

THE ENZYMES OF THE PYLORIC CAECA OF *PISASTER OCHRACEUS* (BRANDT):

AN HISTOCHEMICAL STUDY

by

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ABSTRACT

1. An histochemical study of the enzymes of the pyloric caeca of the starfish *Pisaster ochraceus* was carried out using frozen tissue sections. The enzymes were demonstrated with simultaneous capture (coupling) reactions using azo dye methods. The effects of nutritional state on enzyme patterns were investigated in starfish which had been deprived of food for up to 10 weeks, or allowed to feed normally.
2. An histological investigation was concomitantly carried out on tissue samples from the same animals utilizing paraffin sections. The cellular details were observed during the various nutritional conditions outlined above.
3. The four enzymes tested for (acid phosphatase, alkaline phosphatase, leucine aminopeptidase, and esterase) were all demonstrated within the digestive epithelium. The enzymes appear to be affected more by the nutritional state of the asteroid than by the season.
4. Season of the year has little effect on the cellular inclusions of the caecal tissue, but the nutritional condition of the starfish does have a marked effect.
5. During starvation the zymogen granules are few, and the zymogen vacuoles are small and scarce. However, these inclusions never disappear entirely and are present after 10 weeks of starvation. Immediately after feeding, however, the zymogen granules increase

markedly in number and the associated vacuoles increase in size as well as number.

6. The lipid deposits are not diminished significantly by starvation. Following feeding the lipid deposits and storage granules become very abundant within the tissue.
7. The possible activities of the enzymes are considered in relation to the functions of the pyloric caeca. The phosphatases, especially alkaline phosphatase, seem to perform an important role in nutrient transport. Acid phosphatase and, perhaps to some extent, leucine aminopeptidase may be important in intracellular (lysosomal) digestion, especially in starved animals. Leucine aminopeptidase also functions in extracellular digestion, whereas the esterases probably function primarily in the hydrolysis of the storage products of the pyloric caeca as well as in lipid absorption and metabolism.
8. The isozyme concept is discussed in relation to its bearing upon enzyme localization and function. The localization and level of activity of the enzymes (in particular, alkaline phosphatase) are seen to be influenced by their function(s). Different localization sites possibly indicate different isozymes and reflect different metabolic needs at those sites.

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Chapter I

INTRODUCTION

Of fundamental importance to the biological investigation of any organism is a thorough knowledge of its digestive physiology and enzymology. Among the echinoderms, knowledge of the digestive process is limited in scope and deals mainly with the anatomy and histology of the digestive system, the mode of feeding utilized, compilations of food preferences, predator-prey behavioural relationships, and the inter-relationship that exists between feeding and reproduction (Hyman, 1955; Yonge, 1954 a and b). Some of these aspects have been related to the environment of the organism; many, however, have not.

The present study was undertaken to elucidate by means of specific histochemical methods the localization and possible functions of some of the enzymes present in the pyloric caeca of the asteroid, *Pisaster ochraceus* (Brandt). In the course of the study, the seasonal variation in the enzymes studied and their localization was investigated, as well as the effect that starvation and feeding has upon them. Concurrent to the histoenzymatic study, it was thought to be advantageous to make routine paraffin sections of the caecal tissue to ascertain whether or not the season or the nutritional state of the animal affects the characteristic form and structures of the caecal digestive epithelium.

The problems dealt with in this research can be summarized, therefore, in the following questions:

- 1) What are the histochemically identifiable enzymes present in the pyloric caeca of *Pisaster ochraceus*?
- 2) Where are they localized within the caeca?
- 3) What are their function(s) in the asteroid? and;
- 4) How are these enzymes [their presence or absence, their localization, and their function(s)] affected by the time of year and the nutritional state of the animal?

Historical Review

To provide a background for the present study, a summary is given here of the research done in the field among the various echinoderm classes. The echinoderms suffer from a comparatively cursory examination of their digestive system and its enzymatic constituents. Most studies to date deal with the morphology, histology, and function of the various organs of the digestive tract (Booolootian, 1966; Hyman, 1955). Relatively little work has been done on the actual enzymology of this phylum (Binyon, 1972).

Crinoidea

The Crinoidea are the least studied of all the echinoderms, perhaps because their narrow ecological parameters exclude them from most shallow-water environments neighbouring accessible regions, and because they are difficult to capture alive and maintain for any length of time in aquaria. Consequently, little is known of the actual digestive process. The short section in Hyman (1955) on the digestive

system of crinoids is largely restricted to a morphological description of the digestive tract. Structural details are scanty and superficial, and function appears to be altogether unknown. The gut wall is described as lined by tall ciliated cells interspersed with mucous glands. No other secretory elements have been reported, the functional nature of the diverticula (of *Antedon*) is unknown and the source and nature of the digestive enzymes remain to be investigated. Nothing is known of the mechanisms of uptake, and storage of nutrients (Booolootian, 1966).

The other classes of the Echinodermata are somewhat better known.

Ophiuroidea

A considerable amount of work has been done on the feeding mechanisms of the Ophiuroidea (Vevers, 1956; Magnus, 1962; Buchanan, 1964; Fontaine, 1964, 1965). Preliminary observations have been performed on the basic histology of some of the organs of this very simple digestive system (Booolootian, 1966; Fontaine, 1961; Roubaud, 1965; Schechter and Lucero, 1968). Information on the functional aspects of the digestive system is sparse. Wintzell (1918) identified the digestive enzymes of *Ophiura texturata* as a strong protease acting over a broad pH range, an amylase, and probably also a lipase. Roubaud (1965) (while studying the digestive system of *Ophiothrix quinquemaculata* as a means of perfecting methods of fixation and decalcification of ophiuroid tissue) was able to demonstrate alkaline phosphatase within the digestive epithelium. More recently, the gut

morphology and digestive enzymes of three species of New Zealand ophiuroids (*Ophionereis fasciata*, *Ophiactis resiliens*, and *Ophiopteris antipodum*) have been investigated (Pentreath, 1969). The studies involved the testing of gut extracts with suitable enzymic substrates as well as several histochemical tests. A wide range of digestive enzymes, particularly those hydrolyzing glycosidic linkages were found. Pentreath (1969) noted the presence of esterases and a proteinase (gelatinase), but his tests for cellulase were negative for all three species. The absence of cellulase agrees with the data of Yokoe and Yasamasu (1964) whose survey of cellulase in invertebrates yielded negative results for ophiuroids, asteroids, and holothurians (some echinoids showed the presence of cellulase, others did not).

Holothuroidea

The studies on the Holothuroidea are more thorough, but they are, nevertheless, few and meager in scope. Boolootian (1966) contains a good summary of what is known of the holothurian gut and digestive physiology. Various investigators (Oomen, 1926; Sawano, 1928; Tanaka, 1958; Choe, 1962; Yokoe and Yasumasu, 1964; and Fish, 1967) have studied the nature and activity of digestive enzymes found in several holothuroids. These authors used extracts of gut tissues with various substrates at different pHs to determine what enzyme types were present and their pH optima. They generally agree on the presence in the gut of proteases, esterases, lipases, and various carbohydrases (there is some disagreement on the presence of cellulase, but this may result from the use of different species and methods). Digestion is most favourable in an alkaline medium, the constriction and small intestine being most active in the digestive process. The

enzymes are presumed to be secreted by cells bordering the gut lumen, but these cells remain to be identified. Absorption and nutrient transport is assumed to be more than a matter of simple diffusion, probably being accomplished by amoebocytes moving in a well-developed haemal system, and aided by the perivisceral coelomic fluid as a transport vehicle to all parts of the body (Kawamoto, 1927; Tanaka, 1958). Recent work (D'Agostino and Farmanfarmaian, 1960; Rundles and Farmanfarmaian, 1964) on the absorption and transport of D-glucose in the intestine of *Thyone briareus* indicates that there may exist an active transport system within the gut wall itself. The identity of tissues and organs serving as nutrient stores in holothuroids remains an unsolved problem (Anderson, 1966). Since, however, evisceration is a well-known phenomenon of holothurians, the body wall rather than the gut itself seems a more probable site for nutrient storage.

Echinoidea

The Echinoidea are a more thoroughly investigated group than the preceding three classes. Descriptions of the histological features of the gut in several species have been provided by Holland and Nimitz (1964) and Lewis (1964) who have described the various cell types of the echinoid gut. The stomach lining possesses cells with refractile granules above the nucleus and a small vacuole. These cells are commonly regarded as the source of digestive enzymes (Holland and Nimitz, 1964). In the basal regions of these cells are found the glycogen and lipid stores. Fuji (1961) noted alkaline phosphatase activity at the free border of the stomach epithelium.

The digestive process, at least in the regular urchins, has been reasonably well studied. Though the urchin's normal food is predominantly algal material, Lasker and Giese (1954) noted that *Strongylocentrotus purpuratus* in aquaria will ingest nearly anything. *S. purpuratus* feeds continuously when food is available, food remaining in the gut as long as two weeks (Giese *et al.*, 1958). Feeding efficiency is very high in the urchins; e.g., *S. purpuratus* feeding on *Macrocystis* averages about 80% digestive efficiency (Lasker and Boolootian, 1960; Boolootian and Lasker, 1964).

There have been several studies in which digestive tract extracts have been tested for enzymatic activity. Lasker and Giese (1954), using extracts of the entire gut of *S. purpuratus* at pH 6.8 - 7.0, found enzymes capable of digesting casein, boiled starch, and iridophycin (a carbohydrate product of the red alga, *Iridophycus*). Because they found no activity against intact algal pieces, they postulated that bacteria and other microorganisms might be significant in the breakdown of dietary components for which enzymes are lacking. Eppley and Lasker (1959) found, however, that gut extracts from *S. purpuratus* could hydrolyze or depolymerize agar and alginic acid. Farmanfarmanian and Phillips (1962) considered that bacterial action was not necessarily involved in normal digestion in urchins, because pieces of alga soaked in antibiotics are digested normally and large amounts of algal material are rapidly passed through the gut without bacterial enrichment but with no decrease in digestive efficiency. Several workers (Van der Heyde, 1922; Giese, 1961; Lewis, 1964) have reported a lipase

in sea urchins. Farmanfarmaian and Phillips (1962) (testing extracts of esophagus, stomach, and intestine from *S. purpuratus* for activity against soaked agar and pieces of *Iridophycus*) found that stomach and esophagus extracts affected both substrates, while intestinal extracts had no effect on either. This localization of digestive activity corresponds to the localization of secretory cells. They also showed that, in starved *S. purpuratus* fed ^{14}C -labelled disks of *Iridophycus*, most of the labelled substances are removed in the esophagus and stomach.

