budgets. However, the methods are still very useful in qualitative and comparative studies.

- 3) Results obtained from this method are determined using a standard curve generated from a series of reducing sugars of known concentration. The standard curve generated from different reducing sugars have significantly different slopes (Fig. 3.1) and it is thus crucial that to use the monomer closest to the units in the polysaccharide substrate in question to generate the standard curve.

Since only an indirect measurement of enzyme activity is based on a standard curve is obtained and since it is often impossible to obtain the monomers of these polysaccharides (and therefore necessary to use suitable substitutes), the results may not always accurately reflect true enzyme activity.

As this method measures total reducing sugar content intrinsic reducing sugar levels in both enzyme extracts and substrate solutions must be accounted for in experimental design.

Figure 3.1: Plots of standard curves obtained on different reducing sugars at the same concentrations.



Glycogen and anabolic enzymes:

The enzyme extracts prepared in many polysaccharidase studies (including this study) are crude extracts (e.g. Sova *et al.*, 1970; Elyakova, 1972; Elyakova *et al.*, 1974, 1981; Moldotsov *et al.*, 1974; Scheibling, 1980; Claereboudt and Jangoux, 1985 and Klinger and Diehl, 1985 among others). Apart from the removal of low molecular weight substances by gel filtration or dialysis, the extracts are generally unpurified soups of tissue homogenate which may contain numerous of biologically active compounds. Since glycogen is stored in the tissue of the gut (Chapter 2) one would expect that this glycogen would be found in solution in the enzyme extract. Doezeema and Phillips (1968, 1970) have shown that glycogen occurs in the gut of the urchin *Strongylocentrotus purpuratus* and that various reducing sugars make good precursors

for glycogen synthesis in the gut. It is therefore likely that crude enzyme extracts contain both catabolic and anabolic enzymes. Thus, results may be either inflated, due to hydrolysis of intrinsic glycogen in the extract, or deflated by the synthesis of glycogen from reducing sugars produced during the experiment. Alternatively these two processes could balance each other, resulting in no net effect.

To obviate this problem, it is necessary to determine the level of glycogen in the extract and the potential yield of reducing sugars from its hydrolysis. Thereafter, it is necessary to monitor samples of enzyme extracts for changes in levels of intrinsic reducing sugars under the same conditions and over time periods comparable to those of the enzyme assay. In this way, the effect of reserve polysaccharides in the extracts can be measured and accounted for in the results of the enzyme assays. This procedure has been neglected in all the aforementioned studies.

In the present study, nine naturally occurring and one artificial polysaccharide were incubated with enzyme extracts obtained from the foregut and hindgut of *Parechinus angulosus* in order to determine the range and extent of polysaccharidase activity.

Materials and methods

Specimen collection:

All specimens of *Parechinus angulosus* were collected at low tide at Port Alfred, South Africa (33° 35'S, 26° 55'E; Fig 1.1) and immediately transported (60km) to the laboratory in Grahamstown in well aerated containers of fresh sea water. The specimens were then processed on ice, in the shortest time possible.

pH of the gut:

The pH of the gut was determined by measuring the pH of the gut contents and homogenates of the gut of freshly dissected specimens with a Beckman 3500 digital pH meter. A measurement of pH 6.8 was obtained which corresponds well with those obtained for other echinoids (Ferguson, 1969; Lawrence, 1982). A 20 mM phosphate buffer (6.8) containing 150 mM NaCl (Fielding *et al.*, 1986) at pH 6.8 was subsequently used in all enzyme extracts and all enzyme assay procedures.

Enzyme extract:

The foregut and hindgut of each specimen was dissected out and immediately washed in chilled buffer in order to remove all the gut contents. The foregut and hindgut were then treated separately. The foreguts or hindguts of ten specimens were combined and homogenised in 20 ml cold buffer with an ultra-turax TP18/2 electric homogeniser. The homogenate was centrifuged for 1.5 hours at 4°C at 12000 g on a Sorvall RC-5 refrigerated centrifuge. The supernatant was then dialysed against buffer (in visking tubing with a cut off point of 12000- 14000) at 4°C for 48hours with two changes of buffer. The dialysed extract was diluted to 75ml with phosphate buffer and stored in aliquots of 15 ml at -15°C for no more than one month. Preliminary tests showed that the activity of the extract was unaffected in this period. The protein content of each extract was determined with the Folin-Lowry total protein assay (Lowry, Rosebrough, Farr and Randall, 1951) using Bovine Serum Albumin (BSA) as a standard.

Substrate polysaccharides:

The substrates (Table 3.1) were selected in order to test for digestion of polysaccharides from as wide a range of algae as possible. The substrates can be divided into two main groups in terms of their function.

Table 3.1: SUBSTRATE ALGAL POLYSACCHARIDES.

The algal polysaccharides used as substrates to assay polysaccharidases, the function of each polysaccharide, its constituent monomer (and monomer used to generate the standard curve), the class in which it occurs and source of information are given.				
Polysaccharide	Function	monomer [^]	class ⁺	source [#]
Amylose	Storage	Glucose	Rh/Chl	MS,MC,PN
Glycogen	Storage	Glucose	Rh	MS,PN
Laminarin	Storage/struc	Glucose	Ph	PE,MC,MS,PN
Inulin	Storage	Fucose*	Chl	PE,MS,PN
CM-Cellulose	Structural	Glucose*	Rh/Chl	PE,PD
Fucoidan	Structural	Fucose*	Ph	MC,PD
Carrageenan	Structural	Galactose*	Rh/Chl	MC,PD,PN
Xylan	Structural	Xylose	Chl/Rh	PE,MC,PD,PN
Mannan	Structural	Mannose	Chl	PE,MC,PD
Alginic Acid	Structural	Glucose*	Ph	PE,MC,PD,PN

[^] - The monomers indicated here were used to generate the standard curve for the corresponding polysaccharide.

* - the monomers of these polysaccharides are derivatives of the indicated sugar, but are unavailable and thus the indicated monomer was used to generate the standard curve.

+ - Rh=Rhodophyta; Chl=Chlorophyta; Ph= Phaeophyta

- PE= Percival, 1979; MC= McCandless, 1982; MS= Manners and Sturgeon, 1982; PD= Percival and McDowell, 1982; PN= Painter, 1983.

These are storage and structural polysaccharides which differ in complexity, the former usually being of a simple nature. At least one structural and one reserve polysaccharide from the Chlorophyceae, the Rhodophyceae and the Phaeophyceae was included. All the substrates used in these experiments were of natural origin except for carboxymethyl-cellulose. For the enzyme assays, 1% solutions of the substrates unless otherwise stated were made up in buffer (pH 6.8). In order to dissolve the alginic acid it was necessary to raise the pH to 8.0 by addition of a NaOH solution (1M) resulting in an alginic acid solution of pH 7.

Assay procedure:

All assays were carried out at 20°C.

1) The measurement of glycogen in enzyme extracts:

a) 0.2 ml of foregut or hindgut enzyme extract was added to a test tube containing 0.2ml buffer and 0.4ml of 1.2M HCl. and were boiled in a water bath for 2hours. After cooling, 1ml of a 2% NaOH solution was added to neutralize the solution. To this, 2ml of Nelson's alkaline copper reagent were added and the samples boiled for a further 10 minutes. After cooling, 2ml of Nelson's arsenomolybdate chromogenic reagent was added. The samples were then centrifuged for 1 minute at 500 rpma and the absorbance measured on a Shimadzu UV-150-02 spectrophotometer at a wavelength of 540nm.

b) Two controls were run in each experiment. These were identical to the experimental samples except that the enzyme extract was substituted by 0.1 or 0.2 mg/ml glycogen solution in order to check for complete hydrolysis of glycogen and to a

obtain an empirical glycogen:glucose ratio for that experiment.

c) Standard curves (for the foregut, hindgut and controls) were generated simultaneously using samples identical to the experimental samples except that the buffer contained a known amount of reducing sugar (0.1, 0.2 or 0.3 mg/ml) and that hydrolysis of glycogen was prevented by immediately neutralizing the acid (as previously described above) and by omitting boiling.

d) Separate blanks were prepared for the foregut, hindgut and glycogen controls. These contained 0.2ml extract or glycogen solution, 0.2ml buffer and 0.4ml 1.2M HCl, and were processed as above.

Absorbances were converted to reducing sugar concentrations from the standard curves. Glycogen concentrations were calculated according to ratios derived from the hydrolysis of the known concentrations of glycogen in the controls. These were then corrected for protein content of the samples and expressed as mg glycogen/mg total protein (BSA equivalents)/ml of enzyme extract. The potential yield of reducing sugars after complete hydrolysis of the determined levels of glycogen was calculated using the conversion factor 1.1 (Oser, 1965). The following equations were used:

$$\text{Specific Glycogen Content} = \frac{\left[\frac{\text{glucose yield after hydrolysis}}{\text{empirical glycogen:glucose ratio}} \right]}{\text{protein concentration of extract}}$$

$$\text{Potential Glucose yield} = \text{Specific Glycogen content} \times 1.1$$

2) Blank and standard curve generation for enzyme assay: Fresh reagent solutions were made up prior to each assay and the same reagents were used for the generation of the standard curve. The standard curve was generated immediately prior to each experiment.

The reducing sugar appropriate to the substrate being tested (Table 3.1) was made up in a serial dilution of 0.1, 0.2 and 0.3 mg/ml in buffer and used as follows: Each sample contained equal proportions (0.2ml) of fore- or hindgut enzyme extract, the appropriate substrate solution and a solution of buffer containing a known concentration of reducing sugar. The enzyme extract and substrate solutions were included to account for volume, turbidity and intrinsic levels of reducing sugar of the solutions. The standards (and blank) were prepared in a manner that prevented any reaction of the enzyme with the substrate by adding the enzyme and the reagent simultaneously at time zero and immediately transferring to a boiling water bath.

The blank was prepared simultaneously and contained equal proportions (0.2ml) of enzyme extract, substrate solution and buffer (void of any reducing sugar) and used for the standard curve as well as the enzyme assay. Reducing sugar levels were determined using the Nelson-Somogyi method. A linear regression was performed to obtain the relationship between absorbances reducing sugar concentration. Only those data which produced a correlation co-efficient of 0.9 or more, were accepted for the standard curve.

3) Enzyme assay: After allowing the enzyme extract to stabilize at 20°C for thirty minutes, equal volumes (0.2ml) of fore- or hindgut enzyme extract, substrate solution and buffer (to compensate for volume in the blank and standards) were introduced to a series of test tubes at time zero. The reactions were stopped at the appropriate times ($t = 0, 2, 4, 6, 8, 10, 12, 15, 17, 20, 23, 25, 27$ and 30 minutes) by adding 0.6ml of Nelson's alkaline copper reagent and placing the test tubes (closed with glass marbles) in a water bath at 100°C for ten minutes. After cooling, 1.2 ml of freshly prepared Nelson's arsenomolybdate chromogenic reagent was introduced. The samples were well mixed and centrifuged after which absorbance was measured. The results were converted to reducing sugar equivalents using the standard curves and corrected for protein content of the extract. The results (mg reducing sugar/mg total protein (BSA equivalents)/ml) were used to generate plots of enzyme activity against time.

The data were plotted as a mean of at least three determinations \pm one standard deviation. Best fit regression analyses were performed on the data and the regressions (where obtained) plotted. Where distinct linear and non-linear phases were obtained, the data were split at the inflection point and the non-linear phase fitted to a power function. Enzyme activity at 0, 2, 5, 10, 15 and 30 minutes was calculated with the equation of the fitted curves.

4) Monitoring intrinsic reducing sugars: In order to monitor changes in the level of reducing sugars, both foregut and hindgut enzyme extracts were assayed in an identical manner to that described for the enzyme assays (above) except that no substrate was present. At $t=0$, the reducing sugar level in an incubation

of 0.2ml of enzyme extract and 0.2ml buffer was measured. This was repeated at t = 2, 4, 6, 8, 10, 12, 15, 17, 20, 23, 25, 27, and 30 minutes. Appropriate standards and a blank were prepared. Absorbances obtained were converted to reducing sugar concentrations from the standard curve and corrected for protein content of the extracts.

Results:

Glycogen levels of enzyme extracts:

Calculations of the glycogen contents for two extracts A and B are shown in appendix 2. The results of the determinations of glycogen in the two extracts are presented in Table 3.2. These results represent the maximum (A) and minimum (B) values obtained. While the results for the foregut extract are fairly consistent, a variation in glycogen content in the hindgut extract was noted. This was possibly due to variation in the glycogen content of the gut tissue, which is known to vary according to the physiological state of the specimen (Doezema and Phillips, 1970).

TABLE 3.2: GLYCOGEN CONCENTRATIONS OF ENZYME EXTRACTS:

		EXTRACT A	EXTRACT B
FOREGUT	mg GLYCOGEN/ mg total protein/ml	0.0223	0.0255
	mg GLUCOSE/ mg total protein/ml (potential yield)	0.0245	0.0280
HINDGUT	mg GLYCOGEN/ mg total protein/ml	0.0230	0.0075
	mg GLUCOSE/ mg total protein/ml (potential yield)	0.0253	0.00825

Digestive polysaccharidases:

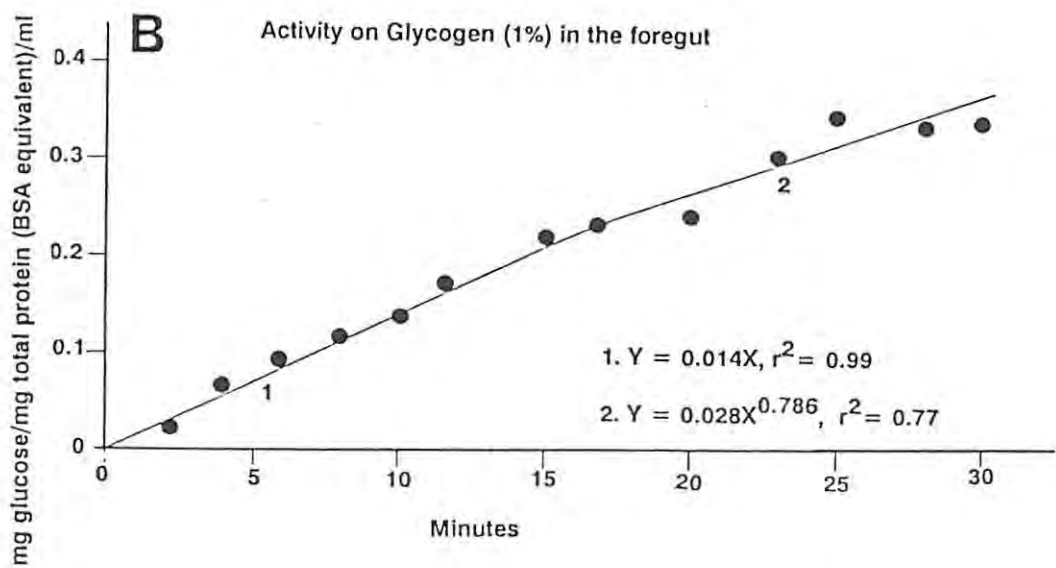
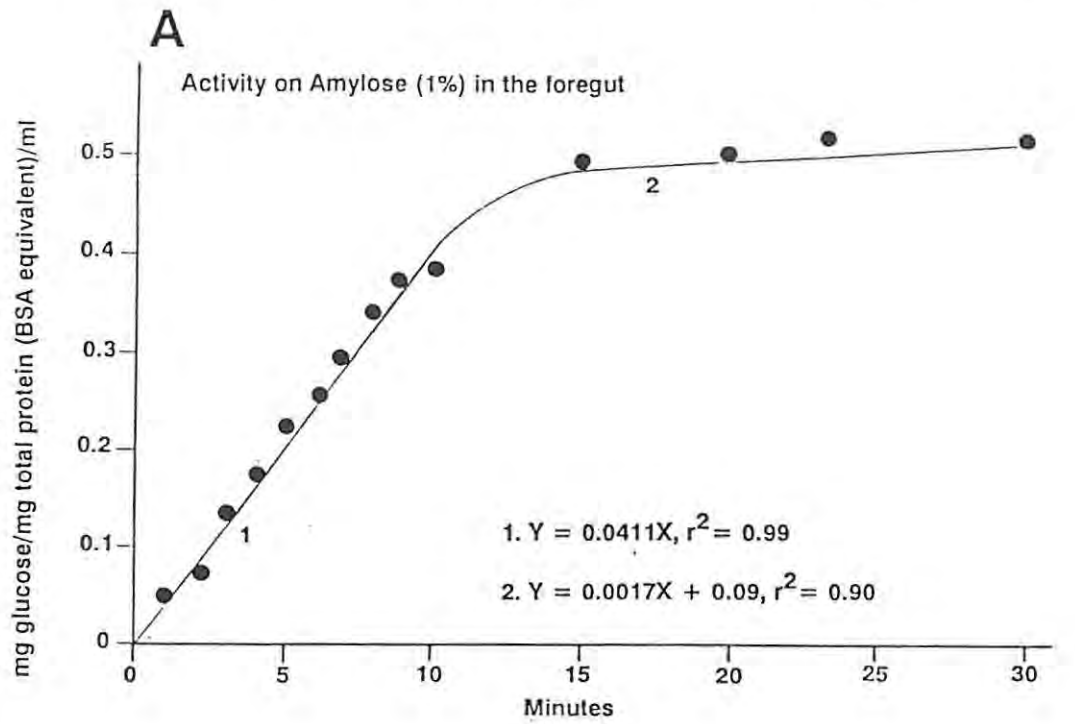
Figures 3.2 (A-J) and 3.3 (A-J) are plots of the enzyme activities recorded on the substrate polysaccharides with regression curves fitted, where obtained. These plots demonstrate that three distinct levels of enzyme activity occurred. Generally, these were end product concentrations greater than 0.1, less than 0.07 or less than 0.01 mg reducing sugar/mg total protein/ml. Different levels were obvious between the substrate polysaccharides and between the foregut and the hindgut.

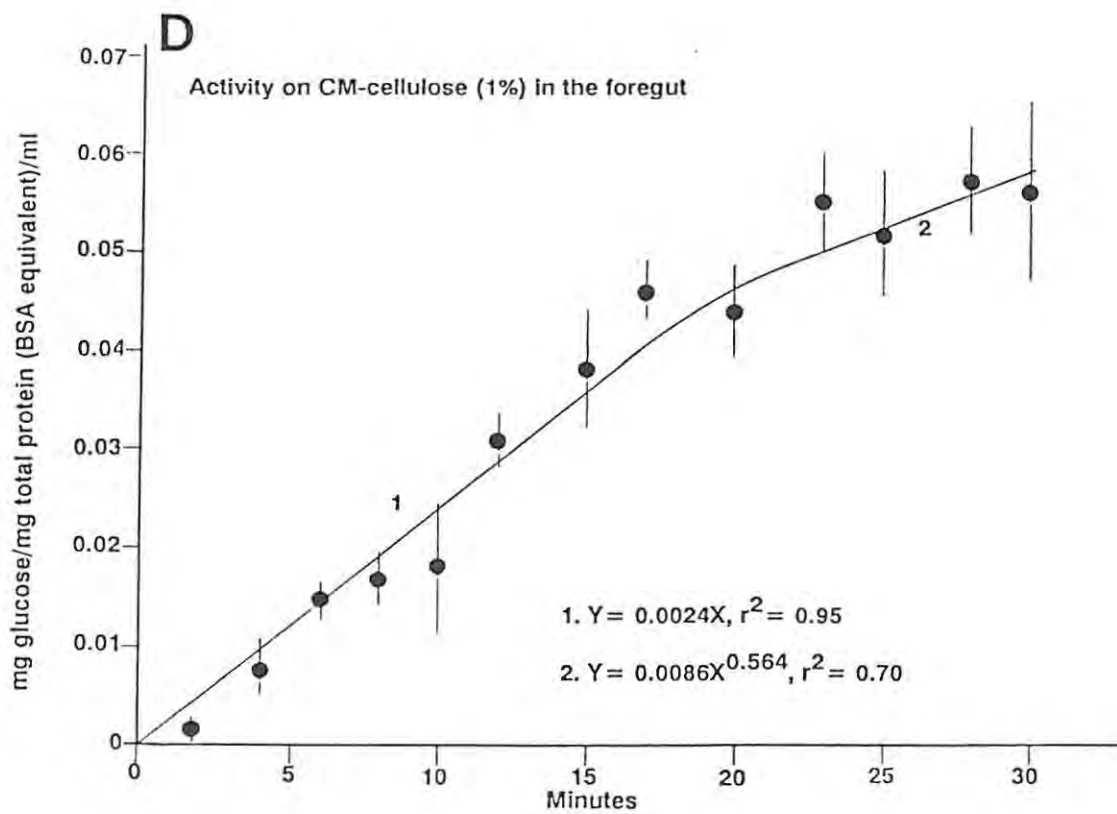
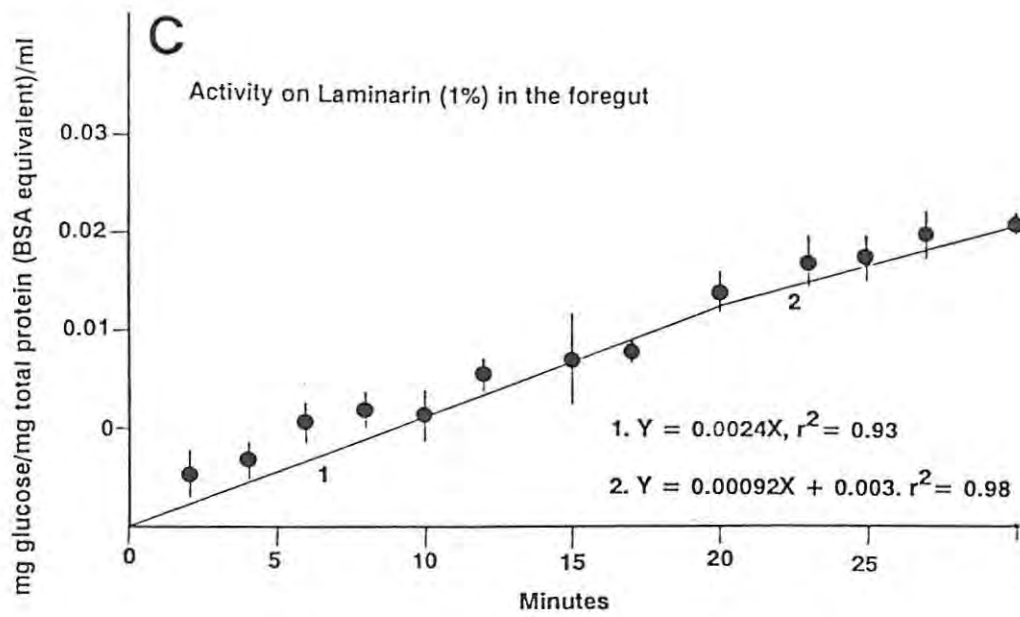
a) Foregut: The highest levels of activity, 0.5mg reducing sugar/mg total protein/ml, produced after 30 minutes, occurred for the storage polysaccharides amylose and glycogen (Table 3.3A). Activity on all the other polysaccharides was an order of magnitude lower; below 0.07mg reducing sugar/mg total protein/ml after 30 minutes.