Lewis (1964), testing extracts of the entire gut of *Diadema antillarum* against starch, gelatin, and olive oil, found indications of a carbohydrase, a protease, and a weak lipase. To localize the production site of carbohydrase, he tested isolated regions of the gut (esophagus, caecum, foregut, hindgut) individually against starch over a pH range. The esophagus, foregut, and hindgut gave negative results while caecal carbohydrase activity occurred at pH 6 and 7 only. The caecal carbohydrase activity acted upon starch, glycogen, sucrose, maltose, dextrin, cellobiose, agar, and algae, but had no effect on lactose, galactose, or cellulose. Therefore, the caecal carbohydrases from *Diadema* attack α -glucoside linkages and also have the capacity to disrupt β -glucoside units.

Little is known of the digestive processes in irregular urchins but, like *Diadema*, the caecum seems to be implicated - not the stomach as in other regular urchins. Yokoe and Yasumasu (1964) were unable to detect cellulase activity in the irregular urchins (*Brissus latecarinatus*,

Clypeaster japonicus, and *Echinarachinius mirabilis*), but they detected cellulase activity in some regular urchins (*Anthocidaris crassispina*, *Pseudocentrotus depressus*, and *Hemicentrotus pulcherrimus*).

The fate of digestive products and their transport to utilization sites have been studied from various aspects, with the vehicle of transport being much debated. To demonstrate the supposed significant role that granular coelomocytes play in the process of uptake and distribution, Stott (1955) and Fuji (1961) administered to urchins inert particulate material in association with nutritive substances. Boolootian and Lasker (1964) claimed to demonstrate glucose histochemically in amoebocytes in the stomach wall of *S. purpuratus*. Because of this finding and the presence of these cells in the haemal connections between the gut wall and the gonads, they concluded that amoebocytes play an important role in transport. However, amoebocytes normally ingest particles as part of their phagocytic activities. Also, the haemal system in the stomach region shows local circulation patterns; therefore the transfer of fluids through this system may assist passage of absorbed nutrients from the gut to the coelomic fluid (Burton, 1964). There is evidence that the perivisceral coelomic fluid is a significant vehicle for distribution of soluble digestion products (Lasker and Giese, 1954; Boolootian and Giese, 1959; Bennett and Giese, 1955; Giordano, Harper, and Filice, 1950). Studies utilizing ¹⁴C-labelled food have added knowledge as to the organic constituents of the coelomic fluid of *Strongylocentrotus purpuratus*, its relationship to the nutritional state of the urchin,

and the role of the fluid in nutrient transport (Farmanfarmaian and Phillips, 1962). Most of the ^{14}C activity is removed in the esophagous and stomach and rapidly reappears in the coelomic fluid, peaking a few hours after feeding. Boolootian and Lasker (1964) performed a similar experiment on the same urchin and detected activity in the coelomic fluid soon after feeding (peak 4 - 6 hours). Mannitol (a sugar-alcohol which is the chief storage product of the brown algae) carries most of the radioactivity. As the activity of the coelomic fluid decreases, the number of amoebocytes in the coelomic fluid and their level of radioactivity increase, reflecting their importance in the transport and storage of nutrients.

Coelomocytes (especially spherule-coelomocytes) have also been linked to digestion and absorption. Péquignat (1966) believes nutrients are absorbed by 'skin digestion' from the external medium by epidermal cells. In regular and irregular urchins this supposedly occurs in cells of the podia and spines aided by spherule-coelomocytes. However, Chia and Fontaine (1968) in an autoradiographic study of the assimilation of dissolved organic molecules in *Cucumaria elongata* and *Amphipholis squamata* question the reality of 'skin digestion'.

The site(s) of storage of nutritional reserves is unknown. Echinoids lack any specialized storage organs, but the gonads may fulfill this purpose (Giese *et al.*, 1958; Lasker and Giese, 1954; Moore, 1937). However, the echinoid gut wall may also be a primary storage organ for nutritional reserves in sea urchins (Fuji, 1961; Giese, 1961; Boolootian and Lasker, 1964; Farmanfarmaian and Phillips,

1962). Several researchers (Greenfield *et al.*, 1958; Laurence *et al.*, 1965; Laurence, 1970; Doezema and Phillips, 1970) have tried to relate the morphology and size of the echinoid gut and gonads to an annual biochemical cycle of their constituents (lipid, glycogen, and other carbohydrates).

Recent work utilizing the techniques of histochemistry and electrophoresis for the demonstration, localization, and characterization of enzymes brings the knowledge of echinoid digestive physiology to its present state. Lyons and Weaver (1962) studied the localization of alkaline phosphatase in adult and larval tissues of *S. purpuratus*. The large intestine and esophagus possess alkaline phosphatase only in coelomocytes, *i.e.*, no epithelial enzyme was demonstrated. The small intestine contains the enzyme both in the coelomocytes and the epithelium. The larvae show alkaline phosphatase activity in the gut tissue soon after gastrulation; in all other tissues the activity is confined to tissue coelomocytes. Lindsay (1970, and personal communication) studied the tissue specificity, localization, genetics, and subunit composition of alkaline phosphatase isozymes in *S. purpuratus* and *S. franciscanus*. He separated and analyzed three isozymes (fast, intermediate, and slow, reflecting their migration pattern) by starch gel electrophoresis. The slow isozyme was localized in the epithelial microvillus border in the proximal portion of the small gut, while the other two forms were localized in the spherule-amoebocytes. The isozyme pattern was complicated by seasonal variation. Lindsay postulates that the isozyme patterns are the product of two non-allelic genes.

Asteroidea

Knowledge of the digestive morphology and physiology of asteroids is mainly based on the important series of publications by J. M. Anderson dealing with the gut of *Asterias forbesi* (Anderson, 1952, 1953, 1954, 1959, 1960, 1962, 1966, and 1967). Other influential studies are those of Mauzey (1966, 1967) on *Pisaster ochraceus* and Chia (1968, 1969) on *Leptasterias hexactis*.

The majority of starfish are carnivores (Feder, 1959; Stephenson and Stephenson, 1961; Wells *et al.*, 1961) but there are a few, like *Henricia leviuscula*, which are particle feeders (Booolootian, 1966). Water temperature and season of the year apparently influence the feeding rate (Mackenzie, 1969). The lining of the digestive tract has various cell types; but those secretory cells termed zymogen cells are believed to be the site of enzyme production. The zymogen cells in most starfish are concentrated in the pouches of the pyloric caeca; however, *Henricia* possesses small numbers in the cardiac stomach and in the epithelium of Tiedemann's pouches. In typical macrophagous sea stars, food is digested as it lies within, or enfolded by, the cardiac stomach which can be everted to surround the food (Smith, 1961; Mauzey *et al.*, 1968; Kim, 1969; Feder, 1955; Hancock, 1958). The powerful digestive enzymes, although acting on food enfolded by the cardiac stomach, are evidently produced not in the cardiac stomach lining but in the glandular pockets of the pyloric caeca. The enzymes are brought to their site of activity by flagellary currents circulating the digestive fluids. Removal of

pyloric caeca renders the starfish unable to digest normally (Anderson, 1960).

Numerous investigators, beginning in the late 19th century, have attempted to demonstrate the kinds of enzymatic activity present in starfish and the conditions under which digestion takes place. Most attempts involved testing gut extracts with various substrates. Frédéricq (1878) found an enzyme capable of digesting fibrin in the pyloric caeca of *Asterias rubens* and reported greatest activity under alkaline conditions. He also found an amylase in caecal extracts but no activity in the fluid contents of the digestive tract or in stomach wall extracts. Chapeaux (1893) found protease, amylase, and lipase activity in pyloric caecal extracts, but he was able, unlike Frédéricq, to demonstrate protease and amylase in cardiac stomach extracts. He considered that some intracellular digestion must occur in the pyloric caeca, since oil droplets and carmine particles were found in the epithelium after feeding experiments. In *Asterias vulgaris*, Stone (1897) found a trypsin-like protease acting in an alkaline medium, a diastase, and a lipase. She concluded that the pyloric caeca were "important digestive glands, very similar in function to the pancreas of higher animals". Van der Heyde (1922) found proteolytic activity in both stomach and pyloric caeca of *Asterias forbesi*, but no amylase or lipase. Though the results have been questioned, his measurements of pH seem reliable; he found pH in the cardiac stomach of *Asterias forbesi* ranging between 7.1 - 7.7, and in the pyloric caeca about 7.3. Similar measurements (Irving, 1926)

on *Patiria miniata* gave figures of 7.3 to 7.5 for the stomach and 6.6 to 7.0 for the pyloric caeca. Apparently, the digestive enzymes in the sea star gut have pH optima below the normal pH of sea water but not in the strongly acid range.

Sawano (1936) improved our knowledge of asteroid proteolytic enzymes by his investigations on *Distolasterias nipon*. He tested extracts of the digestive organs for activity against protein, peptone, polypeptide, and dipeptide and established pH optima. Pyloric caecal extracts were active against all substrates with maximum activity in the pH range from 7.5 - 8.0; the tryptic-like enzyme performance being enhanced by cystine and inhibited by cysteine. He concluded that secretion of the gland cells in the pyloric caeca contains a complete proteolytic system comparable to that of the vertebrate pancreas, with no enzyme acting in the acid range, except one identified as a cathepsin with a pH optimum at 4.8. Remarkable hydrolysis of glycylglycine was observed with extracts of the cardiac stomach. Because of the lack of demonstratable zymogen cells in the cardiac stomach, the enzymatic activity attributed to it by Chapeaux (1893) and Sawano (1936) may have originated in the pyloric caeca and may not have been washed off the stomach epithelium completely.