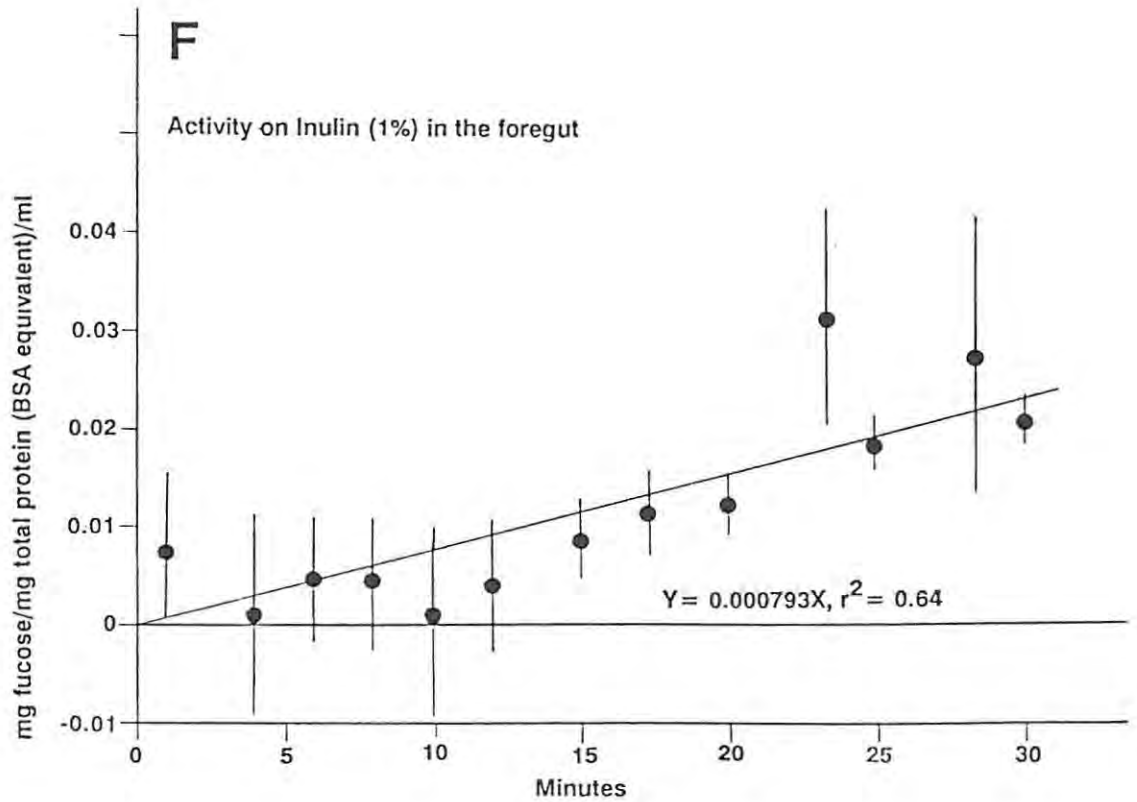
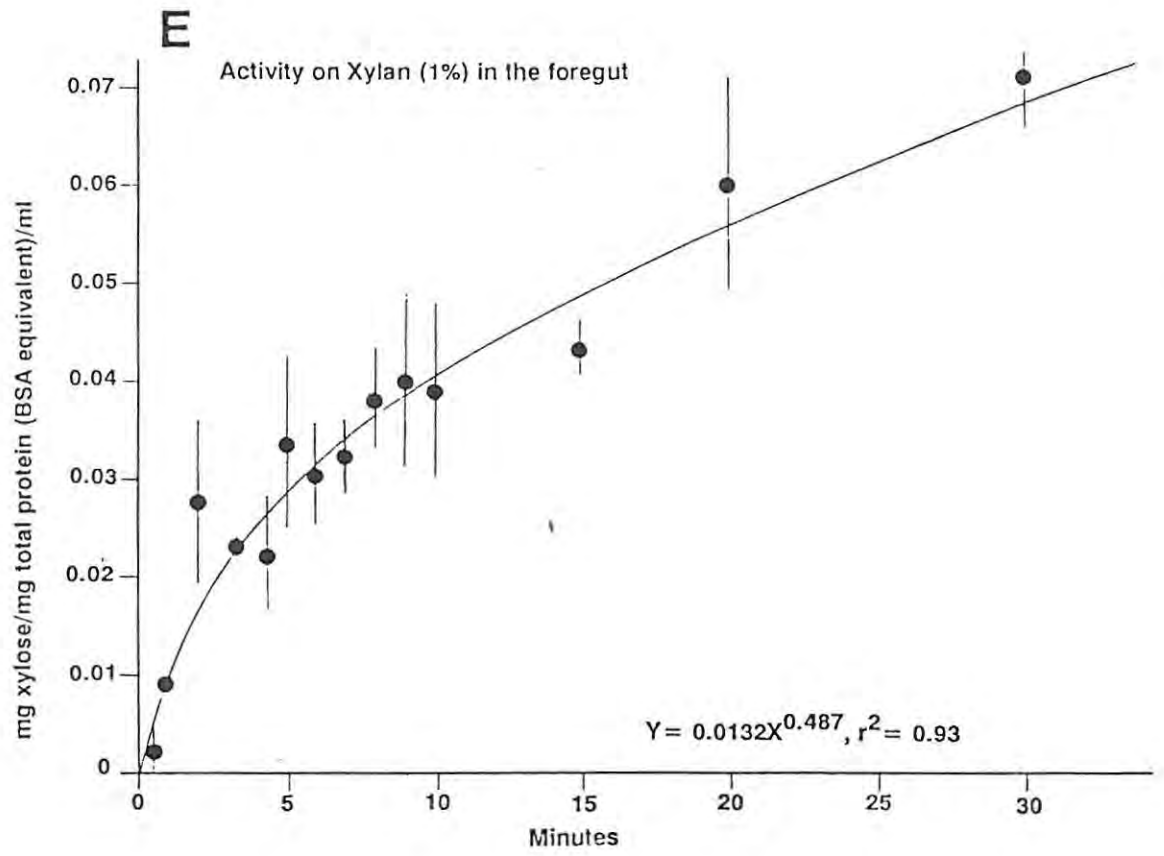
The substrates showing lower levels of activity can be divided into two groups. Assays on one group of polysaccharides (carrageenan, inulin, CM-cellulose, laminarin, fucoidan and xylan) produced end product concentrations of up to 0.07 mg reducing sugar/mg total protein/ml over the incubation period. Alginic acid and mannan produced up to only 0.01 mg/mg total protein/ml, with no correlation between end product concentration and incubation time (Figure 3.2I and 3.2J).

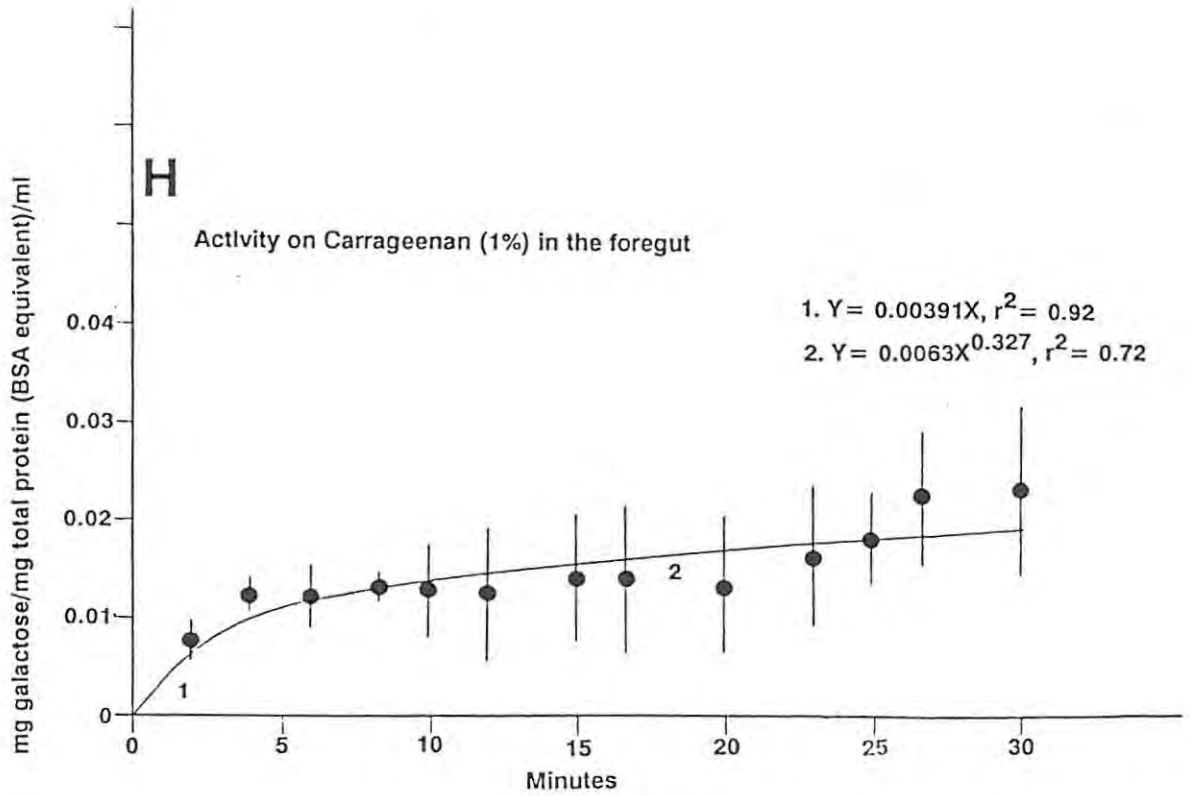
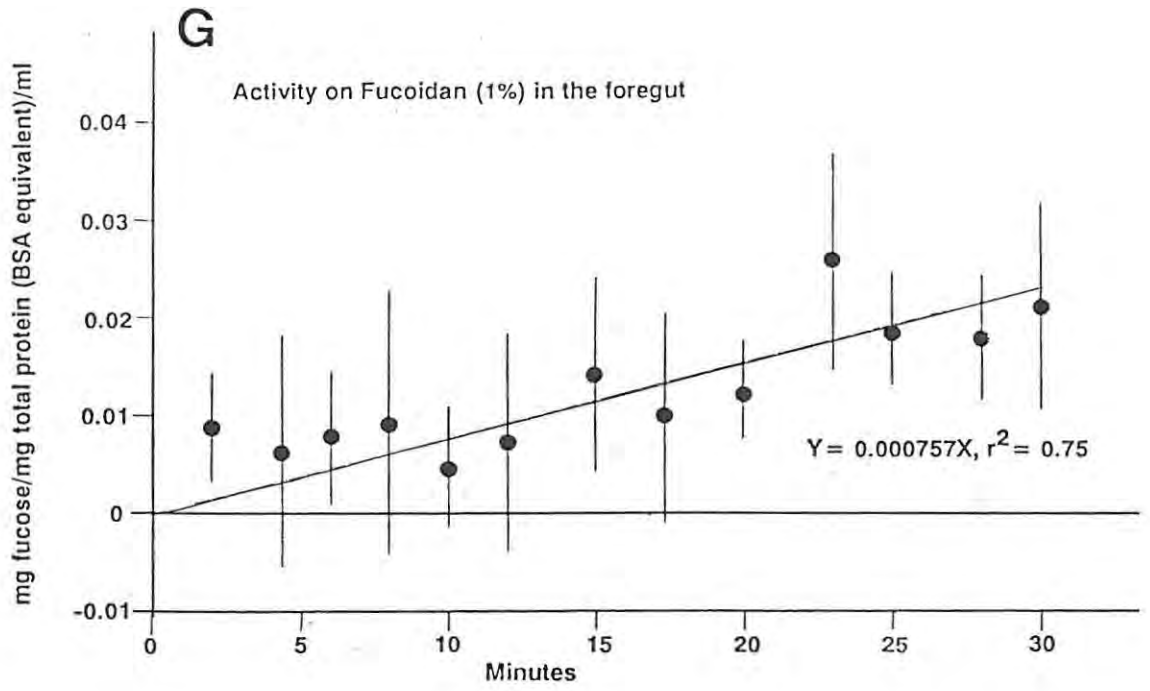
B) Hindgut: Only two levels of activity were apparent for the assays in the hindgut (Table 3.3B and Figure 3.3A-J). Maximum end product concentrations with amylose, glycogen, inulin, fucoidan and xylan was up to 0.05 mg/ mg total protein/ml. None of the other polysaccharides (alginic acid, mannan, laminarin and CM-

Figure 3.2: Enzyme activities on algal polysaccharides for the foregut. Plots are of activity (mg reducing sugar/mg total protein (BSA equivalent/ml) against time. Where obtained, the best fit regression curve(s) are plotted and equation(s) are given.