The more recent work has added some sophistication to our understanding of asteroid digestive biology. Yokoe and Yasumasu (1964), in their survey of the occurrence of cellulase in invertebrates, were unable to detect this enzyme in any of the asteroids tested (*Coscinasterias acutispina*, *Asterias amurensis*, and *Asterias pectinifera*).

In contrast, however, Araki and Giese (1970) found cellulase activity, using carboxymethylcellulose as a substrate, in the pyloric caeca of eight species (*Patiria miniata*, *Pisaster ochraceus*, *P. giganteus*, *P. brevispinus*, *Pycnopodia helianthoides*, *Poraniopsis inflata*, *Luidia foliolata*, and *Dermasterias imbricata*). The cardiac stomachs and coelomic fluids showed little or no cellulase activity. Araki and Giese (1970) also demonstrated laminarinase, lichenase, cellobiase, amylase, and glycogenase activity in all, except *L. foliolata*. Maltase occurred in the pyloric caeca of *Patiria miniata*, *Pisaster ochraceus*, *P. giganteus*, *P. inflata*, and *Luidia foliolata*. Fucoidinase and carraginanase were either absent or slight in these species.

Camacho *et al.* (1970) isolated and purified trypsin-like proteases from the pyloric caeca of *Dermasterias imbricata*. Two different proteins with tryptic-like activity were detected by polyacrylamide electrophoresis and gel filtration. The enzymes apparently exist as inactive precursors in the pyloric caeca, with spontaneous activation taking place at 20°C. Jangoux and Van Impe (1971), in a comparative study of alkaline phosphomonoesterase activities in the gut of several asteroids, found alkaline phosphatase in all the digestive organs, the pyloric caeca showing strong activity. Microphagous sea stars show little enzyme activity in Tiedemann's pouches, but the pyloric complex had a high activity level. Das *et al.* (1972) studied the distribution, specificity, and function of proteases, lipases, and esterases in extracts from the pyloric caeca and skin of several species of starfish by starch gel electrophoresis coupled with

specific staining. Most protease activity was in the caeca; little was found in skin extracts. They concluded that proteases are first formed as inactive zymogens, becoming active after extraction. Distinct trypsin and chymotrypsin-like activities were demonstrated in tissue extracts as well as esterase activity.

Research is being carried out at present in Japan on the isolation and purification of proteolytic enzymes of the pyloric caeca utilizing gel filtration (Sephadex column) technique. It has been possible to identify certain enzymes including lactic dehydrogenases and alkaline phosphatase (T. Sasaki, personal communication).

Kozlovakayo and Vaskovsky (1970), in their comprehensive study of proteinases in marine invertebrates, suggest that proteolytic activity is connected more with systematic position than other factors; the Asteroidea (and also the Crustacea and certain species of Annelida and Mollusca) possess the highest proteolytic activity. Bouillon and Jangoux (1970) carried out an histochemical and histological study on the rectal caeca of *Asterias rubens*. Histochemical examination revealed aminopeptidase granules in the internal epithelium as well as alkaline phosphatase (apical region) and acid phosphatase (basal region) activity. The external epithelium also showed acid phosphatase activity.

Bargmann and Behrens (1968) have performed a light and electro-microscopic study of the pyloric caeca of *Asterias rubens*, including its innervation.

In addition to producing digestive enzymes, the pyloric caeca serve also as the chief organs of absorption and storage (Anderson, 1966). Some glycogen, but relatively much larger amounts of lipid, can be demonstrated histochemically in the caeca. These reserves become depleted over several weeks of starvation in *Asterias forbesi* (Anderson, 1953), or during brooding in *Leptasterias hexactis* (Chia, 1969). Various researchers (Karnovsky *et al.*, 1955; Greenfield *et al.*, 1958; and Rodegker and Nevenzel, 1964) have determined the total lipid and glycogen content in the pyloric caeca of asteroids; approximately 30% of the dry weight of the pyloric caeca in *Pisaster ochraceus* is lipid while glycogen accounts for only 1 - 2% of the dry weight. The importance of the caeca in absorption and storage has been demonstrated by Ferguson (1963, 1964 b) using ^{14}C -labelled nutrients. Allen and Giese (1966) demonstrated the activities of the pyloric caeca in synthesizing nutritional reserves. The pyloric caeca of *Pisaster ochraceus* can incorporate ^{14}C -l-acetate into lipids and ^{14}C -glucose into polysaccharides. Tissues from fed animals carried on lipid synthesis at a faster rate than those from starved animals. Lipogenesis shows seasonal variation; synthetic rate is highest in fall when net deposition of caecal reserves is at its peak.

The problem of how these stored nutrients are mobilized and transported to sites of utilization is especially important in preparation for the breeding season as the gonads increase at the expense of caecal reserves (Farmanfarmaian *et al.*, 1958; and Greenfield *et al.*, 1958). The fluid of the perivisceral coelom seems the most

likely vehicle for nutrient transport, since it bathes almost all the tissues and is kept in vigorous directed circulation by its flagellated lining (Irving, 1924; Budington, 1942). The fluid, however, contains little organic material. Giordano *et al.* (1950) detected five amino acids in the coelomic fluid of *Pisaster brevispinus*; glycine constituting 67.4% of the total.

Ferguson's recent studies (1963, 1964 a, b, 1968) on *Asterias forbesi* provide new insight into the probable mechanism of nutrient transport in asteroids. He demonstrated that the glucose concentration of the coelomic fluid and the total nitrogen of the cell-free coelomic fluid fluctuate, these fluctuations being unrelated to feeding or fasting. The glucose concentration of the coelomic fluid is much less than that found in holothurians and echinoids. Using labelled nutrients Ferguson (1963, 1964 a, b) showed that most radioactivity lodges immediately in the pyloric caeca, but low levels of activity are detectable in the coelomic fluid and coelomocytes soon after feeding and persist for long periods. He also demonstrated that the coelomic fluid does carry nutrients in solution; ¹⁴C-labelled glucose, glycine, or algal protein hydrolysate, injected directly into the fluid of one ray were rapidly distributed throughout the body and taken up by various tissues, especially the pyloric caeca and coelomocytes. Isolated, cultured organs (pyloric caeca, gonads, rectal caeca, and portions of cardiac stomach) can actively absorb labelled glucose and amino acids from dilute solutions in the suspension medium. Because glycine is absorbed very slowly, he concluded that the high

concentration found in the coelomic fluid by Giordano *et al.* (1950) might result because other amino acids competitively inhibit its absorption. There is a rapid turnover of nutrients through the body fluids, soluble nutrients being constantly reabsorbed by the caeca themselves as well as by the other tissues bathed by the fluid. The rate of uptake by the tissue is governed by its metabolic demand. The caeca always contain a vast surplus of stored reserves continually being replenished by absorption from the gut contents. The net movement of material is out of the caeca, into the fluid; the rapid uptake of the other tissues prevents any considerable concentration of organic material in the coelomic fluid. Thus, the asteroid can utilize as its circulatory system a fluid practically identical with sea water, containing such low concentrations of organic compounds that problems of osmotic balance with the external medium are minimized.

Nourishment of the superficial cell layers of the asteroid is probably by the absorption of sugars and amino acids from extremely dilute solutions in the sea water (Stephens and Schinske, 1961; Chaet and Philpott, 1964).

Chapter II

MATERIALS AND METHODS

A. Collection and Handling of Specimens

Starfish of the species *Pisaster ochraceus* (Brandt) were collected at irregular intervals from Saanich Inlet at the Bamberton region near Mill Bay, B. C. The animals were maintained in re-circulating salt water tanks at the University of Victoria, where the water temperature is maintained between 10°C - 15°C. The sea stars were fed fresh mussels (*Mytilus edulis*, *Mytilus californianus*), barnacles (*Balanus cariosus*, *Balanus glandula*), and a few oysters (*Ostrea gigas*) collected from the same area of Saanich Inlet. Mussels seem to be the preferred prey, *M. edulis* more than its congener.

I. Starvation Regime

Freshly collected specimens of *Pisaster* were maintained in 70 gallon re-circulating tanks without food of any kind for definite time periods. The specimens were allowed to acclimatize for 2 days in the tanks with food before the food was removed and the food deprivation commenced. The *Pisaster* were sampled at various time intervals - 2 days, 4 days, 1 week, 2 weeks, 3 weeks, 4 weeks, 6 weeks, 8 weeks, and 10 weeks - to ascertain the effect(s) of starvation on the tissue of the pyloric caeca. Even after 10 weeks of starvation, all were alive and apparently normal, though not very active, remaining attached to the same area of the tank wall (near water inflow) and rarely moving

about. As required, a specimen would be removed from the tank, dissected and the pyloric caeca tissue subjected to the methods described below. Sampling at each time interval was repeated at least 4 times, and the results were similar in each case.

II. Feeding Regime

Freshly collected specimens of *Pisaster* were maintained as described above, but now they were supplied with ample food. When a specimen was noted to be actively feeding, it was removed to another tank with no food. The eversion of the cardiac stomach over the prey was taken as indicating active feeding; usually the valves of the *Mytilus* prey are gaping and bits of *Mytilus* tissue are enfolded by the walls of the cardiac stomach. At definite time intervals after feeding - 10 minutes, 15 minutes, 30 minutes, 60 minutes, 120 minutes, 6 hours, 18 hours, 24 hours, 3 days, 5 days, 1 week, and 10 days - the specimen was removed from the tank, dissected, and the tissue of the pyloric caeca subjected to the methods outlined below. Sampling at each time interval was repeated at least 4 times, with similar results.

B. Handling of Tissues

I. Frozen Sections

Starfish were sometimes relaxed in $MgCl_2$ (7.5% solution diluted 1:1 with sea water), but as the specimens did not move during dissection this was deemed unnecessary. One arm of a starfish was completely excised and the pyloric caeca dissected out. From this, small pieces of the tissue were frozen. Different methods of freezing were tried:

- 1) "Cryoquik";
- 2) "O.C.T." (a commercial embedding medium) embedding, followed by rapid freezing on the quick freeze block in the cryostat;
- 3) quenching of the tissue in liquid nitrogen.