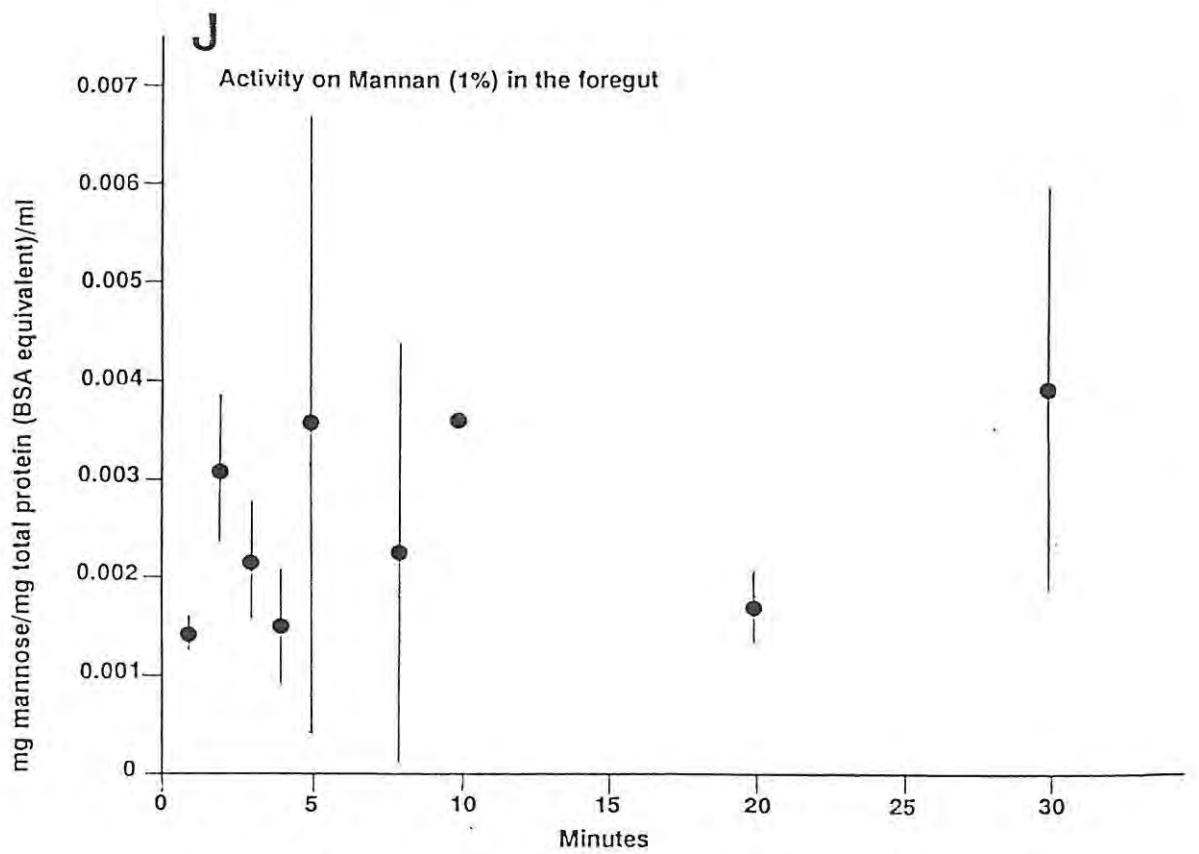
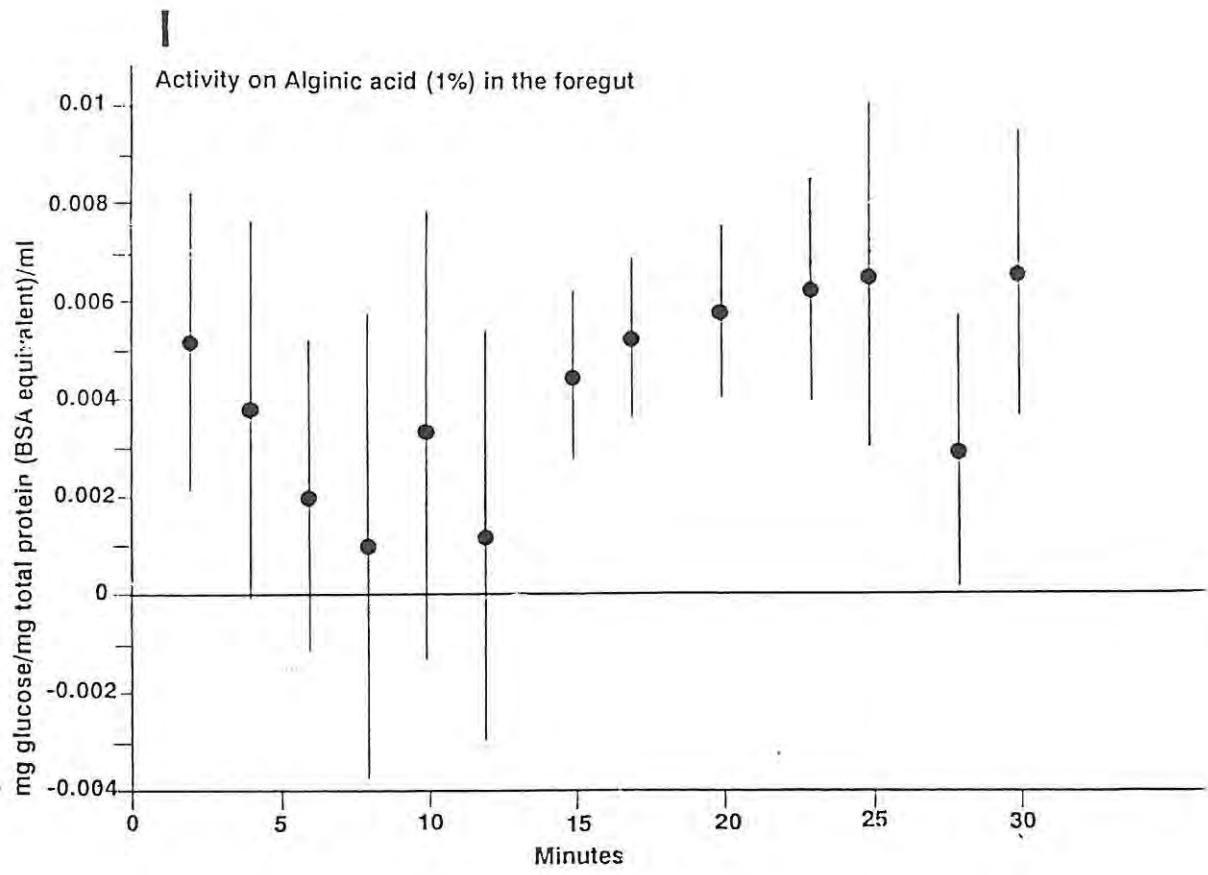
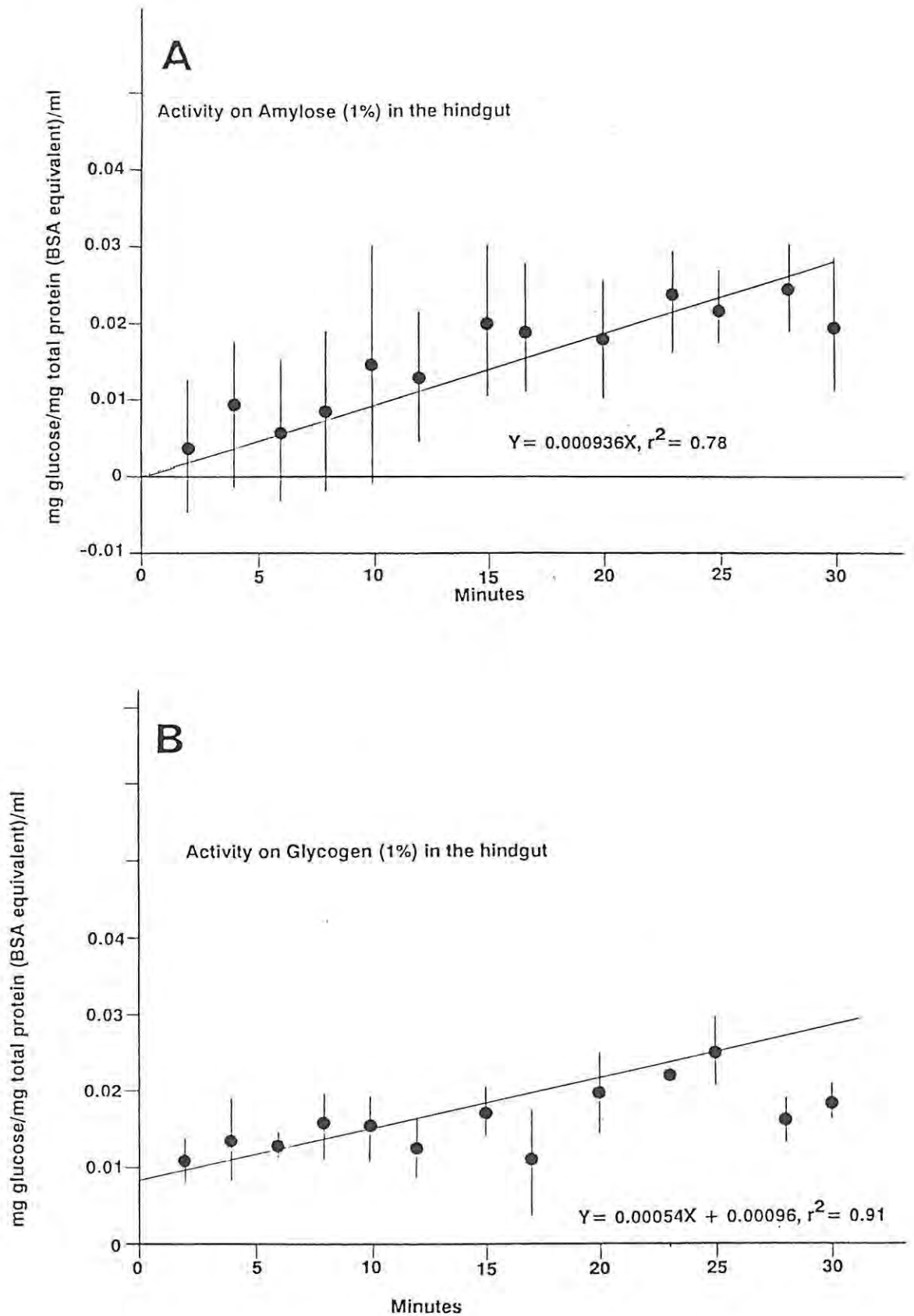
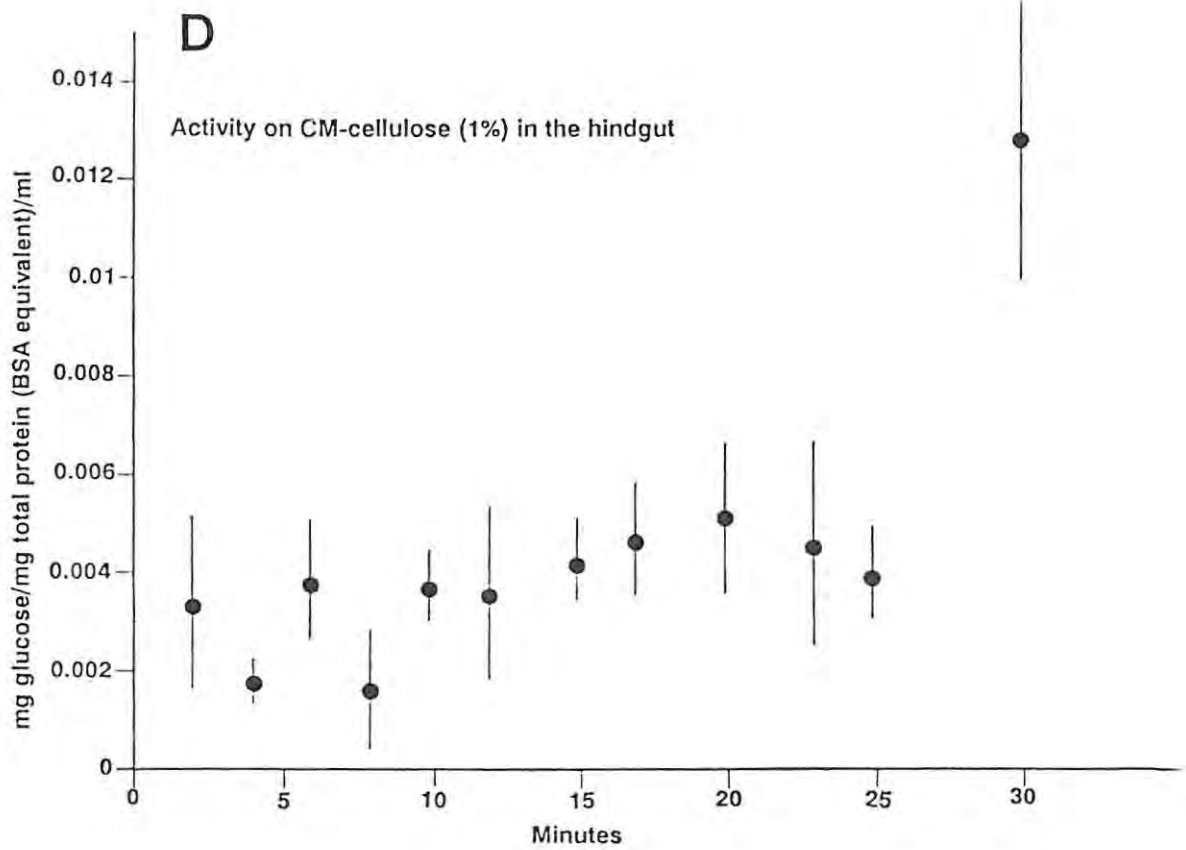
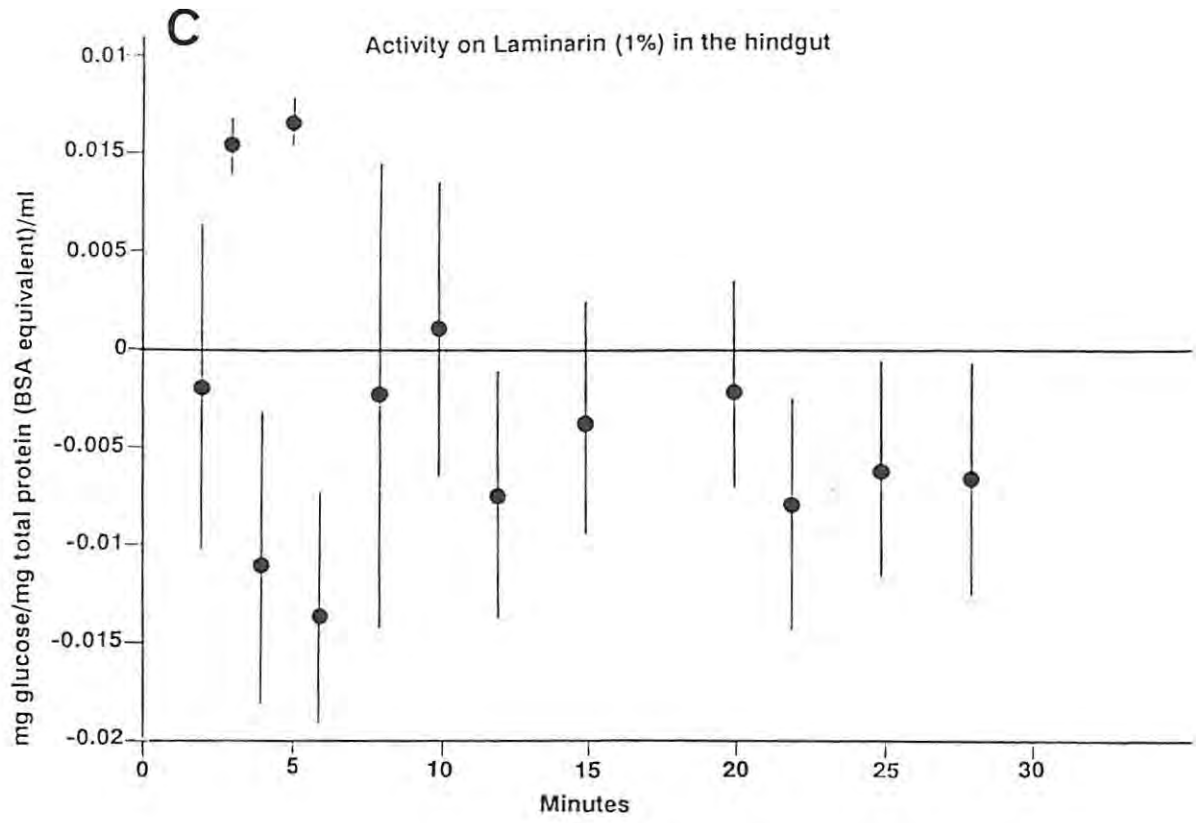
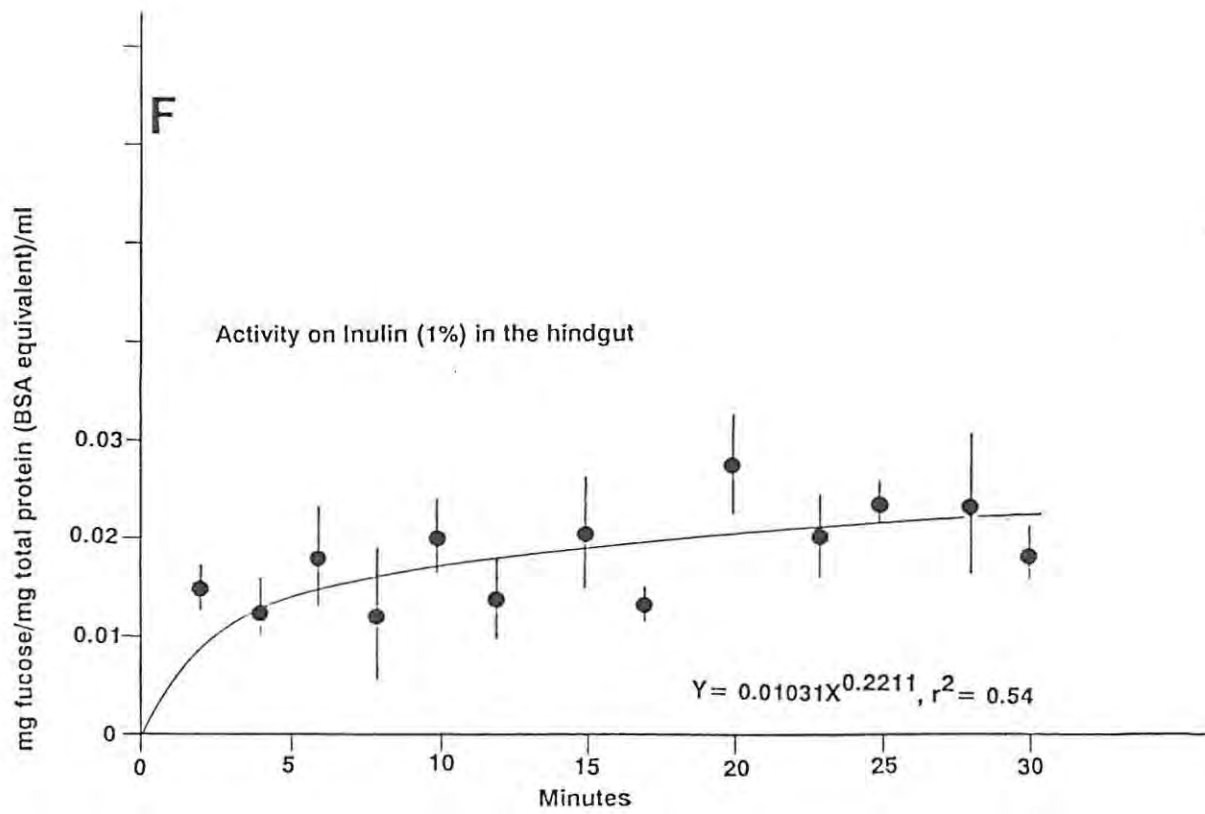
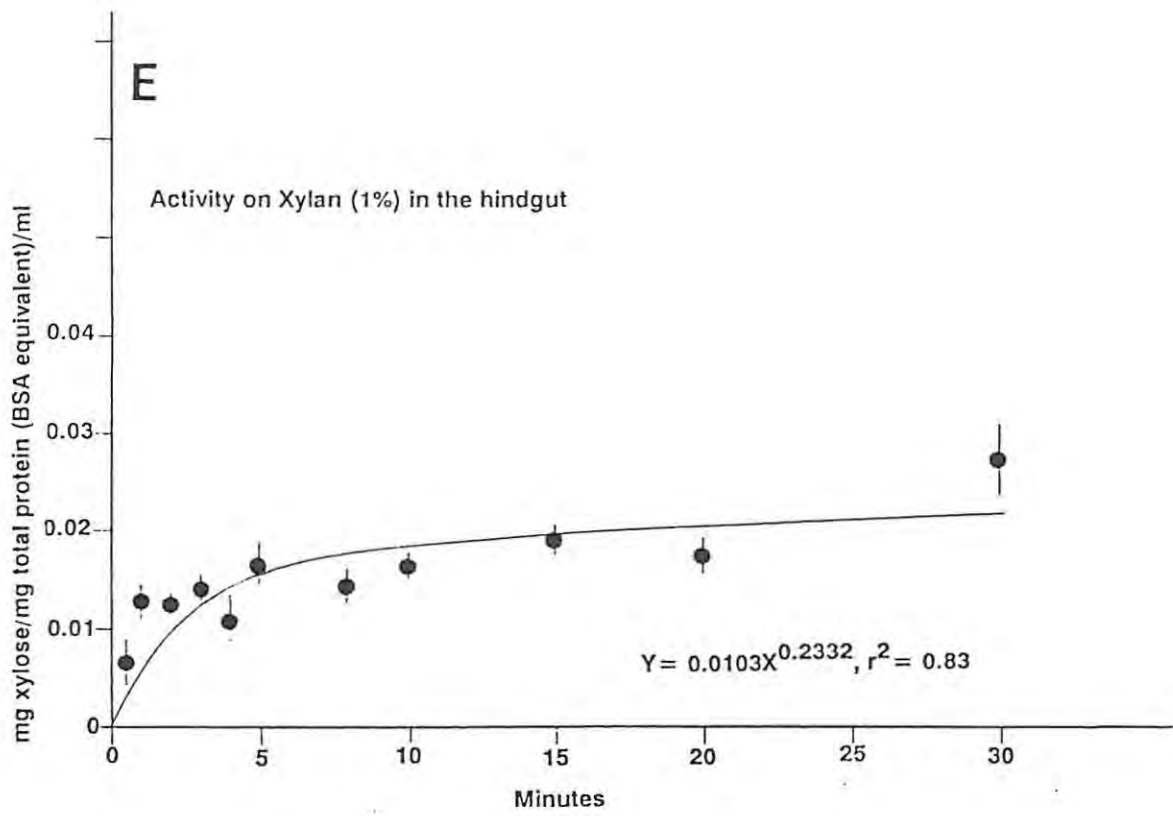
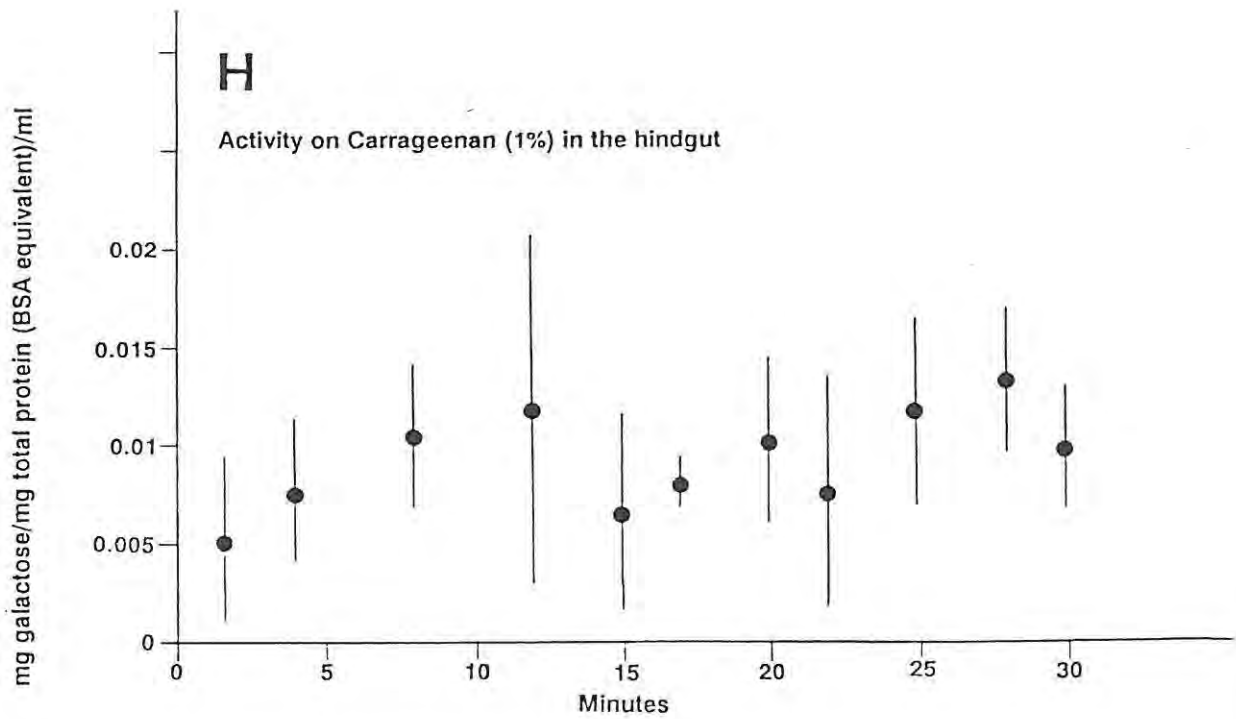
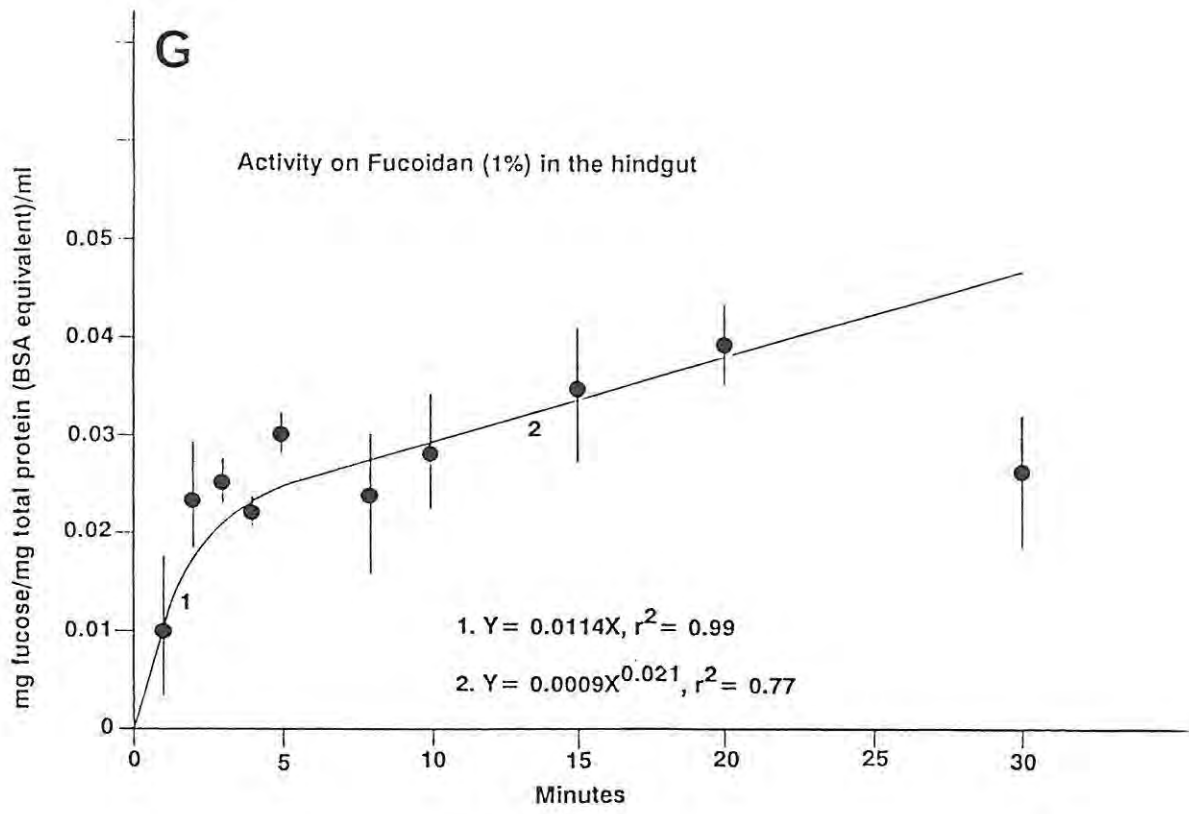


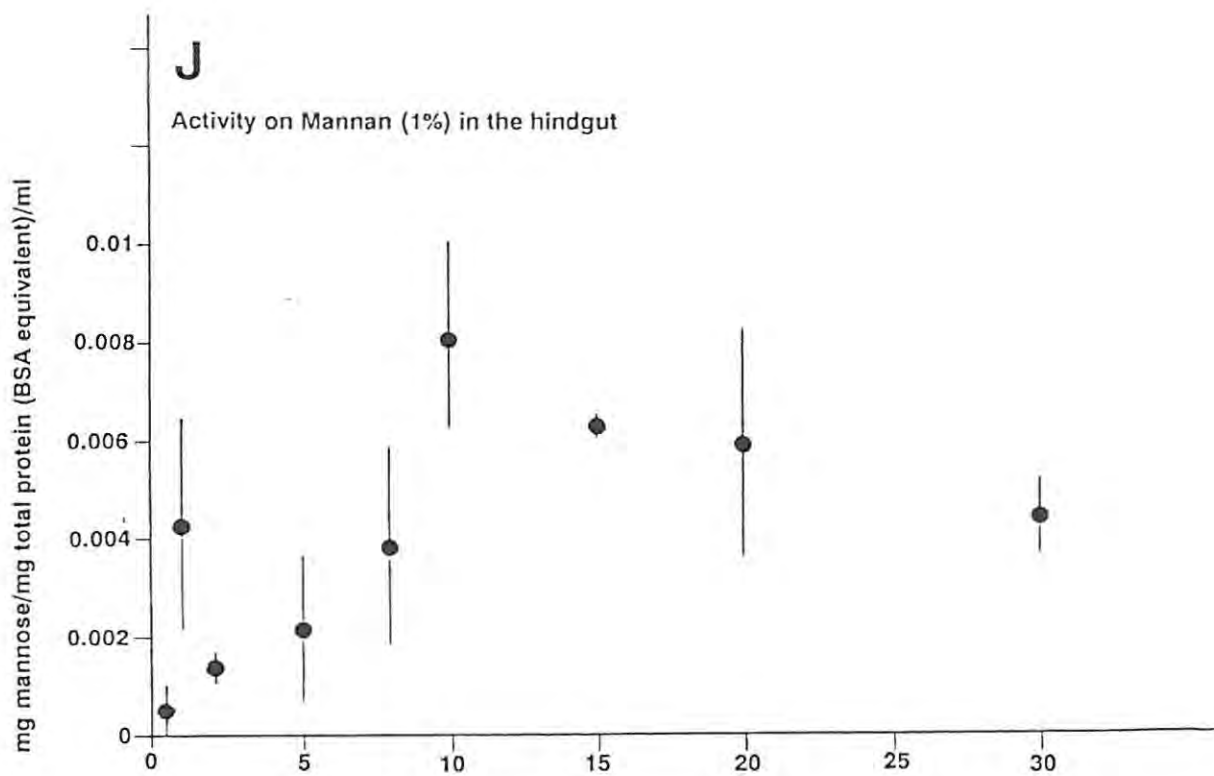
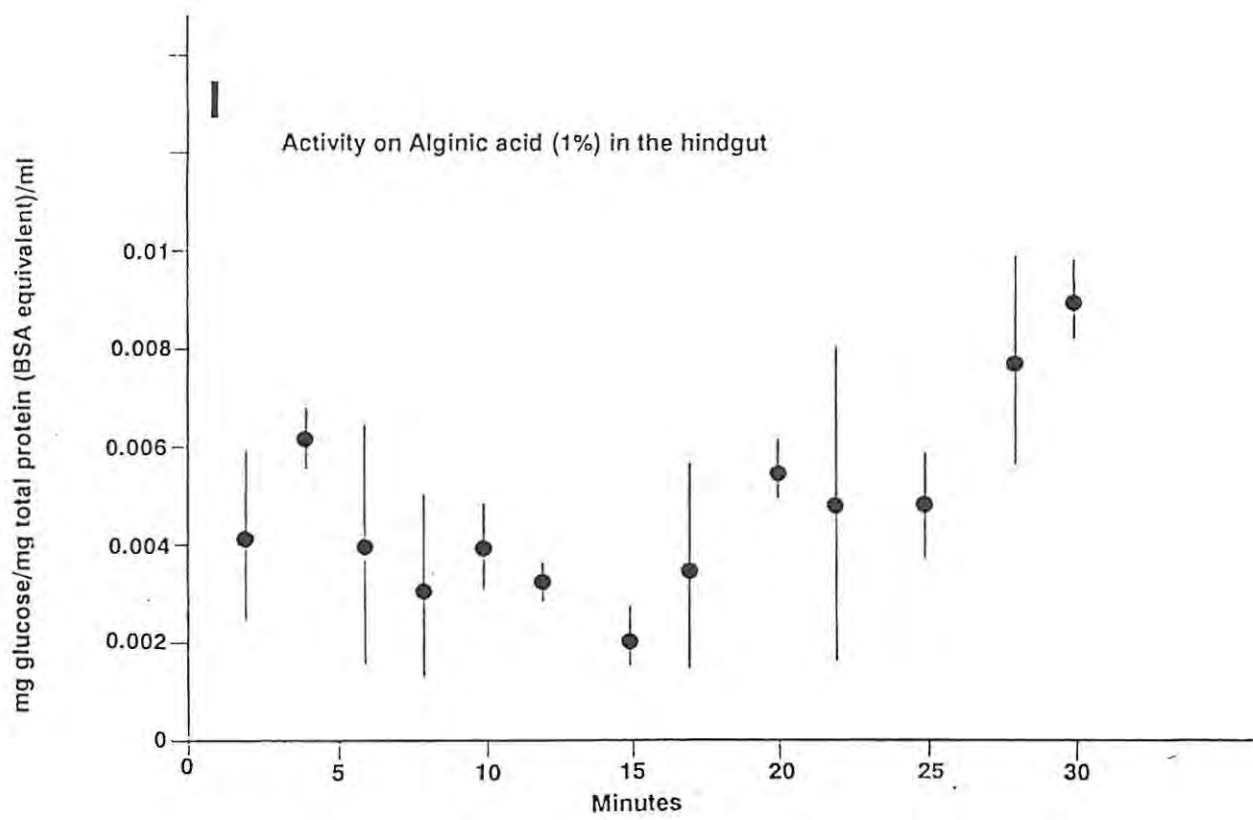
Figure 3.3: Enzyme activities on algal polysaccharides for the hindgut. Plots are of activity (mg reducing sugar/mg total protein (BSA equivalent/ml) against time. Where obtained, the best fit regression curve(s) are plotted and equation(s) are given.











cellulose) produced satisfactory correlations between enzyme activity and incubation time, nor did they produce more than 0.01 mg reducing sugar/mg total protein/ml over this period. The assay on carrageenan produced an initial period of activity for 10 minutes whereafter activity dropped.

These results show that enzyme activity in the foregut was generally higher than in the hindgut. The highest overall activity was produced in the foregut on amylose and glycogen. Activities on these two substrates as well as on xylan was reduced in the hindgut relative to the foregut. Laminarin, CM-cellulose and carrageenan produced low level enzyme activity in the foregut, but activity on these substrates in the hindgut was generally below 0.01 mg/mg total protein/ml and there was no discernable relationship between activity and incubation time. Inulin produced similar activities for both the foregut and hindgut while only fucoidan showed higher activity for the hindgut than for the foregut. Alginic acid and mannan were the only substrates that showed no correlation between activity and incubation time for either the foregut or hindgut. In both cases the maximum end product concentration was below 0.01mg/mg protein/ml.