The liquid nitrogen was found to be the best method and therefore was used for all the experimental work recorded here. It freezes the tissue rapidly with apparently little or no introduction of structural artifact to the tissue.

A small piece (several mm^2) of tissue is mounted on a circular piece of cork which is itself resting in a small amount of water on a metal mounting button. The entire setup is then lowered on a holder into a vessel of liquid nitrogen for several seconds until the tissue is completely frozen. It is then rapidly transferred to a cryostat (International, Model CTD) set between -28° to -30°C and allowed to equilibrate before sectioning at $10 - 12\mu$ with a microtome knife precooled in the cryostat. This cryostat temperature was found to be best after quenching with liquid nitrogen; warmer temperatures either resulted in soft tissue that was extremely difficult to section or in sections that rolled up or fragmented badly. At the beginning the sections were mounted on cover slips coated with glycerine jelly to aid adherence, but this was found to be unnecessary and was subsequently discontinued. The cover slips with the adhering sections were then fixed for approximately 20 minutes at room temperature by placing them in a microstaining jar in the bottom of which was placed a pledget of cotton wool saturated with neutral buffered formalin.

Exposure to formaldehyde gas was the only means of fixation used on the frozen sections. Completely unfixed sections tend to get very soft, and to become detached from the cover slips. After fixation, the sections were processed through the methods listed below.

II. Paraffin Sections

As samples of pyloric caeca tissue were removed for frozen sections, tissues from the same caeca were also taken for paraffin sections. These tissues were immediately fixed in Susa, Romeis', or Bouin's fixatives. Susa fixative preserved cellular detail best and was the fixative most often used. After dehydration and clearing, the tissue was embedded in Paraplast, and sections were cut at 5 - 15 microns. The stains used for histological observation were:

- a) Periodic acid-Schiff (PAS) reaction, with or without salivary amylase controls (Humason, 1967) - demonstrates glycogen and other polysaccharide compounds;
- b) Pantin's Mallory triple stain (Humason, 1967) - demonstrates muscle and connective tissues, cell boundaries, and zymogen (secretory) granules;
- c) Mallory's phosphotungstic acid hematoxylin (PTAH) stain (Culling, 1963) - demonstrates muscular and connective tissue fibres, cell membranes, flagella and their basal bodies, and some cytoplasmic granules, notably storage and zymogen granules;
- d) Alcian Blue (Humason, 1967) - demonstrates acid mucopolysaccharides, *e.g.*, mucous goblet cells;

e) Sudan Black B (Pearse, 1968) - demonstrates masked lipid deposits and paraffin resistant lipids. Sudan Black B staining was also carried out on frozen sections; the method is described further on.

Some paraffin sections were also subjected to a test for acid phosphatase, a modification of Ruyter's (1964) method (Pearse, 1968), however, this did not show localization to be any different from tests using frozen tissue sections and was therefore discontinued after several trials.

Paraffin sections were used in conjunction with frozen sections to observe the effect of feeding and starvation on the characteristic cellular components of the digestive epithelium of *P. ochraceus*.

C. Enzyme Histochemistry

I. General Comments on the Technique

Though it is possible that nearly 900 enzymes exist, there are histochemical methods for less than 80 of these (Pearse, 1968). The four enzymes (acid and alkaline phosphatase, esterase, and leucine aminopeptidase) studied in this research were chosen because of the relative abundance of research already performed on them in a variety of species and tissues.

The main concern in enzyme histochemistry is to preserve the maximum amount of enzyme activity while at the same time maintaining the *in vivo* localization of the enzyme. These two principles are often in conflict, since preservation of localization by fixation results in a reduction of enzyme activity (this is especially true in

the case of the soluble, diffusible, lyo-enzymes where fixation of some sort is absolutely necessary as opposed to the bound or desmo-enzymes). And yet, paradoxically, the preservation of maximum enzyme activity with minimal tissue or cell damage may lead to less accurate enzyme localization. A compromise must therefore exist between these two principles and this leads to some of the restrictions inherent in histoenzymological technique. Another important factor to take into account when assessing the validity of histochemical localization is that diffusion of enzyme occurs to different degrees in different fixatives and it varies for each enzyme. Of course, localization of lyo-enzymes is more affected by diffusion than in desmo-enzymes. False-positive reactions may be confused with diffusion artifacts. An unsuspected affinity of the split product of the substrate or of one of the reagents used for visualization may result in staining that is unrelated to enzyme activity. This type of artifact seldom occurs in techniques employing short incubation times, and control sections are employed to distinguish between false and positive stains.

To assure valid localization and to maintain maximal enzyme activity, it is advisable (if possible) to adhere to the following criteria:

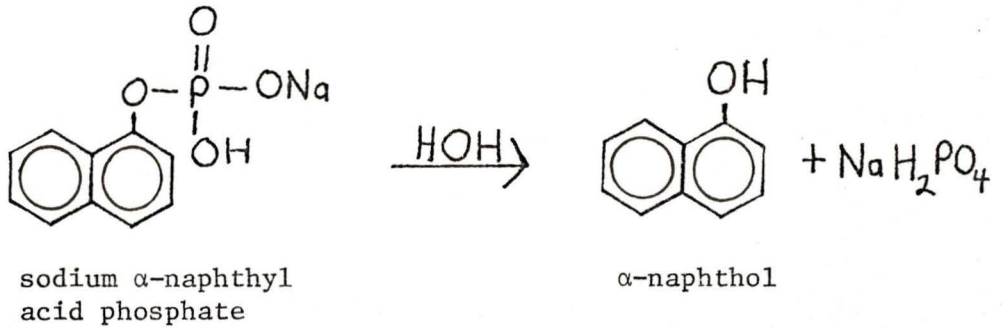
- a) the substrate should be water-soluble and of low molecular weight to ensure rapid diffusion into the tissue and sufficient concentration of substrate for the enzyme reaction;
- b) the substrate should be easily hydrolyzed at the pH and temperature necessary for optimum enzyme activity;
- c) the substrate should possess a lack of polar groups;

- d) the primary (PRP) and final (FRP) reaction products should be insoluble in water and lipid; and,
- e) the PRP, in a capture reaction, must react rapidly with the chosen capture reagent. (In the case of the coupling azo dye methods, this is a diazonium salt.)

In azo dye methods, the alcoholic part of the substrate is coupled with a diazonium salt to form a coloured dye. The resulting dye contains two aromatic nuclei (one from the substrate and one from the diazonium salt which are linked through the azo group (-N=N-). In the simultaneous capture (coupling) reaction the diazonium salt present in the incubating medium combines with the coupler (may be either a naphthol or naphthylamine) as soon as it is released from the substrate by enzymatic hydrolysis. Hydrolysis and coupling take place "simultaneously". In these reactions the main obstruction to accurate localization of the enzyme is diffusion of the PRP. For this reason, the diazonium salt should couple rapidly with the PRP. Electronegative groups in the diazonium ion increase the rate of coupling while the same groups, if present in the coupling component, decrease the rate. Electropositive groups in the coupling component increase the velocity of coupling but, if present in the diazonium component, they cause a decreased coupling velocity (Barka and Anderson, 1963).

As an example of the chemistry involved in a simultaneous coupling azo dye method, the following is the reaction for the demonstration of alkaline phosphatase using sodium α -naphthyl acid phosphate as the substrate and Fast Blue RR as the diazonium salt (Fig. 1):

a) enzymatic hydrolysis of the substrate.



b) simultaneous coupling of naphthol radical (PRP) and diazonium salt to yield FRP (azo dye).

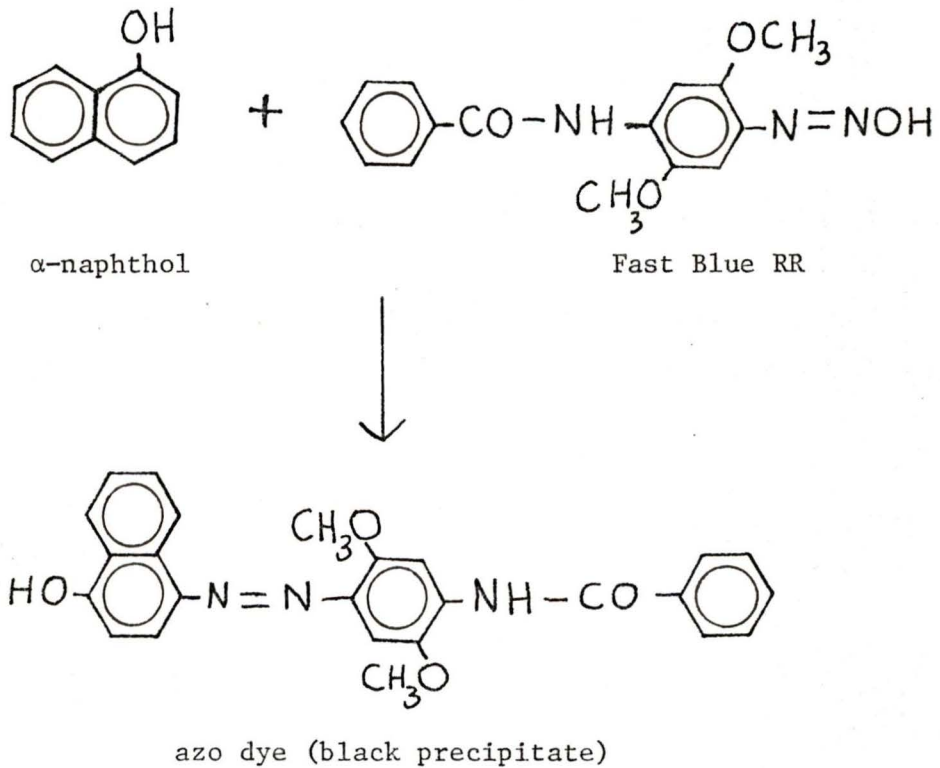


Fig. 1. Reaction series representing the simultaneous azo dye method for alkaline phosphatase.

II. Enzyme Test Methods

All the methods, unless otherwise stated, are taken from Barka and Anderson (1963) and are modifications by them of earlier procedures. The exact reference will be listed with each method. The number that appears after the enzyme is allocated to that particular enzyme by the I.U.B. Commission on Enzymes in 1961. All enzyme substrates and diazonium salts were obtained from the Sigma Chemical Company (St. Louis, Missouri, U.S.A.).

1. The Phosphatases

a) Alkaline Phosphatase (3.1.3.1)

Simultaneous coupling azo dye method using α -naphthyl phosphate (Barka and Anderson, 1963; p. 231-234).

(i) The sections were incubated at room temperature for approximately 45 minutes. This time was found to be optimal for this tissue and test after several trial runs at different times; however, the reaction product begins to appear quite rapidly after incubation is begun (usually within 5 minutes). The incubating medium was prepared as follows: sodium α -naphthyl acid phosphate is dissolved in 0.05 to 0.1M TRIS buffer, pH 9.2, at a concentration of 1 - 2 mg/ml. After the substrate is dissolved, 1 mg/ml of the diazonium salt Fast Blue RR (C.I. 37155) is added. The freshly prepared incubation medium is filtered and poured on cover slips with sections placed in a micro-staining jar.

(ii) Sections washed 2 - 3 minutes in distilled water.

(iii) Then washed 1 - 2 minutes in 1% acetic acid.

(iv) Rinsed in distilled water.

(v) Sections attached to cover slips are mounted on slides in glycerine jelly (Barka and Anderson, 1963; p. 416).

The reaction product that marks the localization of alkaline phosphatase in this method is black in colour.

b) Acid Phosphatase (3.1.3.2)

Simultaneous coupling azo dye method using α -naphthyl phosphate (Barka and Anderson, 1963; p. 242-244).

(i) The sections were incubated at room temperature for approximately 45 minutes (this time was found to be optimal for this tissue and test after several trial runs at various time intervals). The incubating medium was prepared as follows: sodium α -naphthyl acid phosphate is dissolved in 0.1M acetate buffer at pH 5.0 in a concentration of 1 mg/ml. The stable diazonium salt, Garnet GBC (C.I. 37210), is then added in a 1 mg/ml concentration. After mixing, the solution is filtered onto the formalin vapour-fixed sections in microstaining jars.

(ii) The sections washed in running water.

(iii) The sections are mounted on slides in glycerine jelly.

The site of enzyme activity is marked by a reddish brown azo dye deposit, which invariably forms crystalline aggregates. The deposition of the dye usually begins to appear 15 minutes after the incubation period is begun.

The more recent simultaneous coupling azo dye method using substituted naphthols was also employed to test for the localization of acid phosphatase. In this method the substrate naphthol AS-TR phosphate couples with hexazonium pararosanilin leaving the sites of enzyme activity marked by a red azo dye precipitate. Though the method

gives excellent localization with no diffusion or other artifacts, it was found to be no more advantageous than the method listed above using α -naphthyl phosphate, is more tedious and time consuming, and was therefore only used on several occasions to verify the localization obtained with the previous method. As this procedure was not utilized for the majority of the work, a full description of the method will not be given (see Barka and Anderson, 1963; p. 244-246 for complete description of the technique).

2. The Esterases (E.C. 3.1.1.1, 3.1.1.2, 3.1.1.6, 3.1.1.7, 3.1.1.8)

α -naphthyl acetate method (Barka and Anderson, 1963; p. 261-264).

(i) The sections were incubated at room temperature for 5 minutes (esterase activity is markedly high in this tissue). The timing was determined after several test runs to be optimal for this tissue and method. The incubating medium was prepared as follows: to 20 - 25 ml of 0.1 M phosphate buffer (pH 7.5), 0.5 ml of a 1% α -naphthyl acetate solution in acetone and 1 mg/ml Fast Blue B are added (C.I. 37235). After shaking, the solution is filtered onto the formalin vapour-fixed sections and incubation commenced.

(ii) Rinse in water.

(iii) Mount sections in glycerine jelly.

The localization sites of the enzyme are marked by purple-black deposits.

3. Leucine Aminopeptidase (3.4.1.1)

L-leucyl-4-methoxy- β -naphthyl amide method (Barka and Anderson, 1963; p. 286-290).

(i) The sections mounted on cover slips after fixation in formalin vapours are incubated at 37°C for 45 minutes (incubation period was determined to be optimal for this tissue and method after several trial runs at various time intervals). The temperature of 37°C is recommended for the demonstration of aminopeptidase. The substrate stock solution contains L-leucyl-4-methoxy-β-naphthyl amide hydrochloride, 4 mg/ml. To 1 ml of the substrate stock solution is added 5 ml 0.1M acetate (or phosphate) buffer (pH 6.5), 3.5 ml saline (0.85% sodium chloride), and 5 mg Fast Blue B (C.I. 37235).

(ii) Wash sections for 1 minute in saline.

(iii) Place sections for 2 - 5 minutes in 0.5M copper sulphate solution.

(iv) Rinse in saline.

(v) Wash in water.

(vi) Mount in glycerine jelly.

The deposit of red azo dye indicated the site of enzyme activity. Chelation with cupric ions causes a shift of colour from red to purple when β-naphthyl amine and Fast Blue B are used.

D. Lipids: Sudan Black Staining of Frozen Sections

Frozen sections were also subjected to testing with Sudan Black B to ascertain the position and extent of lipid deposits in the digestive epithelium under the varying nutritional conditions. The method (Pantin, 1964; p. 27, 46) is as follows:

(i) Wash frozen sections in water.

(ii) Stain in Sudan Black B (saturated solution, 0.3 - 0.5 gm in 100 ml of 70% alcohol), up to 8 minutes (usually 5).

(iii) Rinse in 70% alcohol.

(iv) Rinse in 50% alcohol, and then water.

(v) Stain in carmalum (usually 3 minutes).

(vi) Rinse in water to remove excess stain.

(vii) Mount in Farrant's medium (Culling, 1963; p. 168).

The lipid globules stain black (purple-black) with this stain.

For each enzyme test carried out, there was also a concurrent control test carried out simultaneously. Control tests consisted of sections incubated in the medium without the presence of the substrate, or sections incubated in a medium which has been boiled for about five (5) minutes prior to the incubation period to denature the enzyme proteins. The effect(s) of inhibitors or promoters of enzyme activity on these enzymes was not ascertained.

Chapter III

THE PYLORIC CAECA: A REVIEW OF THEIR MORPHOLOGY, HISTOLOGY, AND FUNCTION(S).

The digestive tract of asteroids is straight and short, extending from the oral to the aboral side of the disc. The mouth, situated in the center of the peristomial membrane, is provided with a sphincter muscle. The mouth leads into a short, wide esophagus. The esophagus passes into the stomach, a broad sac that fills the interior of the disc, and is often divided by a horizontal constriction into a voluminous oral part - the cardiac stomach - with folded out-pouched walls, and a much smaller, flattened aboral part - the pyloric stomach - to which the ten glandular appendages known as the pyloric caeca (digestive glands, brachial caeca, hepatic caeca, and other synonyms) are attached (Hyman, 1955).

The pyloric caeca of *P. ochraceus* are morphologically typical of five-armed, forcipulate starfish. In each arm are a pair of glandular diverticula, each suspended from the aboral wall by two longitudinal mesenteries. A median duct travels along each caecum and, after uniting with its fellow duct of the same arm, enters the pyloric stomach. A single caecum consists of a longitudinal series of lobules along both sides of the duct. These lobules are supplied with lateral branches of the duct. From the pyloric stomach the short intestine runs to the anus. The intestine possesses diverticula or caeca, called rectal or intestinal caeca.

Histologically, the digestive tract is composed of three layers: an outer mesothelium or peritoneum of simple ciliated cuboidal epithelium; a middle layer of connective, muscular, and nervous tissue; and an inner digestive epithelium. In the pyloric caeca the lining epithelium composes most of the thickness of the wall (average height is 280 microns) and is flagellated.

Mauzey (1966) studying feeding behaviour and reproductive cycles in *P. ochraceus*, made a cursory examination of the histology of the caeca. More detailed studies have been carried out by Anderson (1953) for *Asterias forbesi*, Chia (1969) for *Leptasterias hexactis*, and Chan and Fontaine (1971) for *P. ochraceus*. The first three researchers concur in being able to distinguish four distinct, identifiable cell types for which they use the following standard nomenclature:

- a) special current producers,
- b) mucous cells;
- c) storage cells; and
- d) zymogen (secretory) cells.

The *special current producers* have long flagella that function in maintaining distributive fluid currents in the pyloric caeca. Current producers are characteristic of the pyloric duct and its lateral branches, lining primarily the roof and floor of these ducts. The current producers are liberally interspersed with *mucous cells* that also occur less abundantly everywhere throughout the caecum. The main tissue of the caecum, apart from the current-producing areas, is composed of granular (secretory or zymogen) and storage cells, with some

mucous cells. *Storage cells* are very abundant in the caecal epithelium. They have flagella shorter than those of the current producers and their cytoplasm is full of nutritive stores. Droplets of lipid substances are situated at the basal ends of the cells. They also contain some glycogen. The rest of the cytoplasm is filled, except distally, with numerous storage granules which are most likely a carbohydrate-protein complex (Chia, 1969). The free ends of the storage cells contain numerous fine pigment granules which impart the characteristic greenish-yellow colour to the pyloric caeca. The *zymogen (secretory) cells* occur together with the storage cells but are less abundant than the latter. They can be identified by the presence of coarse PTAH-positive secretory granules and clear vacuoles. These granules are believed to be enzymatic in nature.