Intrinsic reducing sugars and minimum activity levels:

Table 3.2 shows that the maximum potential yield of reducing sugars from intrinsic glycogen is approximately 0.03mg/mg total protein/ml. This amount could account for most of the lower levels of enzyme activities produced in the enzyme assays.

TABLE 3.3A: Specific activities of digestive polysaccharidases for the foregut of *Parechinus angulosus*. All units are expressed in mg reducing sugar produced/mg total protein (BSA equivalents)/ml. Data are calculated from the regression curves in figure 3.2.

Substrate	Time (minutes)						
	0	2	5	10	15	20	30
AMYLOSE	0	0.0823	0.2057	0.4114	0.4899	0.4983	0.515
GLYCOGEN	0	0.0282	0.0705	0.1410	0.2114	0.2618	0.360
LAMINARIN	0.0037	0.0056	0.0083	0.0130	0.0176	0.0222	0.031
INULIN	0	0.0016	0.0040	0.0079	0.0119	0.0159	0.023
CM-CELLULOSE	0	0.0048	0.0120	0.0240	0.0360	0.0467	0.059
CARRAGEENAN	0	0.0078	0.0106	0.0133	0.0152	0.0167	0.019
FUCOIDAN	0	0.0015	0.0038	0.0076	0.0114	0.0151	0.022
XYLAN	0	0.0185	0.0290	0.0405	0.0494	0.0568	0.069
ALGINIC ACID	-	-	-	-	-	-	-
MANNAN	-	-	-	-	-	-	-

TABLE 3.3B: Specific activities of digestive polysaccharidases for the hindgut of *Parechinus angulosus*. All units are expressed in mg reducing sugar produced/mg total protein (BSA equivalents)/ml. Data are calculated from the regression curves in figure 3.3

Substrate	Time (minutes)						
	0	2	5	10	15	20	30
AMYLOSE	0	0.0019	0.0047	0.0093	0.0140	0.0187	0.028
GLYCOGEN	0.0097	0.0107	0.0124	0.0151	0.0176	0.0205	0.025
LAMINARIN	-	-	-	-	-	-	-
INULIN	0	0.0120	0.0147	0.0172	0.0188	0.0200	0.021
CM-CELLULOSE	-	-	-	-	-	-	-
CARRAGEENAN	0.0042	0.0055	0.0074	0.0106	-	-	-
FUCOIDAN	0	0.0228	0.0254	0.0298	0.0341	0.0384	0.047
XYLAN	0	0.0122	0.0150	0.0177	0.0195	0.0208	0.022
ALGINIC ACID	-	-	-	-	-	-	-
MANNAN	-	-	-	-	-	-	-

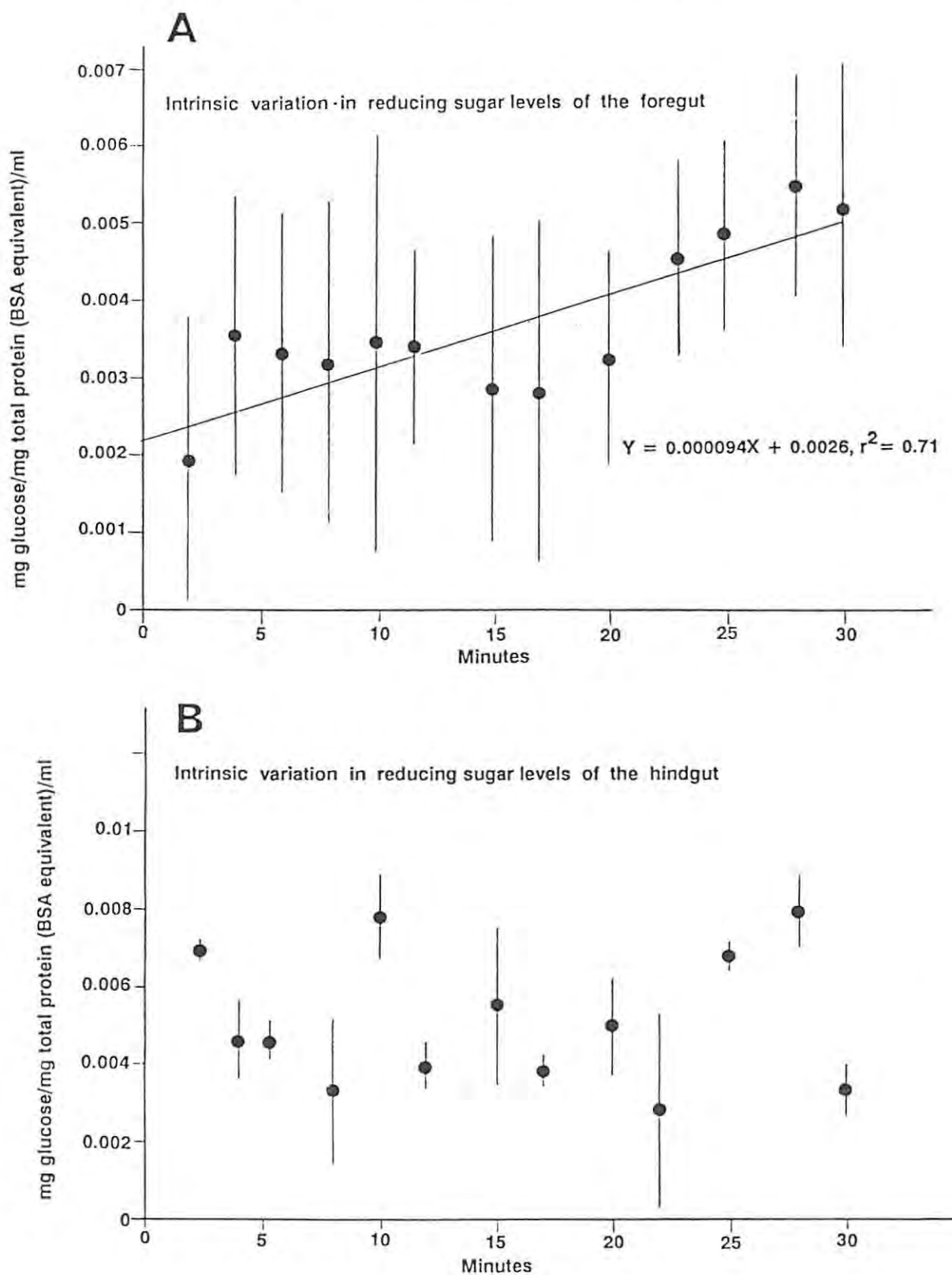
It is therefore important to establish whether the lower level of enzyme activity is real or artifactual.

Further, a cut-off point can be determined above which the level of enzyme activity can be accepted as hydrolysis of the substrate, whereas below this level, the activities are artifactual, indicating no hydrolysis. In order to do this, two experiments were performed. Firstly, changes in intrinsic reducing levels were monitored in enzyme extracts (see page 50). Secondly, a representative substrate which produced lower level enzyme activity at 1% concentration was re-assayed at a concentration of 3%.

The changes in intrinsic levels of reducing sugars with time, of both foregut and hindgut enzyme extracts are shown in Figs. 3.4A and 3.4B. A correlation was obtained for the foregut, but not for the hindgut. In both cases the activity was of the order of 0.01mg reducing sugar/mg protein/ml.

Figure 3.5 shows the reducing sugar concentrations produced by the hydrolysis of a 3% xylan incubation with foregut enzyme extract. Enzyme activity at 3% was almost double that at 1% (Table 3.4). Figure 3.6 shows the regression curves for the hydrolysis of xylan at 3% and 1% by the foregut extract. Also plotted is the regression curve for intrinsic reducing sugar level for comparison.

Figure 3.4: Changes in intrinsic levels of reducing sugars in enzyme extracts. The changes, measured as mg glucose/mg total protein/ml, are plotted against time and where a correlation was obtained the curve is plotted and equation given.



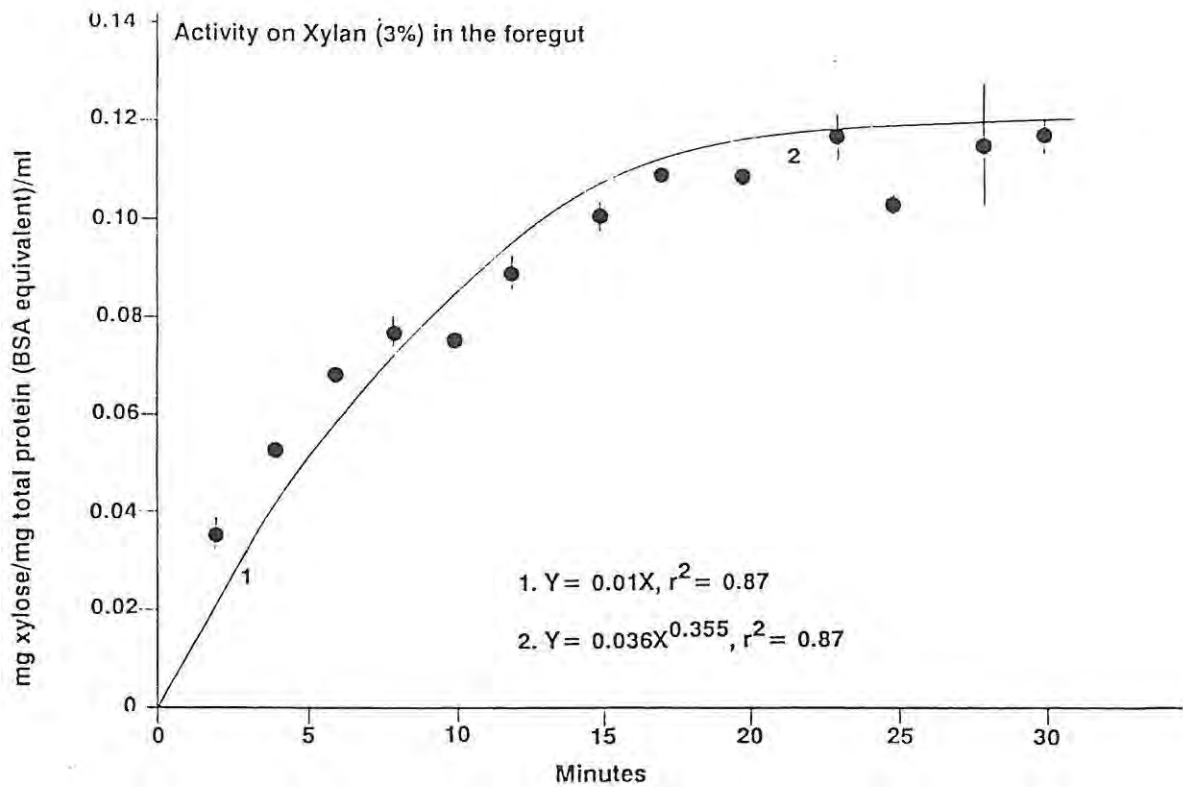


Figure 3.5: Plot of enzyme activity on 3% Xylan for the foregut.

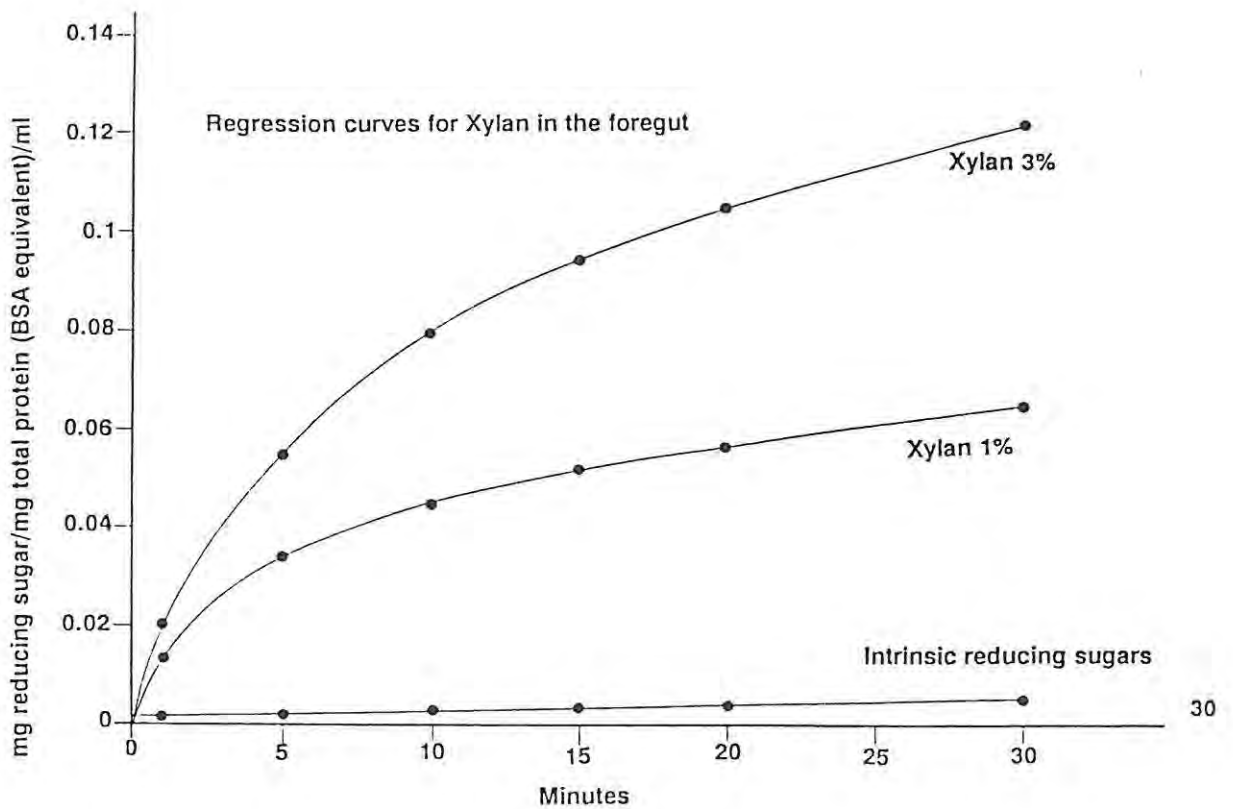


Figure 3.6: Regression curves for Xylan at 3% and 1%, and regression curve for intrinsic reducing sugar changes in the foregut.

TABLE 3.4: Specific activities of foregut enzyme extract on xylan at 1% and 3%.

Xylan concentration	Time (minutes)						
	0	2	5	10	15	20	30
1%	0	0.0185	0.0289	0.0405	0.0494	0.0568	0.0692
3%	0	0.0218	0.0546	0.0822	0.0949	0.1051	0.1214

The potential yield from glycogen in the extracts is approximately 0.03mg glucose/mg total protein/ml which could theoretically account for the lower levels of enzyme activity measured. However, the maximum level of intrinsic reducing sugar measured in the extract incubations can only account for levels of activity below 0.01. Therefore, levels of activity less than 0.01mg reducing sugar/mg total protein/ml can be regarded as artifactual and not resulting from the hydrolysis of the substrate. Thus, while glycogen does occur in the enzyme extracts, its effect on end-product polysaccharidase assays is minimal, but must nevertheless be considered.

The result of the assay on 3% xylan confirms that low level enzyme activity (above 0.01 mg/mg protein/ml) is real and not artifactual since the increase in substrate concentration resulted in an increase in activity that can only be explained as increased hydrolysis. Thus, real activity was detected on all the substrates tested, except alginic acid and mannan, with different degrees of activity in the foregut and hindgut on most substrates.

Discussion

Regional specialization in the gut of *Parechinus angulosus*.

It was concluded from the ultrastructural study of the gut epithelium of *Parechinus angulosus* (Chapter 2) that proteogenic exocrine cells occurred primarily in the foregut. That digestive enzyme activity was higher in the foregut than in the hindgut corroborates this conclusion. Why activity in the hindgut was detected on only some of the substrate polysaccharides tested and why activity on fucoidan was higher in the hindgut than in the foregut is more difficult to explain. One possible explanation is that the ratio of enzyme concentration to total protein concentration may have varied from extract to extract to some degree.

De Ridder and Jangoux (1982) concluded that regional specialization, in terms of function, occurs in the gut of echinoids. This is supported by Bamford (1982) who cites work which showed that, in both *Paracentrotus lividus* and *Echinus esculentus*, galactose and glucose were actively taken up in greater quantities in the hindgut (intestine) than in the foregut (stomach). Also, Doezeema and Phillips (1970) showed that glycogen was synthesized at a greater rate in the hindgut (second circuit) than in the foregut (first circuit).

In the present study, a functional distinction between the foregut and hindgut can also be made with regard to digestive polysaccharidases. It is proposed that the site of primary extracellular digestion is in the foregut while the function of the hindgut is primarily absorptive.

The digestive polysaccharidases of *Parechinus angulosus*.

One consequence of the inconsistency in the methods used to study the polysaccharidases of marine invertebrates is that it is difficult to make direct comparisons between species studied since the units used to express enzyme activities vary. Lawrence (1982) has summarized most of the results obtained in studies on the digestive polysaccharides in the whole phylum, but this provides only qualitative information relating to the presence or absence of the enzymes in question, with only a descriptive reference to their levels of activity. It is apparent from Lawrence's review that, while not ubiquitous, activity on all the substrates (except fucoidan and inulin) tested in this study have been detected in at least one other echinoid. Fucoidan was used as a substrate in only one study where a negative result was attained (Huaug and Giese, 1958). As far as can be ascertained, no assays have previously been performed on inulin.

Storage polysaccharidases

The ability of echinoids to digest the storage polysaccharides of marine algae appears to be ubiquitous. Using a variety of methods, all echinoids tested have shown the ability to hydrolyze amylose, starch, amylopectin and α -glucosides (see Lawrence 1982 and also Elyakova *et al.*, 1981; Klinger, 1984 and Claereboudt and Jangoux, 1985). The ability to utilize glycogen (which is used as an analog of the storage polysaccharides of the Rhodophyta; Painter, 1983) has also been demonstrated for all echinoids tested (Lewis, 1964).

The results obtained for *Parechinus angulosus* in this study show that the α -glycosides amylose and glycogen are hydrolyzed to a greater extent than any of the other storage or structural

polysaccharides. Likewise, most comparative studies on echinoids have shown that α -glucosidase activity (using amylose, amylopectin, glycogen or chromogenic derivatives as substrates) is higher than that for other enzymes (e.g. Elyakova *et al.*, 1981 and Klinger, 1984), although Moldotsov *et al.* (1974) demonstrated only limited α -glucosidase activity.

Laminarinases hydrolyze the β -(1,3)-linked glucan laminarin which is the storage glucan in the Phaeophyceae (Painter, 1983). They are commonly found to occur in the guts of echinoids (Sova *et al.*, 1970; Piavaux, 1977 and Elyakova *et al.*, 1981). The only comparison between activity on laminarin and the other storage compounds has shown that activity is generally lower than for alpha-glucosides, although this was not always the case (Elyakova *et al.*, 1981). This certainly appears to be true for *Parechinus angulosus* where activity on laminarin was an order of magnitude lower than that for the α -glucosides tested.

Structural polysaccharidases

The results obtained in the present study generally agree with those obtained in other studies, and are discussed here for each polysaccharide in turn.

While other β -glucosidases are reported in studies on echinoids (e.g. Moldotsov *et al.*, 1974 and Klinger, 1984) these have not been specified as β -(1,3)- or β -(1,4)-glucosidases. Presumably, the polysaccharidases assayed in these studies are β -(1,4)-linked glucanases which hydrolyze cellulose. Cellulases (also assayed as β -(1,4)-glucosidases, or CM-cellulases) have been found to occur in many, but not all echinoids tested. Except in the study of Moldotsov *et al.* (1974) comparative studies have

shown that these enzymes are limited in their distribution, and where they do occur, their activity is relatively low (Hultin and Wanntrop, 1966; Elyakova, 1972; Elyakova *et al.*, 1981 and Klinger, 1984). CM-cellulase activity in *Parechinus angulosus* is in agreement with these findings.

Moldotsov *et al.* (1974) have produced the only study on xylanases in echinoids. They recorded xylanase activity in the echinoids they studied, but at very low levels. In *Parechinus angulosus* xylanase activity was at the same level as for the beta-glucosides and other substrates tested (see below).

Since an artificial chromogenic substrate was used by Moldotsov *et al.* (1974) to assay xylanase activity, it is impossible to make a valid quantitative comparison between their results and those presented here. However, it is important to note that the concentration of the substrate was 0.1% (w/v) compared to 1% in this study. When the concentration of the substrate was increased to 3%, a two-fold increase in activity was recorded. Thus, attention must be paid to the concentration of the substrates when comparing results. However, in the case of Moldotsov *et al.* (1974) the concentration of their substrates was consistent throughout, making comparison between substrates in that study valid. Also, the fact that they used artificial chromogenic substrates circumvents the problem caused by the hydrolysis of intrinsic reserve substances. Low level activities can only be explained in terms of relatively little hydrolysis of the p-nitrophenol molecule from the xylose monomer, unless this hydrolysis is caused by another factor.

Fucoidan is a term used to describe a range of sulphated fucose polymers which are important structural polysaccharides in the Phaeophyceae (McCandless, 1982). The only record of a study that assayed hydrolysis of this polysaccharide in echinoids is that of Huaug and Giese (1958) who detected no activity for this polysaccharide in *Strongylocentrotus purpuratus*. Another fucan found in marine algae is inulin which is a simple deoxy-fucose polymer found in the Chlorophyceae (Painter, 1983). No studies have been carried out to determine whether this substrate is hydrolyzed in the gut of echinoderms. For both of these substrates hydrolytic activity was detected in the gut of *Parechinus angulosus*. The activity was generally of the same level as that on beta-glycosides and xylan.

Carrageenans are a wide range of galactans that occur extremely variably in the Rhodophyceae and Chlorophyceae (Percival and McDowell, 1982). The hydrolysis of galactans has been assayed by Moldotsov *et al.* (1974) and Klinger (1984) for a variety of echinoid species using artificial chromogenic substrates. In both studies activity was detected in almost all species tested, but was consistently very low. Benitez and Macaranas (1978, cited in Lawrence, 1982) showed that activity on Carrageenan (sic) occurred in the gut of *Diadema setosum* but no further information is provided. The results here show that the levels of activity on carrageenan in *Parechinus angulosus* were also relatively low and only marginally above the minimum level of 0.1mg reducing sugar/mg total protein/ml accepted in this study.

The occurrence of an α - and β -mannosidase was reported by Moldotsov *et al.*, (1974) for a variety of echinoids using an

artificial chromogenic substrate. They showed that the levels of activity of this enzyme was relatively moderate where it occurred. In *Parechinus angulosus* no activity on mannan was detected.

Alginic acid is a mannuronic and guluronic acid polymer and is the principle structural polysaccharide of the Phaeophyceae (Percival and McDowell, 1982). Epply and Lasker (1959) assayed alginase viscosimetrically in the gut of *Strongylocentrotus purpuratus*. Favorov and Vaskovsky (1971) used end-product analysis, viscosimetry and an extinction method to assay alginases in a variety of marine invertebrates. Only weak activity in the gut of the echinoids they studied occurred. In *Parechinus angulosus* no activity was detected on alginic acid.

The interpretation of differential levels of enzyme activity:

The study of Favorov and Vaskovsky (1971) on the alginases of marine invertebrates provides an opportunity to compare methods used to assay polysaccharidases. It is important to note that Favorov and Vaskovsky (1971) detected alginase activity in the gut of the echinoids in their study using viscosimetry but no activity was detected using the Nelson-Somogyi technique. This does not imply that end-product analysis (as measured by the Nelson-somogyi technique using glucose as a standard) is not appropriate for this polysaccharide since the same authors showed that this method worked well for the assay of alginases in the molluscs in their study. However, it does imply that negative results must be carefully considered.

To extend this argument further, a distinction should be drawn between two types of polysaccharidase activity. Most research

has shown that the polysaccharides are either broken down completely resulting in high activity levels (as with the reserve polysaccharides in this study) or only partially, resulting in low (often barely detectable) levels of enzyme activity. It is suggested here that high levels of activity represent the utilization of that polysaccharide by the animal concerned, as a source of carbon.

Low levels of activity require further consideration. Molecular weight is one of factors affecting the gelling properties and viscosity of agars, carrageenans, alginates, fucans and other marine algal structural polysaccharides (Whistler and BeMiller, 1973 and Kloareg and Quatrano, 1988). Thus, even cleavage of these long-chained polysaccharides at one point can significantly reduce their gelling properties and consequently their efficacy in the cell wall.

If cleavage were to occur at a number of points along the polysaccharide chain, the increase in the level of reducing sugars released may be very small (since only a few reducing ends of the sugar monomer will become available), however the secondary and tertiary structure of these molecules may change significantly.

Whether this increase is measurable will depend on the degree of hydrolysis. Thus, low levels of enzyme activity could be interpreted as representing cleavage of long chain polysaccharides to lower molecular weight polymers. The implication for the animal is that this degree of activity would be enough to cause disruption of the cell wall, although not enough to allow utilization of the cell wall itself as a carbon

source. It is suggested that this effect could be measured by employing chromatographic techniques to separate different oligosaccharide fractions after treatment with enzyme extract. In this way a correlation could be obtained between the extent of reducing sugar level increase and the degree of hydrolysis of the polysaccharide.

In summary, *Parechinus angulosus* possesses enzymes that can at least partially digest most of the algal structural polysaccharides tested. Two levels of activity were detected. High levels of enzyme activity occurred on the reserve polysaccharides that occur in the Rhodophyceae and Chlorophyceae. Lower levels of activity were detected on the storage polysaccharides of the Phaeophyceae and on all the structural polysaccharides tested except for alginic acid (which is the principle structural polysaccharide in the Phaeophyceae) and mannan. The implication of these results is that *Parechinus angulosus* has the ability to digest the cell walls of green and red algae. The lack of an alginase implies that they cannot digest the polysaccharide most commonly found in brown algal cell walls.

From Lawrence's (1982) review of digestion in echinoids and subsequent literature such as Klinger, 1984 and Claereboudt and Jangoux, 1985 it is possible to establish which species or genera have received most attention with regard to polysaccharidase activity, and what range of enzyme activity has been detected in those species. Some species have been assayed more than once for the same polysaccharidase, using a variety of methods, in order to make valid comparisons between the levels of activity of different polysaccharidases and different species within each

study. A case in point is *Strongylocentrotus intermedius* which was assayed by Elyakova (1972) for cellulase (using CM-cellulose as a substrate) and by Moldotsov et al. (1974) for beta-glucosidases (using p-nitrophenol-glucosides as a substrate).

The widest range of polysaccharidases has been detected in the *Strongylocentrotus* species. However, these species have also received most of the attention. Most polysaccharidase assays in a particular species have been successful, although in some species only a narrow range have been tested. Some assays have been unsuccessful in some studies and successful in others. *Parechinus angulosus* has been shown here to possess as wide a range as any of the other species assayed. However, to conclude that it possesses a wider range than any other species is not possible. This is because a number of other polysaccharidases which were not included in this study have been assayed elsewhere and also some of the polysaccharidases assayed here were not assayed in other species. More important is the question of which polysaccharidases are not possessed by a particular species since this information could define the limits of its diet.

A final point is that where low levels of activity are detected, these results should be carefully tested for validity. It is shown here that very low levels of activity are probably artifactual and not due to substrate hydrolysis. This may pertain only to studies that use end product analysis as measured by the changes in reducing sugar levels. The use of artificial substrates can help to obviate this problem, but results in a compromise with regard to understanding the digestion of polysaccharides which occur naturally in cell walls.

CHAPTER 4
THE UTILIZATION OF ALGAL POLYSACCHARIDES BY ENTERIC BACTERIA
FROM *Parechinus angulosus*.

Introduction

The ecological significance of microorganisms in the shallow water marine environment has received much attention (see Mann, 1988 for review) and it is clear that they can play an important role in energy flow (e.g. Newell, Field and Griffiths, 1982). Various strains of bacteria isolated from these environments have the ability to utilize structural polysaccharides that occur in sea weeds (Araki, Aoki and Kitamikado 1987; Andrykovitch and Marx, 1988). The occurrence of enteric bacteria has been reported for a wide range of herbivorous marine invertebrates (Sochard, Wilson, Austin and Colwell, 1979 and Dempsey, Kitting and Rosson, 1989) although their precise role remains a point of considerable dispute (Lawrence, 1982).

Microorganisms other than bacteria have also been reported in the guts of invertebrates, but their role is even less well understood. The occurrence of fungi in the gut of the urchin *Lytechinus variegatus* was reported by Wagner-Merner and Lawrence (1980) and Barlocher, Newell and Arsuffi, (1989b) showed that marine fungi play a potentially important role in the nutrition of *Littorina irrorata*. Ciliates and other protozoans have been observed in the gut of urchins (Lynn and Berger, 1973) and their population dynamics in the gut of *Psammechinus miliaris* have been investigated by Bedford and Moore (1985).

The possibility that bacteria are cultivated and utilized as a food source by herbivorous and detritivorous marine invertebrates has rarely been considered. This hypothesis was proposed by Prim

and Lawrence, (1975) but only Fong and Mann (1980) have presented any evidence in support of it. Moldotsov *et al.* (1974) showed that the four species of echinoid tested possessed an enzyme that could break down the artificial substrate p-nitrophenol-acetyl-glucosamine. This indicates the ability to hydrolyze the N-acetyl-glucosamine polymer commonly found in bacterial cell walls. Bedford and Moore (1985) suggested that this was not the case in *Psammechinus miliaris*. Evidence does exist that bacteria are utilized by other marine invertebrates such as anthozoa (Herndl and Velimirov, 1986) and molluscs like the filter-feeding mussel *Choromytilus meridionalis* (Seiderer, Davis, Robb and Newell, 1984 and Muir, Seiderer, Davis, Painting and Robb, 1986) and by herbivorous juvenile abalone which strip bacteria from the surface of algae (Garland, Cooke, Grant and McMeekin, 1985).

The difficulty in understanding the contribution gut microorganisms make to the carbohydrate metabolism of the host animal arises out of the fact that researchers have adopted different approaches.

The first type of approach used by researchers concentrates specifically on the metabolic and digestive capabilities of bacteria isolated from the gut. For echinoderms, Lasker and Giese (1954) showed agarolytic activity in bacteria from the gut of *Strongylocentrotus purpuratus* and Garcia-Tello and Baya (1973) showed that *Loxechinus albus* possessed several enteric agar digesting strains of bacteria. Prim and Lawrence (1975) demonstrated that enteric bacteria from *Lytechinus variegatus* could degrade whole algal plants as well as a variety of

structural polysaccharides from marine algae. Claereboudt and Jangoux (1985) successfully tested the amylolytic ability of bacteria from the faeces of *Paracentrotus lividus*.

Other evidence of this nature was presented by Lasker and Giese (1954) who showed that bacteria in the gut of *Strongylocentrotus purpuratus* were the cause of the degradation of bite-sized pieces of algae and by Bedford and Moore (1985) who showed that young *Psammechinus miliaris* grew faster on rotting algae (with a high level of bacterial infestation) than on fresh algae.

A similar approach has also been used for the enteric bacteria of molluscs. Martinez and Trique (1986) showed that *Teredo navalis* possessed a cellulolytic bacterium. A similar strain was described by Griffin, Freer and Greene, (1987) for the wood-boring worm *Psiloterido healdi*. Minamitake, Natori, Nakajima and Murroka (1986) demonstrated the agarolytic ability of a strain from the gut of the abalone *Nordotis* sp.. Vitalis, Spence and Carefoot (1988) grew enteric bacteria from the gut of *Aplysia juliana* on selective media of marine algal polysaccharides. Chemoautotrophic bacteria were shown to be the source of carbon found in the host tissue of the bivalve *Loripes lucinalis* (Herry, Diouris and Le Pennec, 1989).

In the second approach, researchers have compared the composition of the gut microflora with that of the immediate environment and/or food of the species concerned. A degree of selectivity occurs with respect to the composition of the gut flora in *Loxechinus albus* (Garcia-Tello and Baya, 1973) and *Echinus esculentus* (Unkles, 1977) where it differs significantly from that of the surrounding environment. Bensoussan, Scoditti, and

Bianchi (1979) were able to establish that a difference in the potential utilization of various organic compounds exists between bacteria isolated from the gut and from the sediment surrounding some abyssal asteroids and holothuroids. In contrast, Farmanfarmaian and Phillips (1962) have shown that in *Strongylocentrotus purpuratus* the composition of the gut flora is similar to that which develops on sterily incubated algae from the field.

Specialization of the gut microflora is not limited to echinoderms only. For example, Martinez (1982) isolated 113 strains of bacteria from the gut of the shipworm *Teredo navalis* and was able to show that these strains had metabolic characteristics that were distinct from the 220 isolates obtained from the sea water surrounding these animals. Evidence of unique gut microflora or specialized gut microflora in crustaceans has been presented by Sochard *et al.* (1979) and Dempsey *et al.* (1989).

Experiments in which the gut flora are reduced or excluded using anti-biotics form the third type of approach. These experiments have generally been only partially successful, mainly due either to the difficulty in keeping the animals alive or to incomplete elimination of the gut flora (see Lasker and Giese, 1954 and Yerokhin, 1979). Farmanfarmaian and Phillips (1962) showed that in *S.purpuratus*, digestive efficiency was the same for algae soaked in anti-biotic as it was for untreated algae, however their results do not indicate what effect the anti-biotics had on the gut flora of the urchin. Fong and Mann (1980) showed that when gut flora of *Strongylocentrotus purpuratus* was reduced by the use of anti-biotics, essential amino acids were absent from

the protein of the gonads. Anti-biotics were successfully employed by Vitalis *et al.* (1988) who showed that reduction of enteric bacterial populations resulted in depressed growth of the mollusc *Aplysia juliana*.

Results from work on different phyla have indicated that enteric bacteria do not play a significant role in the carbon metabolism of some marine invertebrates. Farmanfarmanian and Phillips (1962) concluded that, in the urchin *Strongylocentrotus purpuratus* bacteria may occur coincidentally in the gut and that their role in the nutrition of this species is not significant. Friesen, Mann and Novitsky (1986) showed that the cellulolytic ability of the shrimp *Mysis stenolepis* was endogenous and that the density of the gut microflora was of the same order of magnitude as that on the eelgrass detritus on which it feeds. Crosby, Langdon and Newell, (1989) demonstrated that the absorption efficiency of crude-fiber carbon of *Spartina alterniflora* was unaffected by the presence of enteric bacteria in the oyster *Crassostrea virginica*. A non-digestive symbiotic relationship between enteric bacteria and the urchin *Echinocardium cordatum* was suggested by De Ridder, Jangoux and De Vos (1985) who concluded that bacteria-laden nodules in the gut functioned to prevent reduction of ingested sediment.

That microorganisms play a role in the nitrogen metabolism of detritivorous and herbivorous invertebrates is, however, convincingly documented (Mann, 1988). Guerinot and Patriquin (1981a, b) showed that in a number of urchin species, facultatively aerobic bacteria of the genus *Vibrio* were capable of nitrogen fixation (acetylene reduction) and that this nitrogen

could be traced to the host proteins. They also showed that this activity was inversely correlated with the nitrogen content of the food sources of the urchins. Fong and Mann (1980) demonstrated that enteric bacteria were responsible for the synthesis of essential amino acids in the diet of *Strongylocentrotus droebachiensis*. The acquisition of essential micronutrients, other than carbon and nitrogen, in marine detritivores has been discussed by Phillips (1984). He suggested that microbes may be an important source of these nutrients.

Two conclusions can be drawn from the literature with regard to gut micro-organisms in marine herbivores and detritivores. Firstly, enteric bacteria have the potential to provide supplementary digestive enzymes and thus may play an important role in the digestion of algal cell walls, especially in times of food paucity. However, it has not yet been shown that these invertebrates are dependent on enteric bacteria for the acquisition of carbon. For echinoids, Lawrence (1982) points out that bacteria may be important even in a relatively short period of time, but their presence is most significant in those species that retain food in the gut longer periods. Thus, bacteria may be an important aid to a generalist herbivore such as *Parechinus angulosus* in coping with the wide range of algal structural polysaccharides described earlier.

Secondly, it has been shown that at least in the case of sea urchins, the bacteria have a more important role in nitrogen metabolism. The bacterial ability to fix nitrogen and supply the urchin with essential amino acids has tremendous ecological significance such as the maintenance of populations in protein and nitrogen limited environments.

In order to investigate the potential role of enteric bacteria in the carbon metabolism of *Parechinus angulosus* microorganisms were isolated from the hindgut and foregut, and tested for their polysaccharidase activity on a range of structural and storage polysaccharides that occur in marine algae. In addition, gut enzyme extract was tested for lysozyme activity.

Materials and Methods

Specimen collection:

All specimens were collected at low tide at Port Alfred (33° 35'S 26° 55'E; Fig. 1.1). The specimens were placed directly into containers of sea water cooled to approximately 2°C and kept at that temperature for one hour while transferred to the laboratory where they were processed immediately. This procedure prevented defecation by the animals; cooled specimens recovered completely within one hour at room temperature in sea water tanks in the laboratory.

Plate counts:

Five specimens were dissected and the foreguts and hindguts (and their respective contents) placed into two separate sterile test tubes. Five ml of 0.45µm filtered and sterilized sea water was added to the guts and these were then homogenized under sterile conditions at slow speed on an ultra-turax TP18/2 electric homogenizer. A serial dilution of 10^{-3} , 10^{-4} and 10^{-6} was then made and 0.1 ml of each was then plated out on each of five separate plates for each dilution for the foregut and the hindgut. The culture medium used was similar to that of Muir *et al.* (1986) and contained 1.5% (w/v) Agar, 0.5% peptone, 0.1% yeast extract and 0.01% ferric phosphate in sterile sea water

solution (diluted 3:1, sw:dw). The plates were then incubated at 20°C for 24 hours before examination and enumeration of cultures. Gram stains were performed on samples from each colony type. The entire procedure was repeated several times.

The utilization of algal polysaccharides by mixed cultures

The methods used here were adapted from Prim and Lawrence (1975).

1) Preparation of mixed cultures: Mixed cultures of enteric bacteria were obtained by inoculating liquid culture media with 0.1 ml homogenate of foregut (or hindgut) and contents. The media contained 0.5% (w/v) peptone, 0.1% yeast extract and 0.01% ferric phosphate in sterile sea water solution (diluted 3:1, sw:dw). After 24 hours at 20°C, 5ml of each bacterial culture (foregut and hindgut) was sterily transferred to a 10ml centrifuge tube and centrifuged at 5000g for 15 minutes. The centrifugate was discarded and the pellet resuspended in sterile artificial sea water (Parsons, Maita and Lalli, 1984; appendix 3) free of any organic carbon. This suspension was used to inoculate test media.

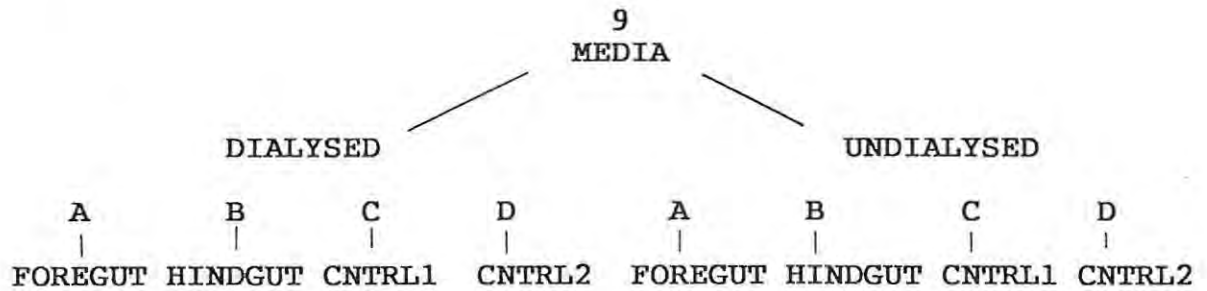
2) Test media: All test media were made up in artificial seawater (adapted from Parsons *et al.*, 1984; appendix 3) diluted with double distilled water (approx. 3:1) to give a final salinity of 26⁰/oo (hereafter referred to as ASW). The minimum medium used here was established by monitoring growth on the following media:

- i) ASW (Parsons' 1 and 11, appendix 3)
- ii) ASW, inorganic nutrients and vitamins (Parsons' 1-4, appen.3)
- iii) ASW, inorganic nutrients, vitamins (Parsons' 1-4, appendix 3) and glucose (0.1% w/v).

Ten ml of each test medium was inoculated with mixed cultures, incubated at 20°C and monitored for growth. No growth occurred on media (i) and (ii) while growth did occur on medium (iii). Therefore all test media used contained ASW, inorganic nutrients, and vitamins (Parsons' 1-4) and the test polysaccharide at 0.5% (w/v) as the only carbon source.

Test media were made up with the algal polysaccharides described previously (Chap. 3; Table 3.1). These were starch, laminarin, inulin, carrageenan, fucoidan, mannan, alginic acid, xylan and Carboxymethyl-cellulose (CM-cellulose). The media were made up and separated into two batches. One batch was dialysed against ASW for 48 hours through a pore size of 12-14000, and then both batches were autoclaved at 121°C for 15 minutes. The effect of autoclaving was checked by measuring reducing sugars (using the Nelson-Somogyi method) in the media before and after autoclaving. Autoclaving did cause a slight increase in reducing sugar concentration in the media. After autoclaving there were less reducing sugars in the dialysed media samples of laminarin, xylan, fucoidan, inulin and mannan than in the undialysed samples. In the CM-cellulose, carrageenan, alginic acid and starch media samples, the reverse was true (See Figures 3.4A and 3.4B).

3) Growth experiment: 5ml of each test medium (dialysed and undialysed) were sterily introduced into four spectrophotometer tubes (ST), resulting in eight sets of nine media each (see diagram below).



These were then grouped to give four sets of 18 ST which were inoculated with either 0.1ml foregut culture (set A) or 0.1ml hindgut culture (set B) or were used as controls which were either incubated with the inoculated media to account for contamination (set C) or kept at 2°C for the duration of the experiment and used as blanks (set D).

The experimental media (sets A and B) and the control (set C) were then incubated at 20°C on a shaker for 72 hours. Absorbance was measured every 6 hours after the first 12 hours at 600nm on a Bosch and Lomb Spectronic 20 spectrophotometer using set D as a blank. Growth of bacteria in the media was measured as a relative increase in absorbance, since bacterial growth caused an increase in the turbidity of the media.

Artifactual growth:

The problem of artifactual growth on the test media, caused by the utilization of extraneous low molecular weight saccharides rather than the test polysaccharide, is exacerbated by two factors. Firstly, dialysis is not completely effective in removing all the low molecular weight substances from the media. Secondly, autoclaving will result in a relative increase in the amount of low molecular weight saccharides found in the media.

However, in the event of such artifactual growth, extraneous (utilizable) sugar will be used up and growth will cease with the result that the level of reducing sugar in the inoculated medium (test) will be lower than in the control media. Growth resulting from the hydrolysis of the substrate will result in no net decrease and possibly even an increase in reducing sugar levels of the medium relative to the uninoculated (control) medium.

Thus the differences between reducing sugar concentrations in inoculated (Test) and uninoculated (Control) media were measured after the above incubation to distinguish between artifactual growth and substrate utilization. The reducing sugar content of 1ml of each medium in set A, set B, set C and set D was measured using the Nelson-Somogyi method. The standards were made up in ASW using glucose at 0.1, 0.2 and 0.3mg/ml and ASW as a blank. For alginic acid, the samples were first neutralized with NaOH and a separate set of standards and a blank, identically treated, was prepared.

Only qualitative information can be obtained from this experiment since the blank and standards can only account for the effect of ASW on the assay and not for the differential turbidity of the substrate polysaccharides. However, the standard curve can provide a means with which to compare the relative levels of reducing sugars in samples of the same inoculated and uninoculated media.

Lysozyme testing:

The method used here to test for lysozyme activity closely followed that of Jolles (1952). The target bacterium used was *Micrococcus leisodeikticus* which was obtained from Sigma chemical

company. Egg white extract was used as a control. Enzyme extracts were obtained from the foregut and hindgut and a serial dilution of 1/10, 1/100 and 1/500 was made with phosphate buffer. The serial dilution of enzyme extract as well as a serial dilution of egg white extract was tested for lysozyme activity by monitoring the attenuation of absorption at 600nm on a Bosch and Lomb Spectronic 20 spectrophotometer in a 2.6ml reaction mixture of a suspension of bacterial cells and enzyme extract at 25°C at pH 6.4.

Results

Plate cultures:

Table 1 gives the values obtained for enumeration of colonies obtained from the serial dilutions of foregut and hindgut homogenates. The plate counts revealed no significant difference ($t= 1.15$, $p>0.05$) between the degree of bacterial colonization of the foregut and hindgut.

Table 4.1: Plate count results for the 10^{-3} , 10^{-4} and 10^{-6} serial dilution of homogenates of the foregut and hindgut. The results are expressed as bacteria per 0.1ml homogenate unless otherwise indicated.

DILUTION	10^{-3}	10^{-4}	10^{-6}
FOREGUT	1.488×10^5	1.62×10^6	1.13×10^7
HINDGUT	2.877×10^6	4.68×10^8	5.66×10^7
CALCULATED TOTAL FORE. (x10/5)	2.96×10^5 bacteria/ml	3.25×10^6 bacteria/ml	2.26×10^7 bacteria/ml
CALCULATED TOTAL HIND. (x10/5)	5.754×10^6 bacteria/ml	2.34×10^8 bacteria/ml	1.13×10^8 bacteria/ml
MEAN FOREGUT TOTAL	8.71×10^7 bacteria per ml of foregut		
MEAN HINDGUT TOTAL	1.17×10^8 bacteria per ml of hindgut		

Seven different colony types grew on the agar plates. The majority of these were Gram negative chain forming cocci. Some Gram negative coliforms were also present. One capsulated strain liquified the agar plates. This strain maintained its agarolytic ability in pure culture, generally causing pits in the agar or liquifying the plate altogether.