The more recent description by Chan and Fontaine (1971) diverges from that of the earlier researchers in that six, not four, distinctive cell types were distinguished. Therefore, their terminology differs somewhat. Chan and Fontaine (1971) differentiate between the mucous cells of the duct epithelium and those of the diverticular epithelium. They found that, except for the height, the *duct mucous cells* are morphologically identical with the *diverticular mucous cells*. Their secretions, however, were found to be histochemically distinct. The duct mucous cell secretes a substance tentatively identified as an epithelial sialomucin (PAS- and Alcian Blue (AB)-positive and strong gamma-metachromasia with toluidine blue). The substance secreted by the diverticular mucous cell, however, appears to be a highly sulphated

acid mucopolysaccharide, similar to mast cell heparin. Its characteristic features are PAS-negative and AB-positive reactions and an intense gamma-metachromasia with toluidine blue (Chan and Fontaine, 1971). They thus discerned two histochemically distinct mucous cell types; this difference probably went unnoticed by Anderson (1953), Mauzey (1966), and Chia (1969) because they failed to utilize the same histochemical reactions. Chan and Fontaine's (1971) description of the *special current producers* and the *storage cells* concurs with that given by the previous authors. However, where Anderson (1953) uses the terms zymogen cell and secretory cell synonymously to describe cells secreting coarse, PTAH positive granules associated with clear vacuoles, Chan and Fontaine (1971) use the two terms to describe two distinct and different cell types in the diverticular epithelium. The *zymogen cells* of Chan and Fontaine are concentrated in the rounded, acinus-like termini of the diverticular lobules. They are slender cells filled with coarse (2 micron) PTAH positive granules. They also give a positive DMAB-nitrite reaction, indicating a high concentration of tryptophan. This perhaps indicates that the granules are precursors of digestive enzymes rich in tryptophan. Anderson (1953), and Chia (1969) reported vacuoles associated with the zymogen granules of their starfish species. Chan and Fontaine (1971) did not find vacuoles in the zymogen cells of *Pisaster ochraceus*, but found them in what they called secretory cells. These secretory cells were also abundant in the acinus-like termini of the diverticular lobules. Their contents are distinctly granular in appearance. These granules are negative to PTAH and DMAB-nitrite

methods, but rich in -SH and -S-S- groups. The cells are tapered at both ends, swollen in the middle, and usually have a large vacuole in the lower third of the cell. Chan and Fontaine believe this may be an enzyme secreting cell with a product chemically distinct from the zymogen cell. Contrary to what Chan and Fontaine have found however, I have observed cells in all regions of the epithelium which have PTAH positive granules and large vacuoles associated with them (Fig. 28). I consider that the presence or absence of a vacuole and its size may be a reflection of the nutritional state of the animal. The nutritional state could also affect the outcome of an histochemical reaction (*i.e.*, whether or not the PTAH and DMAB-nitrite reactions are negative or positive), however, there is no evidence to support this suggestion. Enzyme production and localization may be controlled by the nutritional condition of the animal. Therefore, I will use the terms zymogen cell and secretory cell synonymously as Anderson does, unless otherwise stated, to denote a cell with coarse, PTAH positive granules which are usually associated with a vacuole.

Anderson (1953) has assigned to the pyloric caeca four specific functions: (a) digestion (probably the zymogen cells synthesize enzymes); (b) absorption; (c) storage; and (d) transfer of nutrients to the coelomic fluid and hence its distribution to the body in general.

The evidence, while incomplete, indicates that digestion occurs in both stomach and pyloric caeca (the pyloric caeca being the probable site of enzyme synthesis) and that food particles are swept into the latter and there undergo extracellular or intracellular digestion, or

both (Hyman, 1955). Digestion products may be passed through the caeca into the coelomic fluid or into the haemal system of the pyloric caeca, which communicates with the main haemal system by way of the gastric haemal tufts, or may be stored in the epithelium of the caeca (Hyman, 1955). According to Anderson (1953), much of this epithelium is devoted to food storage and contains glycogen, neutral fat, and a polysaccharide-protein complex (storage granules that are PTAH and PAS positive) resistant to diastatic digestion. The food stores of the pyloric caeca are utilized during gonad maturation in *Pisaster ochraceus* (Greenfield *et al.*, 1958) or brooding in *Leptasterias hexactis* (Chia, 1969). The amoeboid, phagocytic coelomocytes that are abundant in the haemal system appear to constitute a principal mechanism for the elimination of unwanted material in the asteroids; possibly the pyloric caeca are also excretory in addition to their other functions (Hyman, 1955).

Chapter IV

OBSERVATIONS AND RESULTS

Throughout this chapter an effort has been made to assess the level of enzyme activity for the four enzymes studied. It should be noted that this assessment of the level of activity is entirely based on subjective criteria and is not verified by quantitative analyses. Judgment of activity is therefore, because of its subjective nature, open to error.

A. Starvation Regime

Constant reference to Table 1 will prove helpful in this section. Table I lists the localization sites of the four enzymes in the pyloric caeca at progressive intervals without food and notes the comparative levels of enzyme activity for acid and alkaline phosphatase.

As was noted in the introduction, the zymogen cells in the digestive epithelium are the presumed sites of enzyme synthesis. If this hypothesis is valid, then it follows that during starvation, when enzymes are not 'needed' for digestion, that the granules (and their vacuoles) should decrease in number, perhaps even disappearing altogether. Lipid deposits and the nutritive stores of the epithelium should likewise be affected.

If an animal is starved then the following alternative possibilities exist:

1. The zymogen granules could decrease in number and perhaps disappear because (a) there is no food to be digested, or (b) the granules may only be produced on demand.

TABLE I

STARVATION REGIME: Localization of enzymes in the pyloric caeca of *Pisaster ochraceus* at progressive periods of food deprivation.

Time Without Food	LOCALIZATION SITES			
	Aminopeptidase	Esterase	Acid Phosphatase	Alkaline Phosphatase
2 day	marginal	diffuse	mesothelial marginal *	mesothelial (+++) marginal (+)
4 day	marginal	diffuse	*mesothelial *	mesothelial (+++) marginal (+)
1 week	marginal	diffuse	mesothelial *	mesothelial (+++) marginal (+)
2 weeks	marginal	diffuse	mesothelial (++) *marginal (+++)	mesothelial (+++) marginal (+++)
3 weeks	marginal	diffuse	mesothelial (+++) *marginal (+)	mesothelial (++++) marginal (++)
1 month	marginal	diffuse	mesothelial? *marginal	mesothelial (+) marginal (+++)
6 weeks	marginal	diffuse	mesothelial (+) *marginal (+++)	mesothelial (+++) marginal (+)
8 weeks	marginal	diffuse	mesothelial (+++) *marginal (+)	mesothelial (+++) marginal (++)
10 weeks	marginal	diffuse	mesothelial (+++) *marginal (+)	mesothelial (++++) marginal (+)

Legend: * = granular staining reaction throughout the cytoplasm.
 ++++ = very strong positive reaction.
 +++ = strong positive reaction.
 ++ = moderate positive reaction.
 + = weak positive reaction.

2. The zymogen granules may decrease somewhat in number, but always remain present, *i.e.*, there is a quantitative increase when the asteroid is actively feeding, though some will be present at any time.
3. The zymogen granules do not decrease in numbers; they are always present in relatively the same amount (even during the reproductive season or when food is scarce).

Examination of the paraffin sections should indicate which condition occurs in *Pisaster*. In this section the results of the tests using both frozen and paraffin sections will be discussed in order to verify which one of the possibilities explained above actually occurs in *Pisaster*.

I. Frozen Sections

a) Acid Phosphatase (AcP)

In the simultaneous coupling azo dye method using α -naphthyl acid phosphate, the site of enzyme activity is marked by a reddish-brown azo dye deposit. Localization of the sites of AcP activity with this method is good though there is slight diffusion of the azo dye. The level of activity of AcP remained fairly constant for the entire 10 weeks of starvation. The reddish-brown azo dye which marks the site of AcP activity was found mainly outlining the mesothelial region of the digestive epithelium (Figs. 2, 3), though occasionally the enzyme is also localized along the marginal edges of the ducts in the caeca (Figs. 2, 3). In most of the sections, there was also a distinct granular staining reaction throughout the cytoplasm of the digestive epithelium, mainly in the submarginal region but this did not hinder

Figures 2 - 3

Figure 2. Cryostat section (12 μ) from a *Pisaster* starved for 3 weeks showing acid phosphatase activity in the mesothelial region of the caecal (some lesser activity along the marginal edge of the duct). Note the granular staining throughout the cytoplasm, especially in the region of the nuclear zone and above.

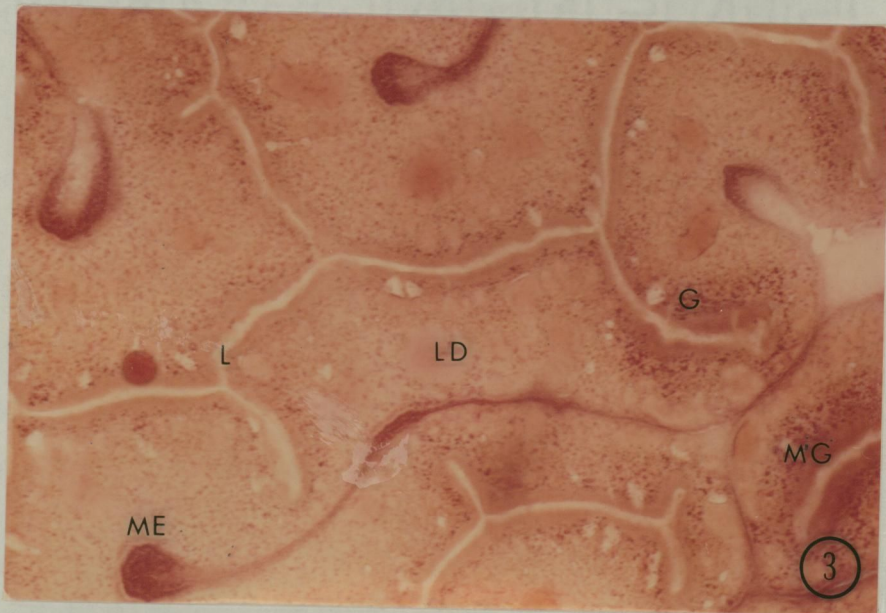
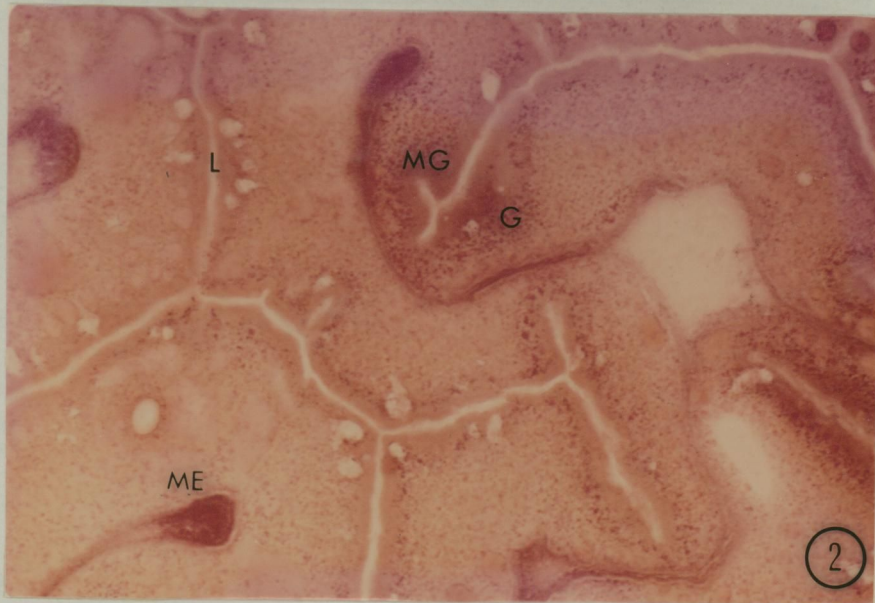
Fresh frozen section; α -naphthyl phosphate; pH 5.0;
Garnet GBC. x 100.

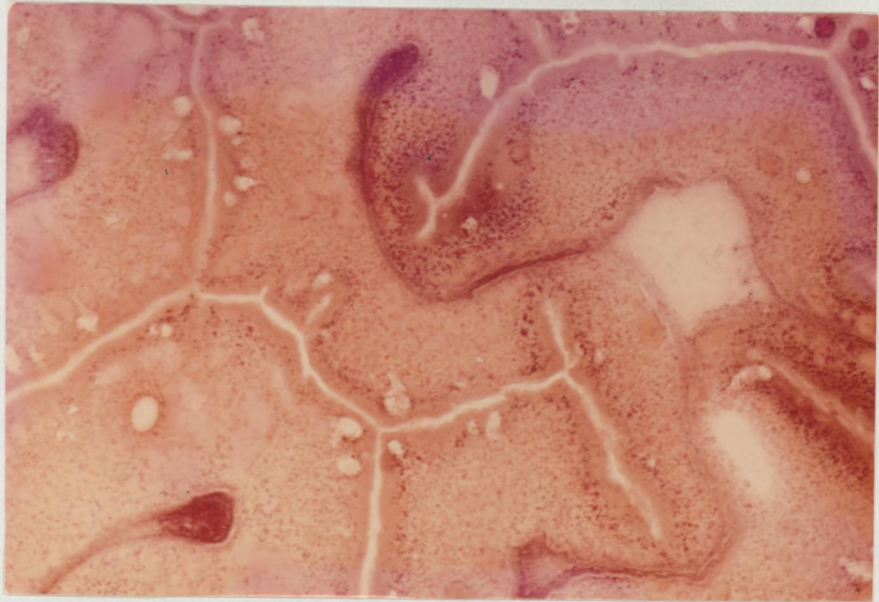
L - lumen of the duct
ME - mesothelial activity
MG - marginal activity
G - granular staining

Figure 3. Cryostat section (12 μ) similar to above but after 8 weeks of starvation. It also shows mesothelial acid phosphatase activity and lesser marginal activity. Note pinkish staining lipid deposits.

Fresh frozen section; α -naphthyl phosphate; pH 5.0;
Garnet GBC. x 100.

L - lumen
ME - mesothelial activity
MG - marginal activity
G - granular staining
LD - lipid deposits





the identification of the other sites of enzyme activity. The length of the starvation period has little effect on either the localization or the level of activity of AcP.

b) Alkaline Phosphatase (ALP)

In the simultaneous coupling azo dye method using α -naphthyl phosphate, the site of enzyme activity is marked by a black azo dye deposit. Localization of ALP with this method is excellent; there are no problems with diffusion of the dye. The level of activity of ALP, unlike AcP, varies. It is the only enzyme of those investigated which does vary its activity level. This variation apparently reflects the function(s) of the enzyme and is probably influenced by the metabolic and nutritive state of the starfish. This phenomenon will be dealt with more thoroughly in the Discussion section following. The black azo dye deposits have their greatest activity level localized mainly along the mesothelial regions of the caecal epithelium although, as with AcP, there is also some localization of the enzyme along the marginal edge of the duct (Fig. 4). The level of activity at the two sites of localization changes (see Fig. 4 where mesothelial activity is greater than marginal activity). The localizations also seem to reflect its function(s); this will also be discussed more fully in the next section. The length of starvation does not appear greatly to affect localization of the enzyme; but, it may have some bearing on the level of activity (Table I).

Figures 4 - 5

Figure 4. Cryostat section (12μ) from *Pisaster* starved for 3 weeks showing alkaline phosphatase activity sites. The activity is mainly mesothelial but there is also some activity along the marginal edge of the duct.

Fresh frozen section; α -naphthyl phosphate; pH 9.2;
Fast Blue RR. x 100.

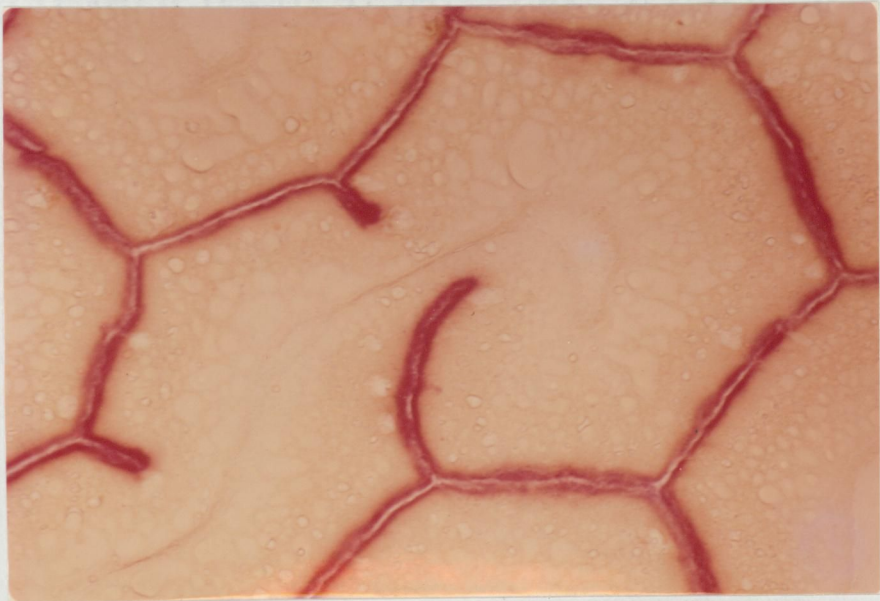
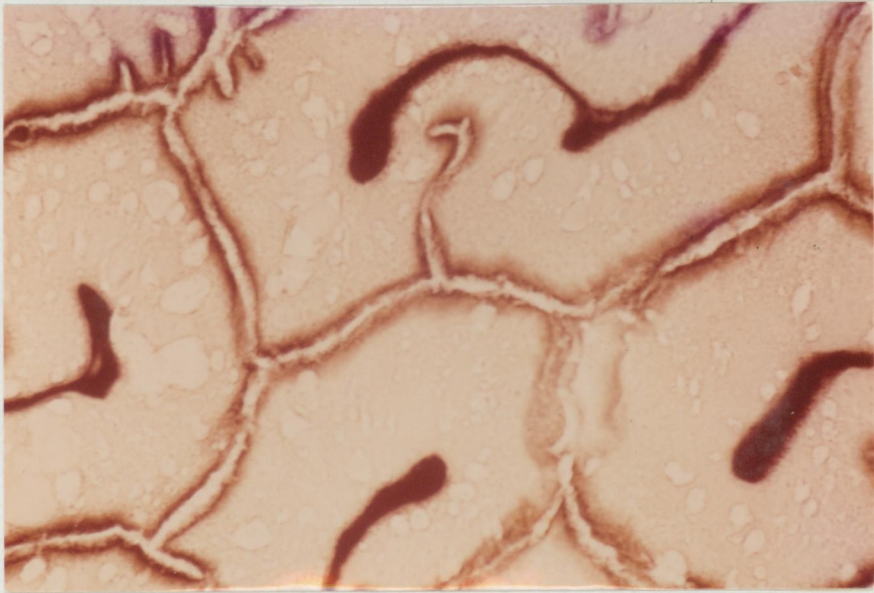
L - lumen
ME - mesothelial localization of enzyme activity
MG - marginal activity site

Figure 5. Cryostat section (12μ) from a *Pisaster* starved for 4 weeks showing leucine aminopeptidase activity. Activity is always localized along the marginal edge.

Fresh frozen section; L-leucyl-4-methoxy- β -naphthyl amide
HCl; pH 6.5; Fast Blue B. x 100.