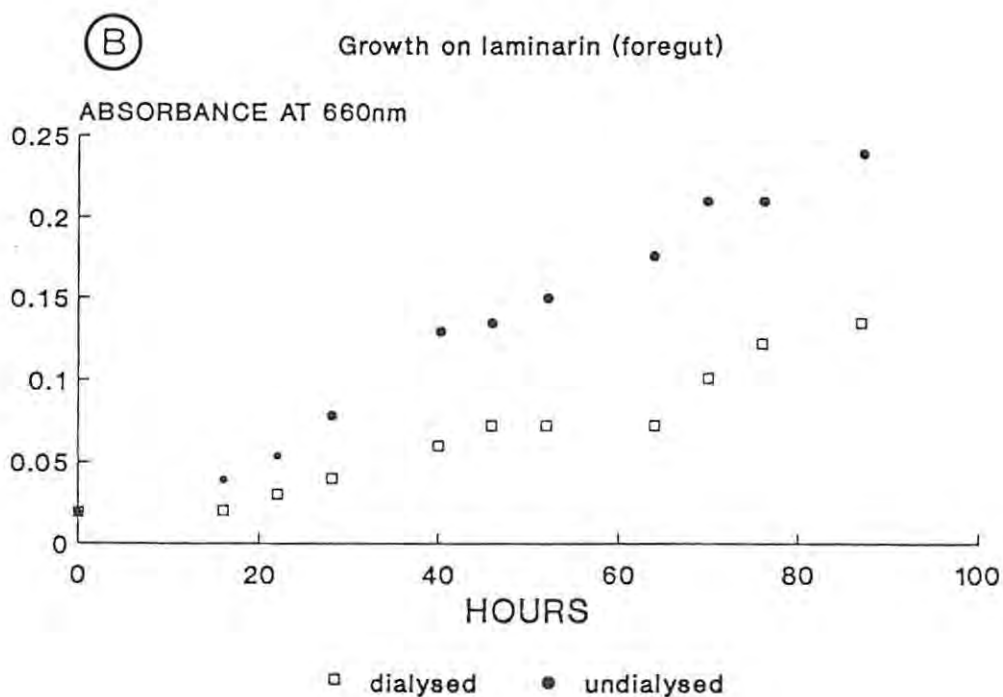
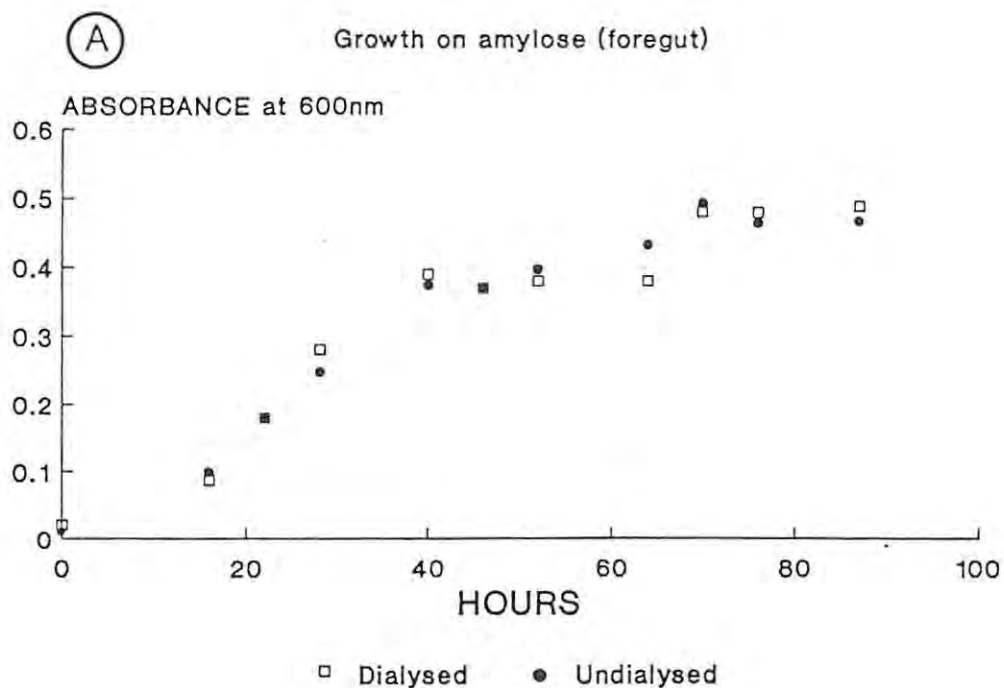
Bacterial growth on selective media

Figures 4.1A- 4.1I (foregut) and 4.2A- 4.2I (hindgut) are plots of the growth responses of bacteria (measured as absorbance) on the indicated polysaccharide media. Each figure displays two plots, one for growth on dialysed medium and one for growth on undialysed medium.

Growth occurred on all polysaccharides tested, with more growth on undialysed media than on dialysed media (except in the case of CM-cellulose for the foregut). Three types of growth pattern emerged. Firstly, for six substrates (CM-cellulose, inulin, carrageenan, xylan, mannan and fucoidan) a brief initial period of growth occurred, followed by cessation and maintenance of the bacterial population at a maximum absorbance of about 0.05 for both undialysed or dialysed media (in both the foregut and hindgut experiments).

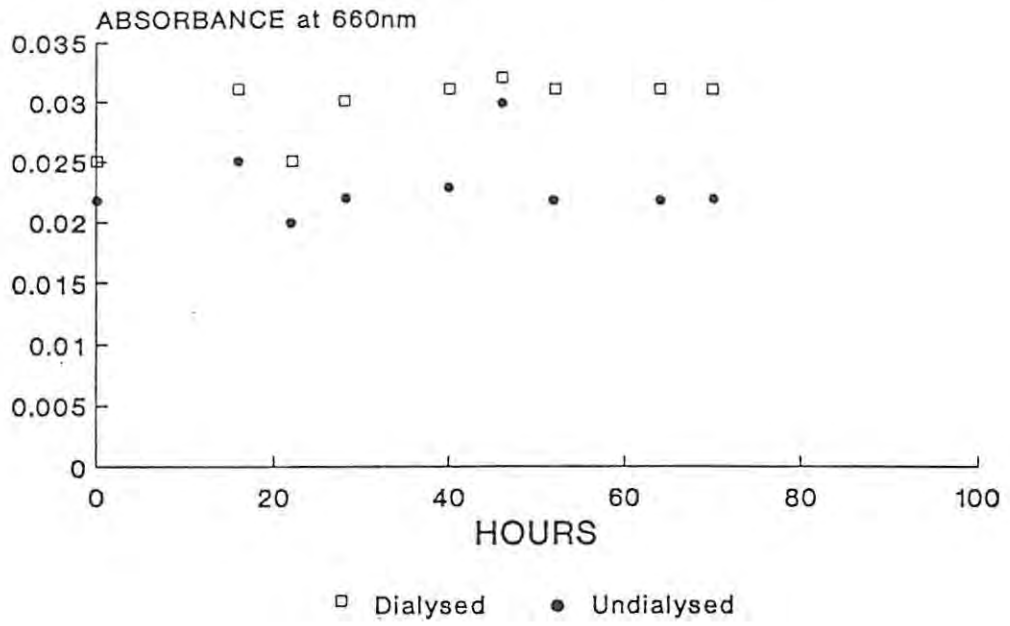
Secondly, for laminarin and starch a continuous growth pattern emerged. On laminarin a final level of about 0.3 for undialysed media and 0.1 for dialysed media was attained. Growth was highest on starch (approximately 0.5) with no distinction between dialysed and undialysed media.

Figure 4.1: Growth of mixed cultures obtained from the foregut on selective media. Plots for growth on dialysed (\square) and undialysed (\bullet) media are given for each algal polysaccharide.



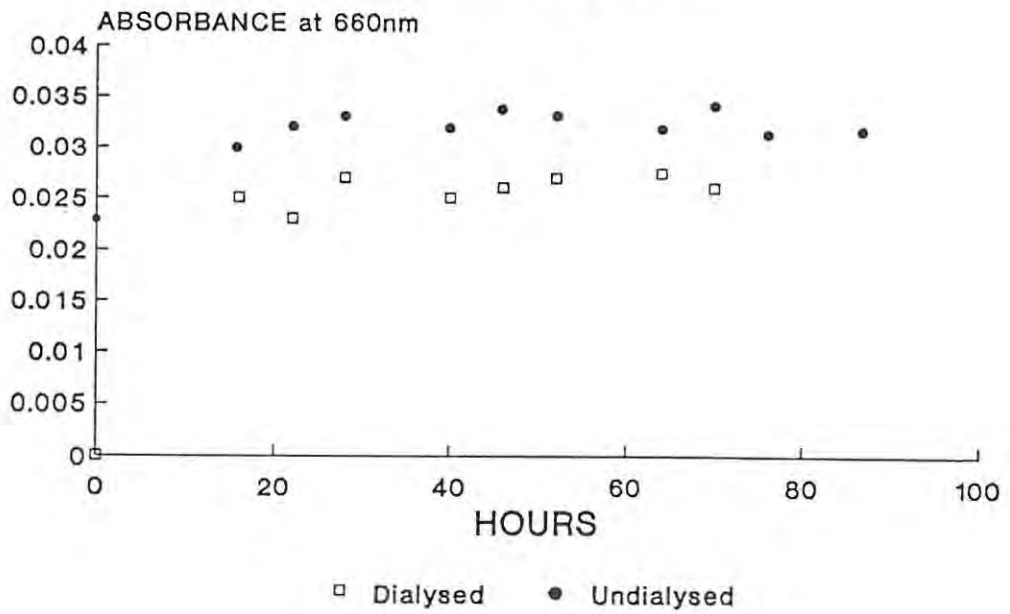
Ⓒ

Growth on CM-cellulose (foregut)



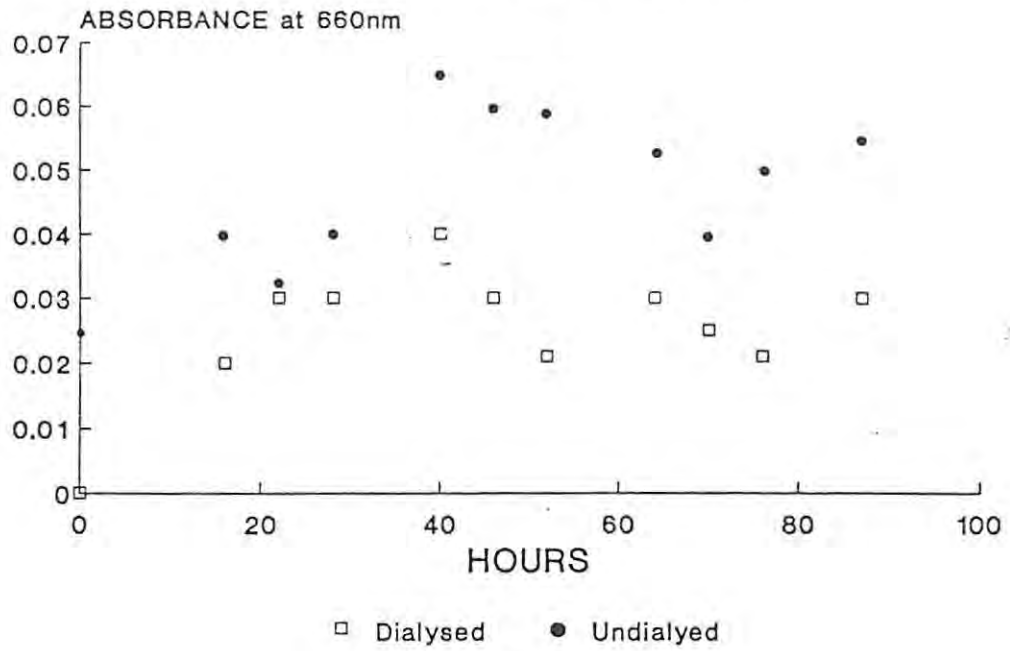
Ⓓ

Growth on xylan (foregut)



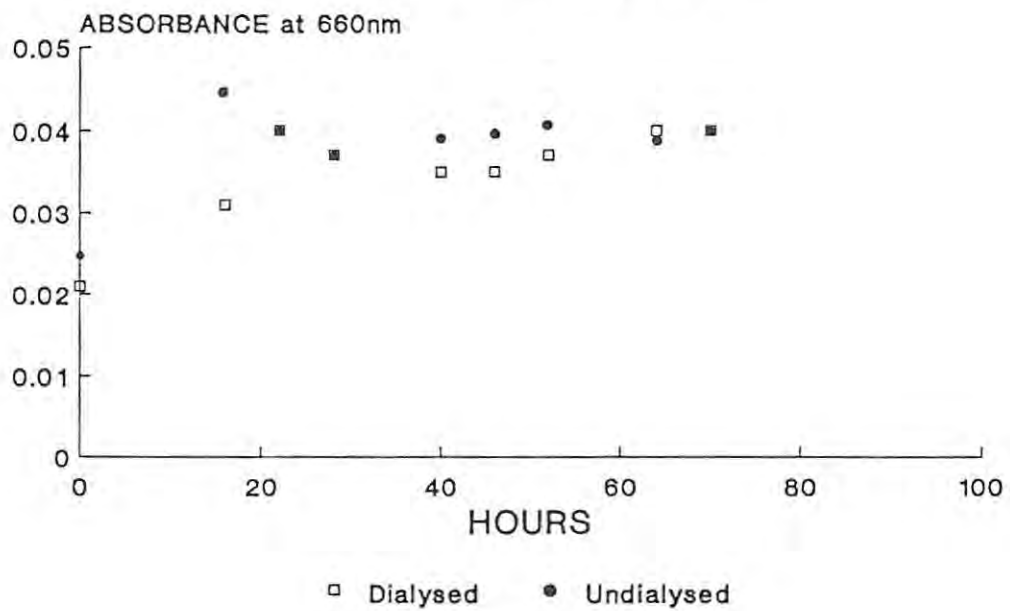
(E)

Growth on fucoidan (foregut)



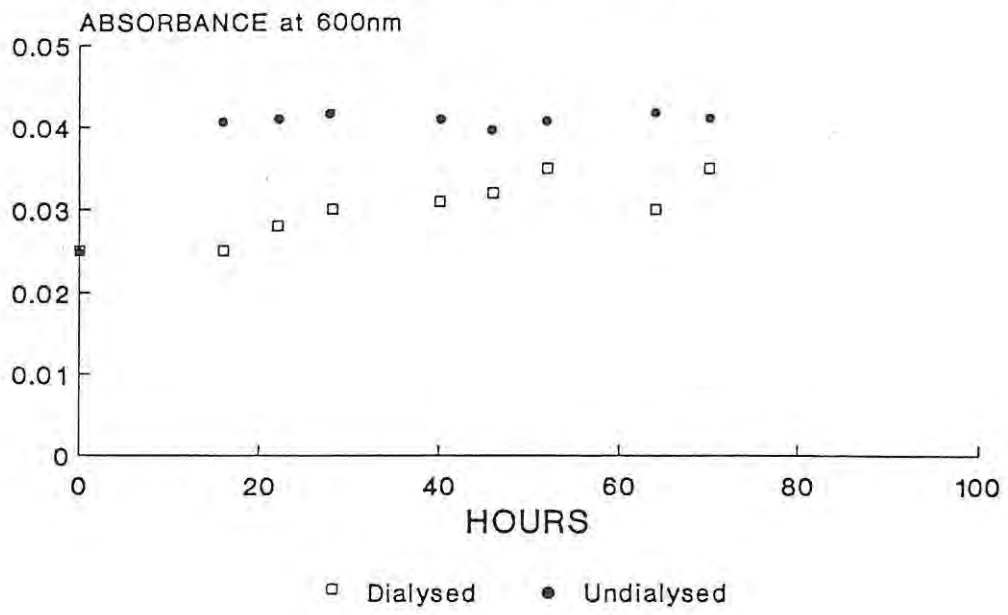
(F)

Growth on carrageenan (foregut)



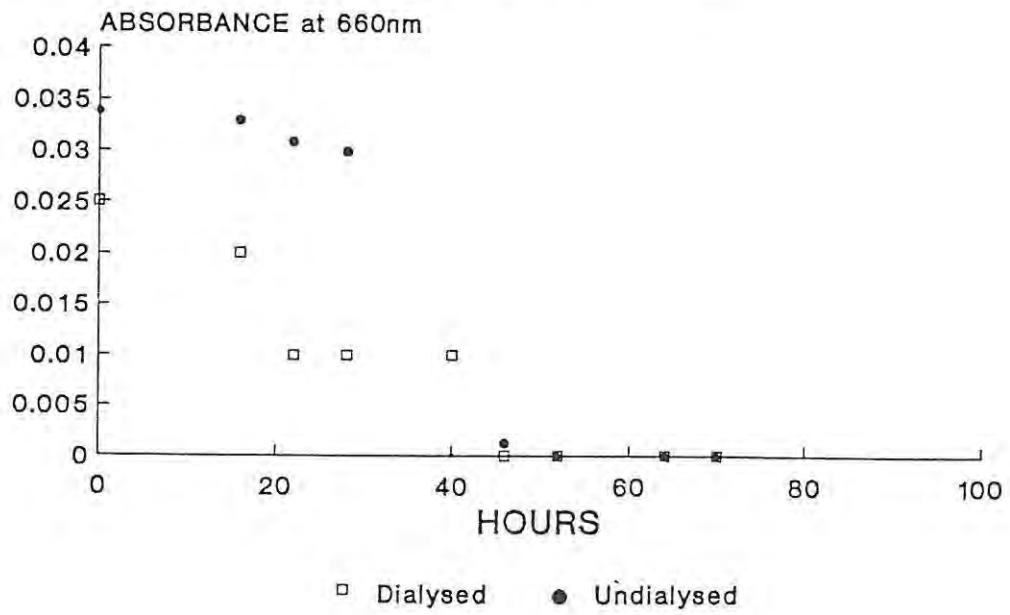
(G)

Growth on inulin (foregut)



(H)

Growth on alginic acid (foregut)



①

Growth on mannan (foregut)

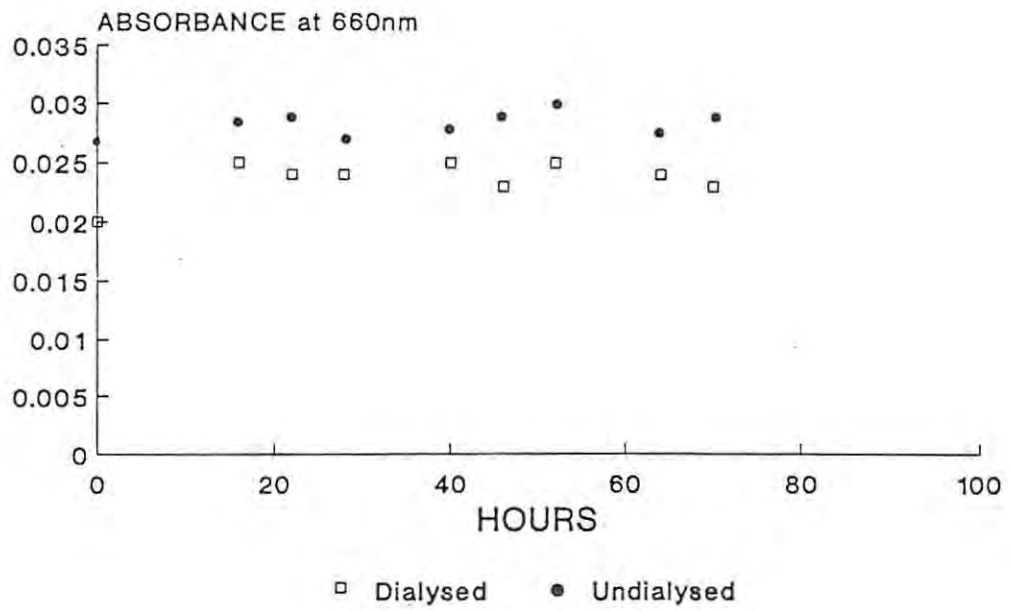
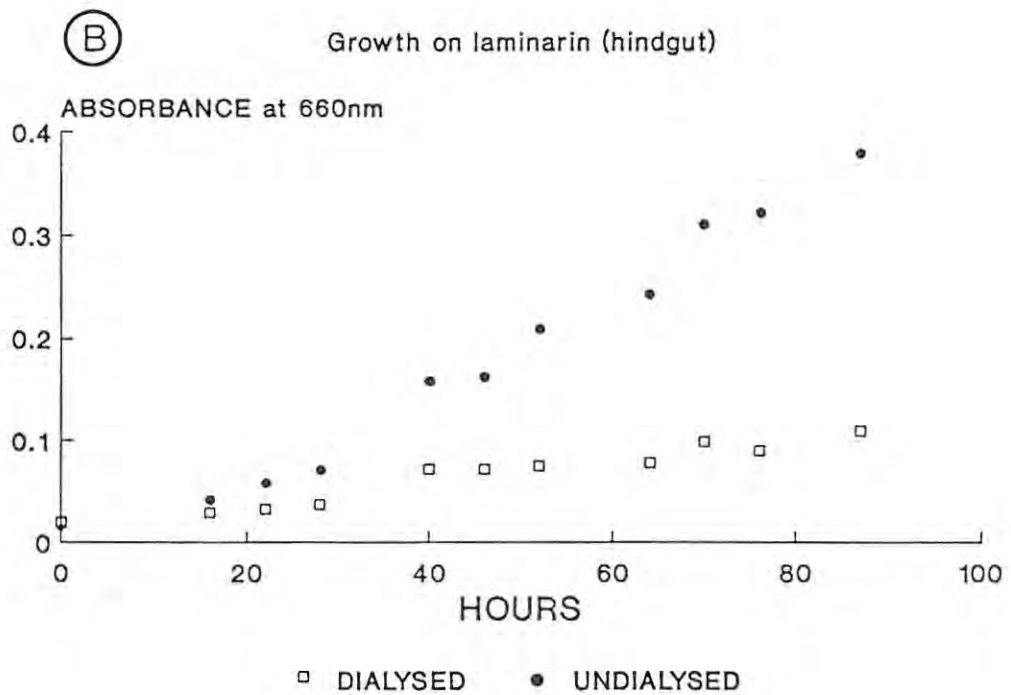
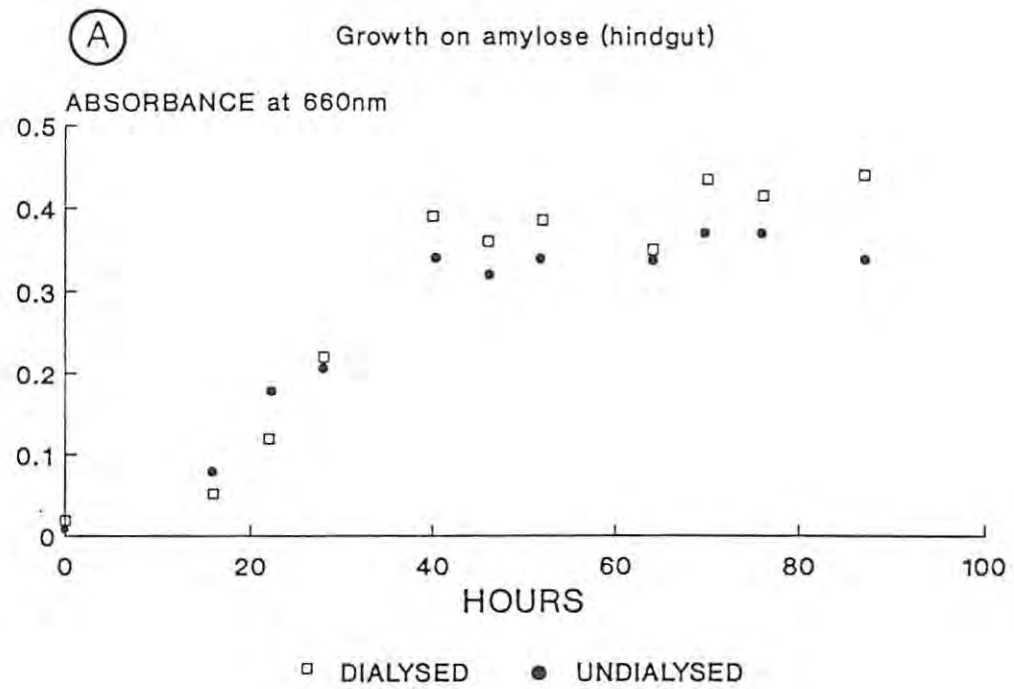
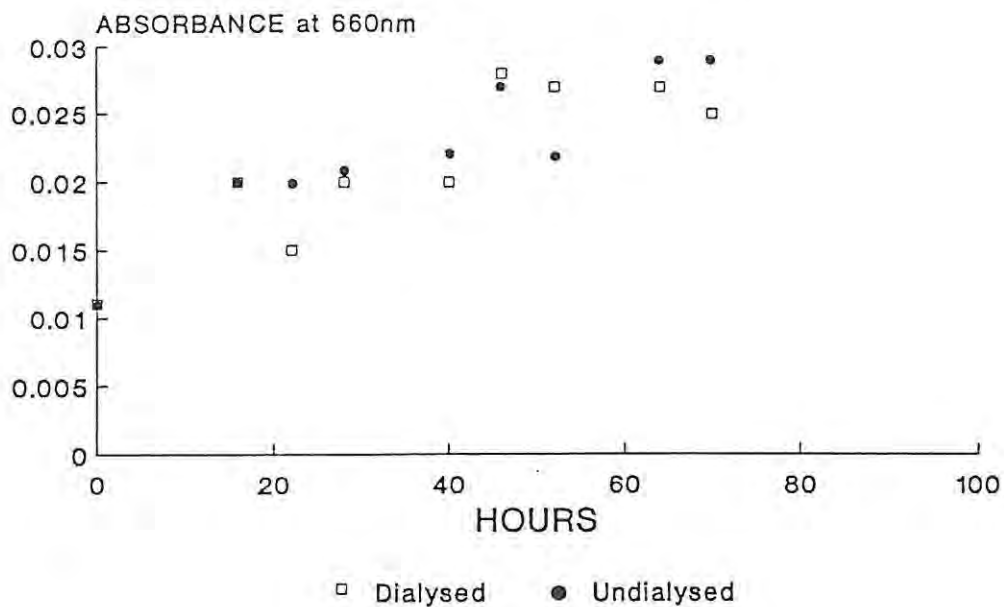


Figure 4.2: Growth of mixed cultures obtained from the hindgut on selective media. Plots for growth on dialysed (\square) and undialysed (\bullet) media are given for each algal polysaccharide.



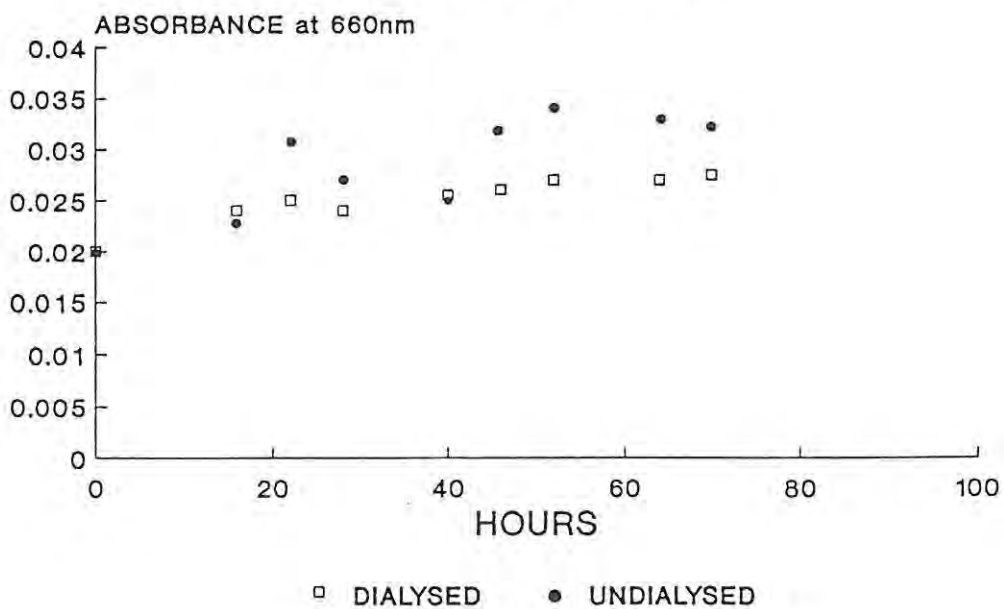
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Growth on CM-cellulose (hindgut)



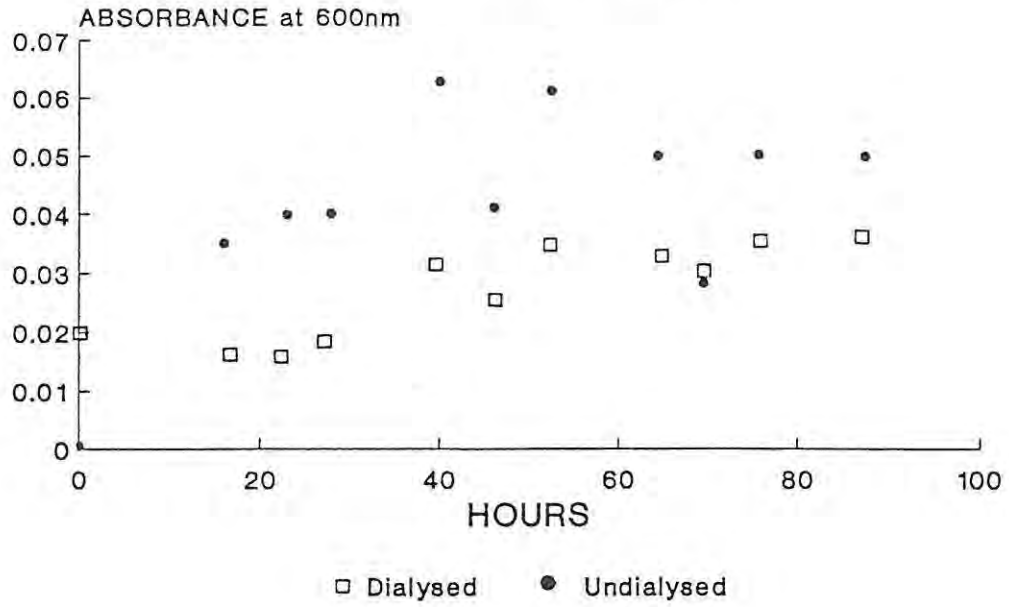
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Growth on xylan (hindgut)



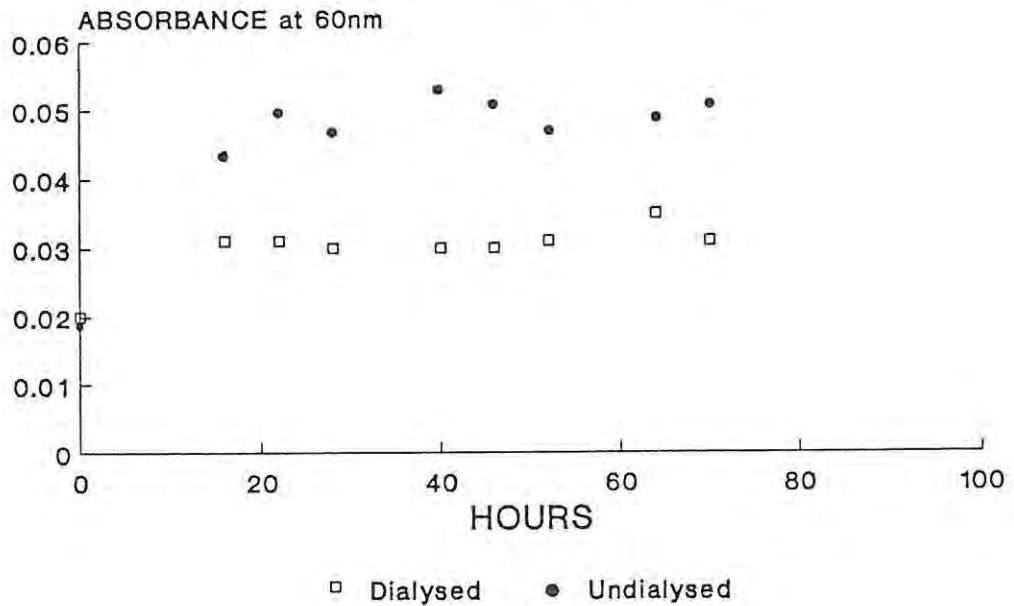
(E)

Growth on fucoidan (hindgut)



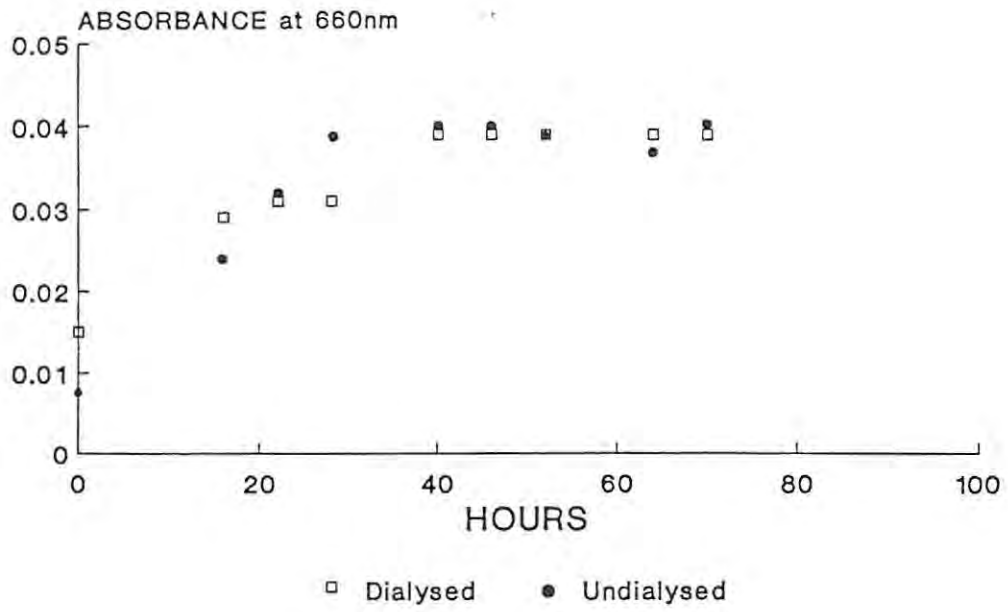
(F)

Growth on carrageenan (hindgut)



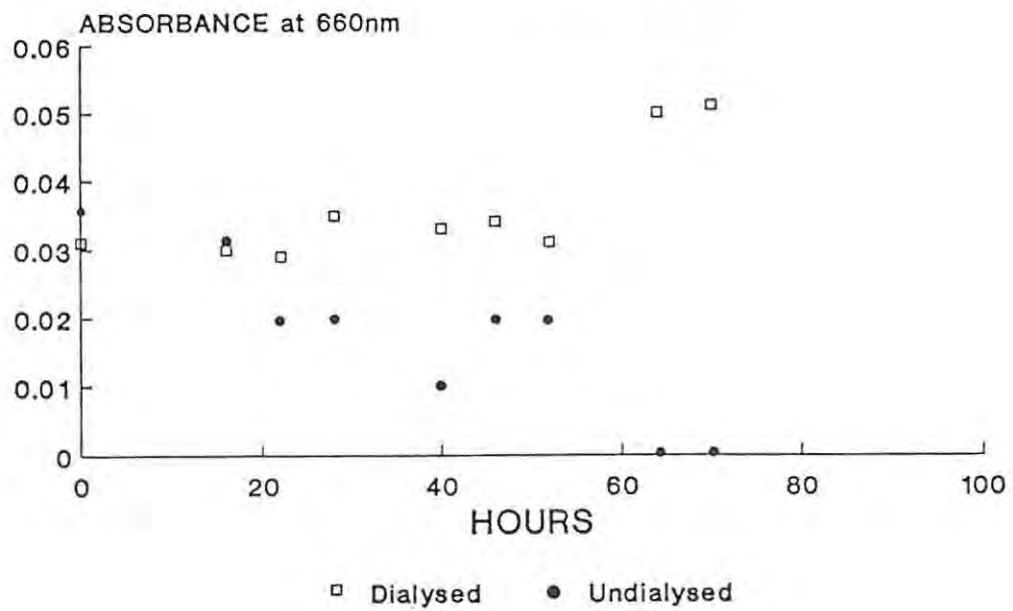
(G)

Growth on inulin (hindgut)



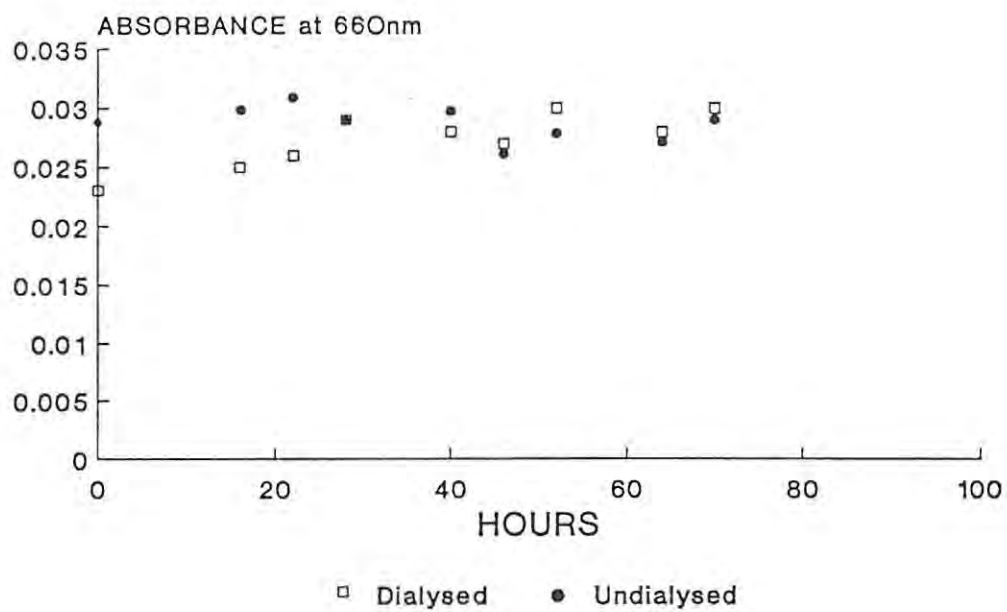
(H)

Growth on alginic acid (hindgut)



①

Growth on Mannan (hindgut)



The third, and anomalous, pattern of growth occurred on alginic acid. Hydrolysis of the alginic acid suspension caused a decrease in turbidity due to hydrolyzed fractions going into solution. Hydrolysis of alginic acid was confirmed by measuring the increase in reducing sugars in solution after inoculation and incubation of bacteria (see below). This was the case for all alginic acid media except for the dialysed hindgut medium (see Figures 4.1H and 4.2H) in which an increase in absorbance was measured. Growth on this medium followed the first growth pattern.

The effect of inoculation on reducing sugars levels in the media:

1) Control media: Figure 4.3A (foregut) and 4.3B (hindgut) shows the levels of reducing sugars in control and test media. The effect of dialysis on reducing sugar levels in the media was unclear as autoclaving had a varying effect on the polysaccharides. For some control media reducing sugar levels in the dialysed portion were lower than in the undialysed portion (laminarin, xylan, fucoidan, inulin and mannan). In other control media (CM-cellulose, carrageenan, alginic acid and starch) the reverse was true.

2) Test media: For the test media, two types of result were obtained. Firstly, for five media (xylan, carrageenan, CM-cellulose, fucoidan and mannan) the reducing sugar level decreased relative to the controls, after incubation. These results were consistent in all four treatments (hindgut, foregut, dialysed and undialysed) and are caused by the utilization of extraneous reducing sugars by bacteria resulting in the depletion of reducing sugars in the inoculated media. All these media that showed a relative decrease in reducing sugar levels produced the

first type of growth pattern described above.

Secondly, for starch, laminarin and inulin, reducing sugar levels were higher after incubation than in the controls. These results were also consistent for all four treatments. In the case the starch media, reducing sugar levels were more than twice those of the controls for both the foregut and the hindgut. For inulin, the increases were less than 15%. Inulin was the only one of these substrates that showed low levels of growth.

For laminarin, the effect of dialysis was marked. In foregut cultures (Fig. 4.3A) both the test media showed reducing sugar levels 100% greater than their respective controls at the end of the experiment. It is important to note that a small difference in reducing sugar concentration in the controls (undialysed > dialysed by 5%) resulted in a substantial difference in the tests (74%, undialysed > dialysed). A similar effect was apparent for laminarin in the hindgut (Fig. 4.3B).

The results for alginic acid (Figs 4.3A and 4.3B) show that this polysaccharide was hydrolyzed in three out of four of the incubations and corroborates the results obtained in the growth experiments. For both the foregut test media, and one hindgut test medium (dialysed) the reducing sugar yield was approximately twice that of the respective controls.

However, the hindgut dialysed test medium (Fig. 4.3B) yielded relatively (6%) less reducing sugar than the control. This corresponds well with the growth experiment results obtained on alginic acid in the hindgut (Fig.4.2H). In this experiment the increase in absorbance in the dialysed test medium corresponds

Figure 4.3A: Reducing sugar levels measured as glucose equivalents in control media and test media at the end of the growth experiment for the foregut.

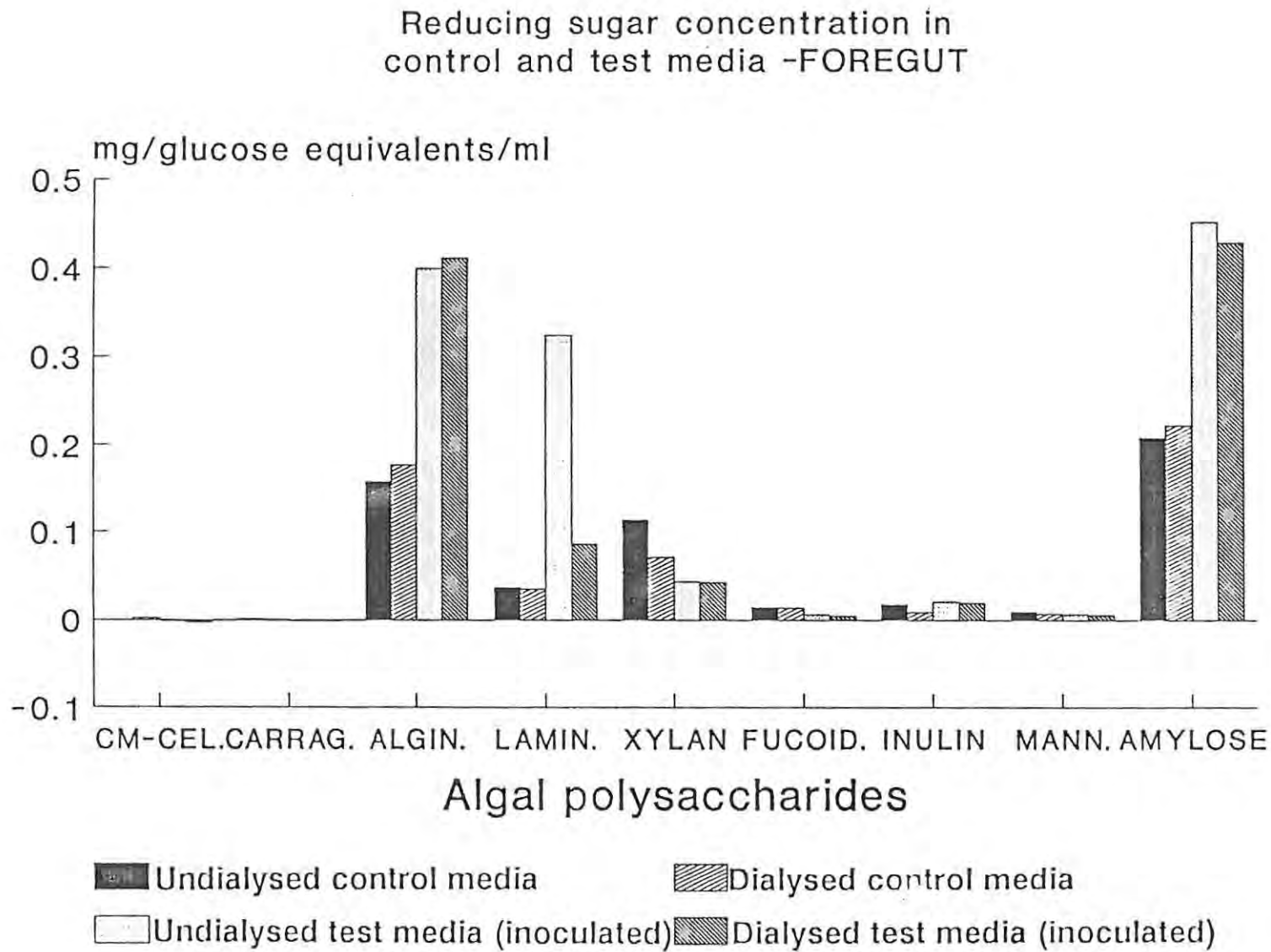
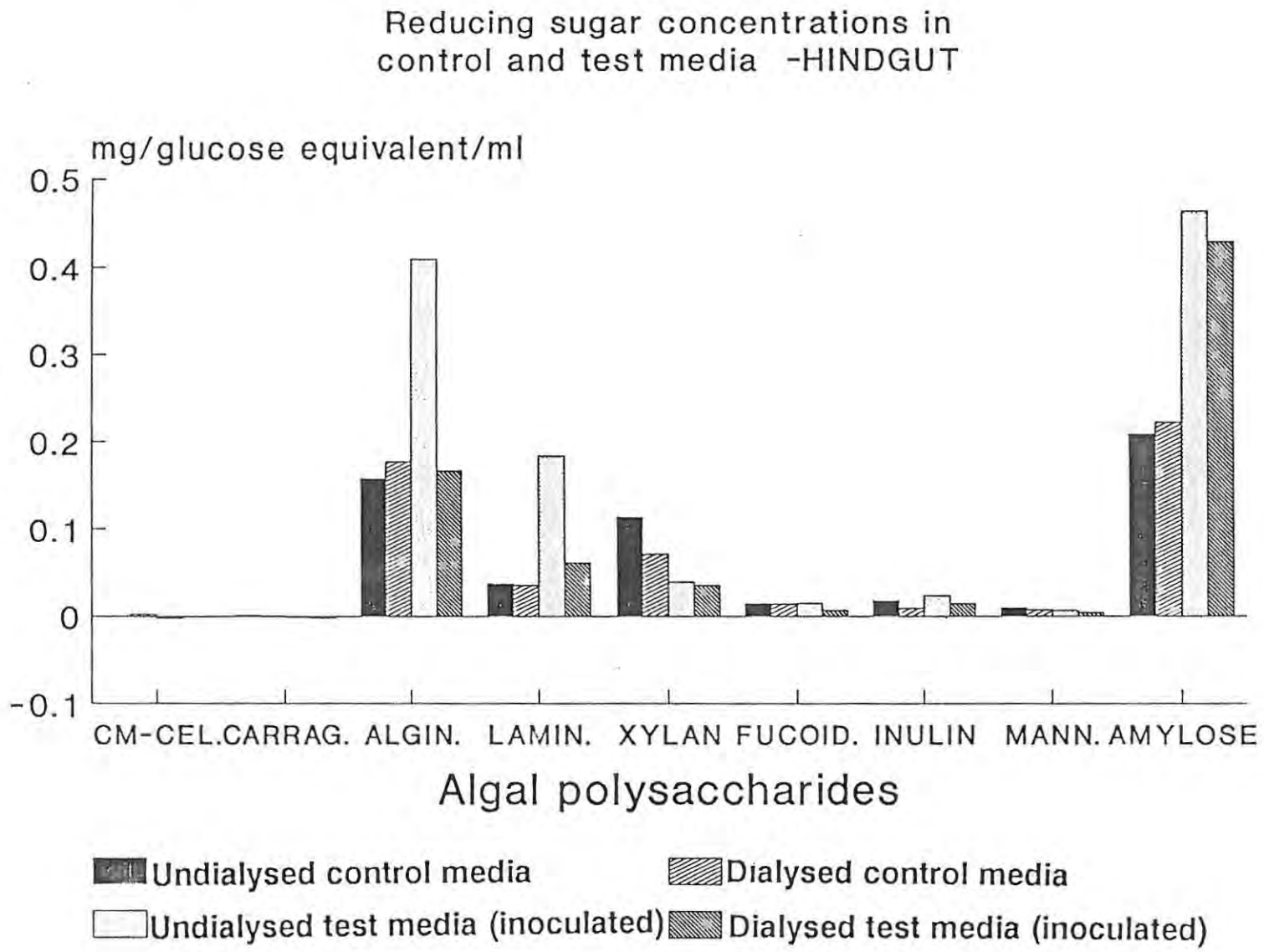


Figure 4.3B: Reducing sugar levels measured as glucose equivalents in control media and test media at the end of the growth experiment for the hindgut.



to a decrease in the reducing sugar levels, while a decrease in absorbance in the undialysed test medium corresponds to an increase in the reducing sugar level.

The implication of this is that, in the dialysed media, the bacteria grew on the extraneous sugar in the medium, thereby causing an increase in turbidity and a decrease in the final concentration of reducing sugars. In the undialysed sample, the bacteria hydrolyzed the alginic acid, causing it to dissolve which resulted in a decrease in absorbance and an eventual increase in reducing sugar levels due to the hydrolysis of the polysaccharide.

Lack of hydrolysis in the dialysed hindgut incubation is probably not an effect of dialysis itself. It is more likely to be an artifactual result of synergistic or antagonistic interactions in that particular culture.

However, this experiment confirms that where a decrease in levels of reducing sugars in the inoculations relative to the controls occurred, the bacteria were utilizing the extraneous sugars rather than the substrate polysaccharide and so growth was artifactual. For inulin, a relatively small increase in reducing sugars in inoculated samples may indicate a weak ability to hydrolyze this polysaccharide which was not discernable from the growth experiment.

In summary, the results of the growth experiments show that in all but one case, undialysed media promoted a higher level of growth due to the presence of utilizable extraneous reducing sugars and probably low molecular weight oligosaccharides. The

measurement of reducing sugars in the the media revealed artifactual results in CM-cellulose, carrageenan, xylan, mannan and fucoidan where extraneous reducing sugars were taken up by the inoculum resulting in limited growth and decrease in reducing sugars in these test media relative to the controls. On starch, inulin, alginic acid and laminarin, growth did occur and hydrolysis of the polysaccharides was confirmed by an increase in reducing sugars in test media. Clearly results from growth experiments on substrate polysaccharides cannot be taken in isolation since growth is not necessarily based on substrate utilization.

The effects of dialysis are difficult to interpret from these results, but do show that extraneous sugar in the substrate polysaccharides can have a marked effect on results. In the case of laminarin and alginic acid final growth levels (as measured by reducing sugar levels in test media) were higher in the undialysed media. This is probably due to faster initial growth of the inoculum in the undialysed medium because of the relatively higher proportion of extraneous reducing sugars. No significant distinction can be made between results obtained for the foregut and hindgut.

Lysozyme tests:

Several repeats of this test revealed no lysozyme activity in either the foregut or the hindgut. The viability of the enzyme extracts was verified in each case by testing for amylase activity. The effectiveness of the each assay was also tested using egg white extract.

Discussion

Experimental design:

The results obtained in these experiments highlight the importance of experimental design when working with bacteria. The *in vitro* experiments performed here used mixed cultures which can only superficially account for possible synergistic or antagonist effects which may occur in the environment of the gut. Selective culture of particular strains cannot be dismissed and how representative the mixed cultures were of the natural gut population is not known. Therefore, from these experiments, conclusions can only be drawn with regard to what bacterial activity has been positively identified. The absence of enteric bacterial activity on substrates tested here does not necessarily imply its absence in the gut. Also, whether the bacterial activity is important to the urchin physiologically is undetermined and can only be considered conjecturally.

Results obtained here do indicate that the enteric bacteria in the gut of *Parechinus angulosus* can utilize the storage polysaccharides of green, red (starch and inulin) and brown (laminarin) algae. Alginic acid was the only structural polysaccharide that was hydrolyzed in the growth experiments indicating bacterial ability to break down the cell walls of brown algae. The hydrolysis of agar is an important observation since it implies that the bacteria have the ability to break down structural galactans of red algal cell walls, although no hydrolysis of carrageenan was detected.

Prim and Lawrence (1975) concluded that bacteria from the gut of *Lytechinus veriagatus* could utilize laminarin, starch, agar, carrageenan, alginic acid and agar, but not cellulose. The

utilization of carrageenan, alginic acid, laminarin and cellulose (using pure cultures) was determined on the basis of cloudiness or surface or bottom growth in the medium. It is shown here that Prim and Lawrence's results should be treated with caution since growth in their experiments may have been artifactual. The agarolytic ability of bacteria from the gut of *Loxechinus albus* was demonstrated conclusively by Garcia-Tello and Baya (1973).

Retention time of food in the gut and bacterial numbers.

The retention time of the food in the gut may have an important influence on the efficacy of bacterial activity. Short retention time has been cited as evidence against bacterial involvement in herbivorous digestion in echinoids (Thayer, Bjorndal, Ogden, Williams and Ziemann, 1984). Retention time has not been measured for *Parechinus angulosus*, but the high number of enteric bacteria measured would probably offset the limitation of a short retention time.

Densities of the order of 1×10^8 bacteria per ml in the gut of *Parechinus angulosus* compares favourably with measurements for other urchins. Lasker and Giese (1954) counted approximately 10^6 bacteria per ml in *Strongylocentrotus purpuratus* although this varied with diet. In the gut of *S. droebachiensis* Fong and Mann (1980) measured up to 6×10^9 bacteria per ml. These high numbers of bacteria would lend support to Lawrence's contention (Lawrence, 1982) that bacteria may be important in echinoderms even in a relatively short space of time.

Coincidental enteric digestion by bacteria.

The ability of bacteria from the environment to break down algal structural polysaccharides has been reported by Araki et al.

(1987) and Andrykovitch and Marx (1988). The latter study showed that bacteria from a *Spartina alterniflora* salt marsh were able to utilize agar, alginic acid, CM-cellulose, laminarin, starch and xylan (among others) but not carrageenan, inulin and natural cellulose.

Specific or generally occurring infections of algal macrophytes, especially detrital algal material, can be expected to have a high complement of bacteria associated with it (Bedford and Moore, 1984 and Thayer *et al.*, 1984) and it is reasonable to assume that these bacteria will be able to degrade that algal material. If that algal material is ingested by the echinoid then the infection will also be ingested. Thus, bacteria in the gut could have an environmental origin and therefore the bacterial breakdown of algal structural material in the gut could occur coincidentally.

It is the specialization of the gut flora relative to that of the environment that is important in this regard. If the bacteria are truly enteric and do not occur in the immediate environment or food, then a symbiotic role is more likely. Unkless (1977) showed that for *Echinus esculentus* the gut flora were selected from the environment to give distinctly different proportions of genera than for the surrounding environment.

Competition for carbon.

It is clear (from chapter 3) that the bacteria are not essential to *Parechinus angulosus* in order to hydrolyze most of the algal structural polysaccharides tested. It appears that the urchin and its enteric bacteria may, in fact, compete for the same source of carbon (namely starch and laminarin).

One potential benefit of the enteric bacteria to the urchin, in terms of its carbon metabolism, is that the bacteria can hydrolyze alginic acid. Whether the urchin depends on bacterial lysis of brown algal cell walls or whether a mechanical mechanism will suffice is unknown. Since the enzymatic ability to digest the phaeophytic storage polysaccharide laminarin (Chap.3) is relatively low, bacterial digestion of brown algae will be of relatively little advantage in terms of carbon acquisition. Thus, unless the diet of the urchin is dominated by brown algae, it may be compete with its enteric bacteria for carbon. On the other hand, if the diet contains predominantly brown algae then the presence of enteric bacteria may be advantageous.

Digestive efficiency.

It is generally accepted that, although urchins are not dependent on enteric bacteria for the digestion of algal structural material, the bacteria can help to increase digestive efficiency of the host, especially in times of food paucity (Prim and Lawrence, 1977; Lawrence, 1982 and Thayer *et al.*, 1984).

An important and novel study in this regard was conducted by Bedford and Moore (1985) on the differential digestion of rotting and fresh *Laminaria saccharina* by the echinoid *Psammechinus miliaris*. They suggested that bacteria may play a role in making algal protein more easily available and thereby reduce the need to digest the organic carbon. Absorption efficiency of organic carbon was lower for rotting kelp since the carbon content of this kelp is lower than that of fresh kelp, due to the the action of bacteria. This partially explains why there is a shorter retention time for rotting kelp than for fresh kelp.

The acquisition of bacterial nitrogen

Bedford and Moore (1985) point out that the lack of a direct role for bacteria in the carbon metabolism of *Psammechinus miliaris*, does not preclude a role in its nitrogen metabolism. For example, bacteria are a source of nitrogen for *Strongylocentotus droebachiensis* (Fong and Mann, 1980) and *Tripneustes ventricosus* (Guerinot and Patriquin, 1981a, b). *Parechinus angulosus* does not possess lysozyme activity so, if enteric bacteria do have a role in its nitrogen metabolism, its means of acquisition of remains unclear.

CHAPTER 5

GENERAL DISCUSSION AND CONCLUSIONS

In this study emphasis was placed on investigating enzymatic and bacteriological mechanisms of food digestion in *Parechinus angulosus*. It appears that these mechanisms do not operate in isolation and that a combination is employed. In this final chapter the results obtained will be synthesized and discussed in terms of the feeding biology of *Parechinus angulosus*.

Ultrastructural observations show that the mucosal epithelium of *Parechinus angulosus* is typically digestive. The foregut is primarily responsible for the production of hydrolytic enzymes while the hindgut has an absorptive function. The experiments in this study have shown that the foregut of *Parechinus angulosus* possesses enzymes that as a whole, display a wide spectrum of hydrolytic activity. It is evident that enzyme activities are greater in the foregut than in the hindgut, with highest activities being attained on algal storage polysaccharides.

Enteric bacteria from *P.angulosus* utilized a narrower range of algal polysaccharides in these experiments than the urchin was able to hydrolyze. The bacteria grew most effectively on the storage polysaccharides, however, did not grow on most of the algal structural polysaccharides tested. Significantly, the enteric bacteria grew well on alginic acid whereas the urchin could not hydrolyze this important brown algal structural polysaccharide. The implications of these findings to the feeding biology of *Parechinus angulosus* need to be carefully considered.

Enzyme spectra and diet:

Klinger (1984) considered the correlation between digestive enzymes and diet in echinoids. He concluded that, for the variety of echinoids that he studied, the urchins possess carbohydrases "broadly appropriate, but not particularly tailored to their diet" (Klinger, 1984; p.599). Kristensen (1972) pointed out that highly purified enzymes that attack a wide range of substrates with the same linkages are well known. This leads to the contention that generalized enzymes, capable of hydrolysing common bonds between the monomers of different structural polysaccharides may occur in generalist herbivores as mixed function oxidases do in insects (Strong, Lawton and Southwood, 1984).

The possession of a spectrum of enzymes that are capable of specifically lysing a wide range of algal polysaccharides (on a one enzyme for one polysaccharide basis) must be regarded as a highly specialized adaptation to a generalized diet. It is more likely that the wide range of digestive enzyme activity detected in *Parechinus angulosus* is the result of the action of the same, or a small number of enzymes. These enzymes would be capable of attacking a specific type of linkage that occurs, at least occasionally, within all the substrate polysaccharides that elicited enzyme activity. The activity of these enzymes (or affinity to the substrate) would then depend on the number of these bonds in the polysaccharide chain. This could, at least in part, explain the low level activity that occurred on some substrate polysaccharides as opposed to the hydrolysis (high level activity) of others.

Whether polysaccharidase activity in *Parechinus angulosus* is indeed the result of specific enzymes or enzymes with a wide range of lytic ability is difficult to conclude from these results. The substrates used differ in their constituent monomers and/or in the type of linkages. The glucans (CM-cellulose, amylose, glycogen and laminarin) have more in common with each other chemically, than with the other substrates. However, activity was markedly variable on these substrates. Cellulase and laminarinase (beta-glucans) activity was of the same order as for non-glucans (e.g. xylan) whereas on amylose and glycogen (alpha-glucans) activity was distinctly higher. This suggests that a generalized glucanase is responsible for the activity on all the glucans with the differential activity being the result of the chemical differences in the linkages between the monomers of the respective polysaccharide chains. A detailed chemical analysis of the substrates would be necessary before a conclusion could be drawn in this regard since these substrates can vary in their specific chemical structure according to their source and depending on how they were obtained.

The algal cell wall is a complicated structure (Kloereg and Quatrano, 1988), generally comprising several different heteropolymers of a variety of monomers (Painter, 1983). Thus the relative digestibility of algal cell walls can be expected to be far more variable than *in vitro* experiments suggest. Nevertheless, even partial breakdown of structural polysaccharides may result in sufficient disruption of the cell wall to allow access to the cell contents.

The role of bacteria:

The lytic ability of bacterial polysaccharidases to hydrolyze specific structural polysaccharides such as xylan (Araki *et al.*, 1987) or alginic acid (shown here) is likely, even though certain strains of marine bacteria have shown the ability to digest a wide variety of structural polysaccharides (e.g. Andrykovitch and Marx, 1988). Bjorndal (1980) suggests that the more specific the diet of a herbivore, the more important the gut microflora are as an aid to digestion, since less variability in the diet will allow the development of a more specialized gut microflora.

Lawrence (1982) concluded that echinoids are generalists but eat predominantly what occurs in their immediate environment at any particular time. While *Parechinus angulosus* is a generalist, it does not necessarily consume a variety of algae at any one time or place. In other words, the variety of algae in the diet of *P.angulosus* will depend on the variety in the habitat where it occurs. The diet of *P.angulosus* more is specialized in western Cape kelp beds (where it consumes mainly *Ecklonia maxima*) relative to elsewhere on the coast (Fricke, 1979; Stuart and Field, 1981; pers. obs.). If Bjorndal's hypothesis is correct, then the gut microflora of *P.angulosus* could have a more important role in digestion in the western Cape populations.

The dominant kelp species on the South African coast are the phaeophytes *Ecklonia maxima* and *Laminaria pallida* (Field *et al.*, 1980). On the east coast (where this study took place) the only kelp species that occurs is *Ecklonia biruncinata*. This macrophyte does not form kelp forests such as those of *E. maxima* but grows fairly abundantly in isolated pockets (Phillips and McQuaid, in prep). *E.biruncinata* is not consumed by *P.angulosus*

(pers. obs.).

Food preferences and digestibility:

A food preference study by Anderson and Velimirov (1982) on 13 species of rhodophyte and phaeophyte algae from the kelp forests of the western Cape revealed that the preferences of *P. angulosus* fell into three categories: preferred, intermediate and non-preferred algae. *Ecklonia maxima* was part of the group of non-preferred algal species. All the algae in the preferred category (5 spp.) were rhodophytes, those in the intermediate category (2 spp.) phaeophytes and those in the non-preferred category (6 spp.) a mixture of the two. It is therefore not surprising that *Ecklonia biruncinata* is not consumed on the east coast as the urchins are presented with a wider variety of algae than in the kelp forests (Bolton, 1986).

Anderson and Velimirov (1982) correlated feeding rate with relative "astringencies" (a measurement of phenol concentration) in algal species. Polyphenols have been implicated as the main factor determining the palatability to *P. angulosus* (Anderson and Velimirov, 1982) and other echinoid species such as *Arbacia punctulata* (Hay, Lee and Guieb, 1986).

It is remarkable that in *P. angulosus* from western Cape kelp forests, feeding continuously on *Ecklonia maxima*, absorption efficiency was on average 84% (Buxton and Field, 1983). Such a high absorption efficiency (*c.f.* Larson, Vadas and Keser, 1980 and Thayer *et al.*, 1984) can be explained in two ways. Firstly, it is possible that the western Cape urchins possess an alginase in contrast to the results obtained in this study for *P. angulosus* on the east coast. This would imply, either that there is a genetic

difference between the western and eastern Cape populations, or that an enzyme switch is responsible for this difference.

Secondly, the gut microflora could be responsible for the breakdown of *Ecklonia maxima* (and the consequent high measure of absorption efficiency). Evidence presented here supports the latter theory since enteric bacteria could utilize alginic acid whereas this substrate was not hydrolyzed by the urchin. It is therefore suggested that the reason *Ecklonia spp* and other brown algae are not preferred by *Parechinus angulosus* is only partly due to their unpalatability, and also due to their intrinsic undigestibility by the urchin itself. This is supported by the fact that *P.angulosus* does indeed consume mainly *Ecklonia maxima* in the kelp beds (Fricke, 1979) even though it is not preferred. Unfortunately no data exist on the diet of *P.angulosus* for populations other than those in the western Cape kelp beds, although they have been observed eating a wide variety of algae in the eastern Cape (per. obs.)

For *Parechinus angulosus*, feeding on kelp or other brown algae seems to be a compromise between what is available and what is preferred, as in *Strongylocentrotus spp.* (Vadas, 1977). Vadas (1977) showed that food preferences were not correlated with calorific value of the food but rather with absorption efficiency. Hughes (1980) suggests that the "prey value" of marine algae to herbivores depends not only on their energy value but also on the digestibility and chemical defences of the algae, and that the ability of urchins to digest such a wide variety of algae is probably due to the digestive capacity of the gut microflora. This study has shown that *Parechinus angulosus* can

digest red and green algae, but not brown algae. This contrasts with the findings of Lubchencho and Gaines (1981) who concluded that in general, urchins can digest reds and browns. In *P. angulosus* the digestion of brown algae seems to be dependent on the action of gut bacteria, but this represents a compromise since the presence of these bacteria, while providing the urchin with a means of digesting the algae, also introduces a source of enteric competition. This is evident from the fact that the bacteria can utilize the same storage polysaccharides (carbon source) as the urchin.

Digestibility is only one criterion for assessing the quality or value of algal food. The chemical composition of the algae such as the relative proportions of organic nitrogen (protein) and lipid could also be considered as criteria (Lawrence, Regis, Delmas, Gras and Klinger, 1989). The chemical composition of algae on the South African coast is known to vary between species (Field, Griffiths, Griffiths, Jarman, Zoutendyk, Velimirov and Bowes, 1980) and seasonally within species (McQuaid, 1985 and Phillips and McQuaid, in prep). Guerinot and Patriquin (1981a,b) showed that nitrogen fixing activity by bacteria in the gut of various echinoids was distinctly seasonal, suggesting that this process was correlated with seasonal changes in food quality. Bacteria have also been shown to play a vital role in the acquisition of essential amino acids in *Strongylocentrotus droebachiensis* (Fong and Mann, 1980). While the role of bacteria in nitrogen metabolism has not been investigated for *Parechinus angulosus* it is known that the nitrogen content of the kelp *Ecklonia maxima* does vary seasonally (Probyn and McQuaid, 1985) which suggests that the bacteria have

a potentially important role in the nitrogen metabolism in a manner similar to that described by Guerinot and Patriquin (1981a, b).

Conclusions:

In conclusion, the ability of *Parechinus angulosus* to digest a defined range of algal structural polysaccharides implies that different mechanisms will be employed on different diets of marine algae. The role of enteric micro-organisms is potentially more important as an agent of digestion when the urchin is feeding on brown algae. Thus, *Parechinus angulosus* employs enzymatic means to gain access to the contents of green and red algal cells. To digest brown algae the urchin requires enteric bacteria to hydrolyze alginic acid. The consumption of brown algae such as *Ecklonia maxima* by *Parechinus angulosus* on the western Cape coast is not preferred because of unpalatability (Anderson and Velimirov, 1982) and also, probably, because of an inability to digest it without relying on enteric bacteria.

Why *Parechinus angulosus* lacks an alginase yet possesses other polysaccharidases is an important question. Alginic acid is a unique algal polysaccharide in that it has a fundamentally different chemistry from the other polysaccharides (Percival and McDowell, 1982). Despite this, some marine invertebrates do possess alginases (Huaug and Giese, 1958 and Favorov and Vaskovsky, 1971). An alternative explanation is that *Parechinus angulosus* may not have evolved an alginase because kelp species have proven unpalatable and are therefore not preferred (Anderson and Velimirov, 1982).

It is clear that a comparative study of the eastern Cape and

western Cape populations would be invaluable. A comparison of digestive mechanisms, absorption efficiencies and gut residence times on different diets as well as comparative food preference studies for both populations is required to increase our understanding of the eco-physiology of *Parechinus angulosus*.

It is suggested that the main area of emphasis of research should in general be placed on a mechanism to assess digestibility of algae in echinoids and herbivores in general. The estimation of absorption efficiency as an index of digestibility using variations of the Conover ration method (Conover, 1966) of comparative organic contents of the food and faeces does not distinguish between digestion by enteric bacterial and digestion by the animal. It is important to know how representative the relative change in organic content is of the eventual gain to the urchin in terms of energy and protein.

APPENDIX 1: STAINING PROTOCOL FOR IMMUNOCYTOCHEMISTRY

STEP	CHEMICAL	TIME
1	BUFFER	4 x 1minute
2	NORMAL GOAT SERUM	10 minutes
3	NORMAL GOAT SERUM	10 minutes
4	BUFFER	4 x 1minute
5	PRIMARY ANTI-BODY	120 minutes
6	BUFFER	4 x 1minute
7	BUFFER	4 X 1minute
8	NORMAL GOAT SERUM	10 minutes
9	GOLD BUFFER	4 x 1minute
10	SECONDARY ANTI-BODY	120 MINUTES
11	BUFFER	4 x 1minute
12	URANYL ACETATE	5 minutes
13	WASH	

APPENDIX 2: DETERMINATION OF GLYCOGEN IN ENZYME EXTRACTS:

	UNIT	A	B
GLY.C1	mg glycogen/ml	0.2	0.208
GLY.C2	mg glycogen/ml	0.4	0.416
GLU.Y1 (SD)	mg glucose/ml	0.2058 (0.007)	0.1763 (0.0125)
GLU.Y2 (SD)	mg glucose/ml	0.4118 (0.01)	0.3596 (0.005)
CONRATIO (SD)	(yield/glycogen)	1.029 (0.0001)	0.85615 (0.008)
YIELD.F (SD)	mg glucose/ml	0.089162 (0.007)	0.09304 (0.006)
YIELD.H (SD)	mg glucose/ml	0.062608 (0.008)	0.019303 (0.00)
GLYCONC.F	mg glycogen/ml	0.086633	0.108679
GLYCONC.H	mg glycogen/ml	0.060843	0.022546
PROCONC.F	mg protein/ml	3.88	4.27
PROCONC.H	mg protein/ml	2.77	2.57
FSPEC	mg glycogen/ mg total protein/ml	0.0223	0.0255
HSPEC	mg glycogen/ mg total protein/ml	0.0230	0.0075
POT.YF	mg glucose/ mg total protein/ml	0.0245	0.0280
POT.YH	mg glucose/ mg total protein/ml	0.0253	0.00825

KEY

GLY.C1 - 1st known concentration of glycogen.

GLY.C2 - 2nd known concentration of glycogen.

GLU.Y1 - Glucose yielded from hydrolyzation of GLY.C1

GLU.Y2 - Glucose yielded from hydrolyzation of GLY.C2

CONRATIO- Conversion ratio obtained from yield/concentration.

YIELD.F - Glucose yield from hydrolyzation of foregut extract.

YIELD.H - Glucose yield from hydrolyzation of hindgut extract.

GLYCONC.F-Equivalent glycogen concentration in foregut extract.

GLYCONC.H-Equivalent glycogen concentration in the hindgut.

PROCONC.F-Total protein concentration of foregut extract.

PROCONC.H-Total protein concentration of hindgut extract.

SPEC.F- specific concentration of glycogen in foregut extract.

SPEC.H- specific concentration of glycogen in hindgut extract.

POT.F- potential glucose yield from glycogen hydrolysis (foregut)

POT.H- potential glucose yield from glycogen hydrolysis (hindgut)

APPENDIX 3: PARSONS' ARTIFICIAL SEAWATER (S=30.5⁰/oo)

(After Parsons *et al.*, 1984)

Solution 1

Solution 2

<u>Chemical</u>	<u>Concentration(g/kg)</u>	<u>Chemical</u>	<u>Concentration(g/kg)</u>
NaCl	20.758	MgCl ₂ .6H ₂ O	9.395
Na ₂ SO ₄	3.477	CaCl ₂ .2H ₂ O	1.316
KCl	0.587	SrCl ₂ .6H ₂ O	0.0214
NaHCO ₃	0.170		
KBr	0.0845		
H ₃ BO ₃	0.0225		
NaF	0.0027		

Solutions 1 and 2 are made up separately and diluted to a final volume of 1 litre.

Solution 3:

Solution 4:

<u>Chemical</u>	<u>Concentration (g/l)</u>	<u>Chemical</u>	<u>Concentration (g/l)</u>
NaNO ₃	4.667	Thiamine	0.01
NaSiO ₃	3.000	Vitamin B ₁₂	0.002
Na ₂ glyceroPO ₄	0.667 X	Biotin	0.001
Na ₂ EDTA	0.553 X		
H ₃ BO ₃	0.380		
Fe(NH ₄) ₂ (SO ₄) ₂ .6H ₂ O	0.234		
FeCl ₃ .6H ₂ O	0.016		
MnSO ₄ .4H ₂ O	0.054		
ZnSO ₄ .7H ₂ O	0.0073		
CoSO ₄ .7H ₂ O	0.0016		

The silicate must be neutralized before: To a solution of 100ml of silicate containing 30g/l, add 20ml 1N HCl and dilute immediately with others. The NaEDTA, FeCl₃, and the Fe(NH₄)₂(SO₄)₂.6H₂O must be added before the remaining trace metals Mn, Zn, and Co. Those chemicals marked X were omitted for the purposes of these experiments. To make AFSW add 10ml solⁿ 3 and 1ml of solⁿ 4 to a 1litre mixture of solⁿ's 1 and 2.

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