L - lumen
M - mesothelial region





c) Leucine Aminopeptidase (LAP)

The L-leucyl-4-methoxy- β -naphthyl amide method for the detection of LAP is an excellent method showing very marked diffusion hindrance to the final reaction product (FRP), a deposit of bright red azo dye indicating the site of enzyme activity. The FRP is microcrystalline and insoluble in nature. LAP is, unlike ALP, very stable both in level of activity and in localization sites. Throughout the entire 10 weeks of the starvation the level of activity did not appear to change. The bright red azo dye deposits were always localized along the marginal edges of the pyloric caeca ducts (Fig. 5). Therefore, it seems that starvation, even for a prolonged period of time, does not affect the localization, nor the level of activity of LAP.

d) Esterases (Es)

Most methods employed to test for esterase are misleading to some extent and difficult to interpret because they tend to be so non-specific (Pearse, 1972). For instance, the α -naphthyl acetate method for esterase employed in this study reacts on non-specific esterases, lipases, and both types of cholinesterase. It is therefore not intended to be specific but is used as a tool for routine survey of esterase activity. Using the azo dye Fast Blue B, the sites of enzyme activity are purple-black. During the entire 10 weeks, the level of activity of the esterases did not appear to change appreciably. The localization, due to the non-specificity of the method, was not as clearcut as it was for the other enzymes studied. Es activity was always diffusely spread throughout the cytoplasm of the epithelium

(Fig. 6), being more concentrated in the region of the nuclear zone and perhaps to some extent in the upper area of lipid deposits. The length of starvation, as with LAP, did not seem greatly to affect either the localization nor the activity level of the esterases.

e) Lipid Deposits

When Sudan Black B is used to stain frozen sections for lipid deposits, these stain a dark red to purplish-black colour. The lipid stores of the pyloric caeca are in the form of large purplish globules which are located in the basal regions of the epithelium below the level of the band of nuclei (Fig. 7). At no time, even after 10 weeks of starvation, were these lipid stores depleted. In fact, the stores seemed to remain almost as abundant at the end of the starvation period as at the beginning. It should be noted that this experiment was carried out during the time of the year when *Pisaster* spawns (May - August). It is generally held that the stored nutrients of the pyloric caeca of asteroids are utilized by the gonads in the development of the gametes during the reproductive season (Farmanfarmaian *et al.*, 1958; Greenfield *et al.*, 1958). However, even though it was the spawning season for *P. ochraceus* and they were starved for up to 10 weeks, the lipid deposits showed no major signs of depletion. They appeared to have sufficient lipid stores in the caecal epithelium to carry on for some time under these experimental conditions.

Figures 6 - 7

Figure 6. Cryostat section (12 μ) showing esterase activity in a *Pisaster* starved for 3 weeks. Note the diffuse staining reaction.

Fresh frozen section; α -naphthyl acetate; pH 7.5; Fast Blue B. x 100.

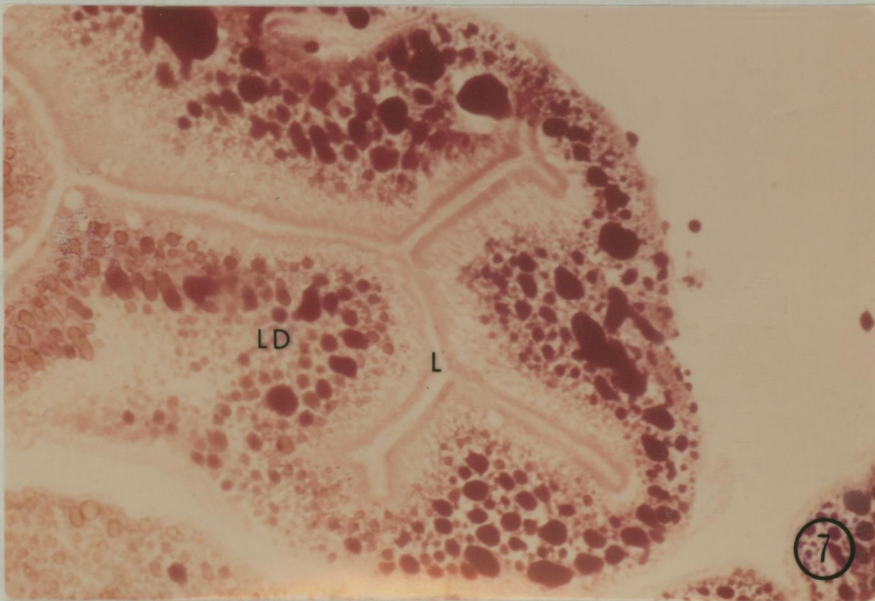
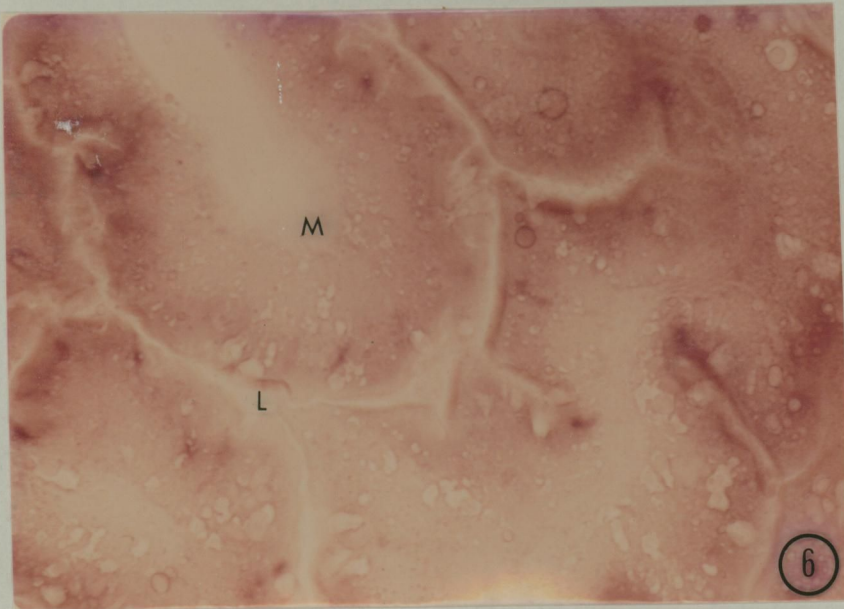
L - lumen

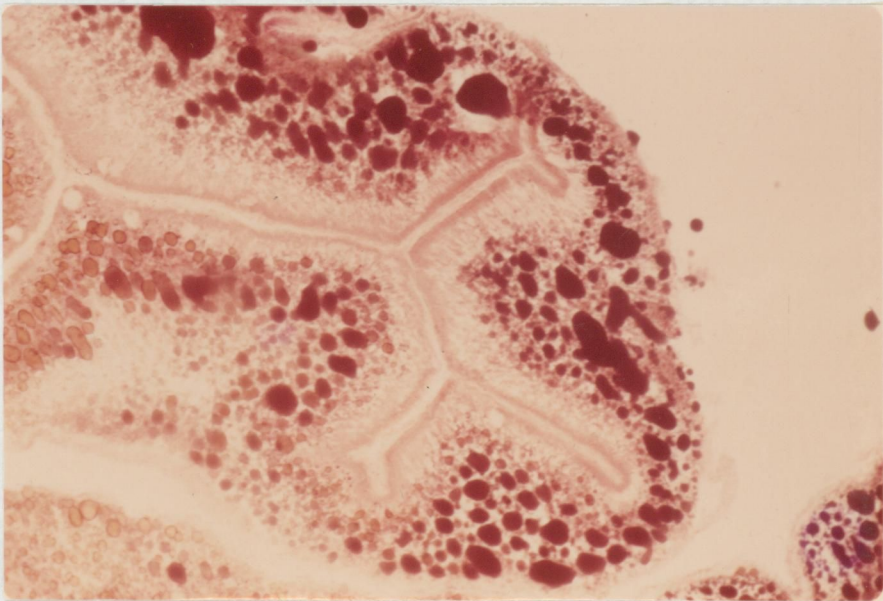
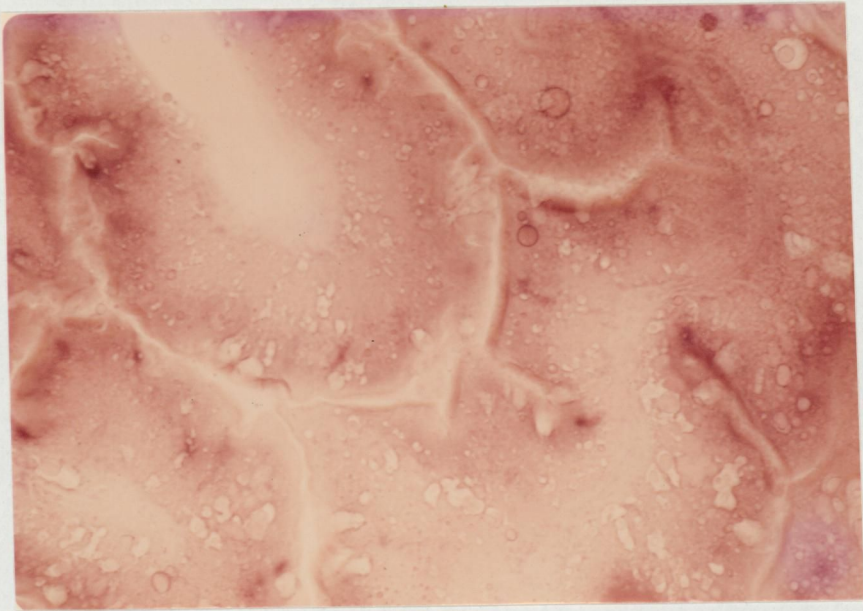
M - mesothelial region

Figure 7. Cryostat section (12 μ) stained with Sudan Black B (Mayer's Carmalum counterstain) from a *Pisaster* starved for 5 weeks. Note the abundant basally located lipid droplets. x 100.

L - lumen

LD - lipid droplets





II. Paraffin Sections

The zymogen cell is flask-shaped, its greatest diameter is at the level of the vacuole. It lacks the apical brush border of the storage cell. The zymogen cells are characterized by the presence of secretory granules and clear vacuoles (Fig. 11). The secretory products consist of long strings of zymogen granules passing from the lower region of the middle zone of the epithelium (below the nuclear zone) towards the marginal edge of the duct. They pass through the apical region of the cells where the pigment granules are found but never are they seen actually free in the lumen. The presumption is that they disintegrate upon emerging from the secretory cells and release enzymes into the fluid contents of the cavity. A vacuole, if present, is located beneath the string of granules, with the nucleus of the zymogen cell at the basal end immediately beneath the vacuole and granules. The nuclei are oval or round. Occasionally a few zymogen granules can be observed below the vacuole.

Zymogen cells with their accompanying granules and vacuoles are readily demonstratable using Mallory's phosphotungstic acid hematoxylin (PTAH) or Pantin's Mallory's triple stain. Zymogen granules were always present in the caecal epithelium throughout the entire 10 weeks of starvation (Figs. 8, 9, 10, 11). As will be seen in the following section, the number of granules is less during starvation than when the asteroid is actively feeding; nevertheless, granules are always present and do not disappear even after the animal is not feeding. This statement is true at least up to ten weeks of

Figures 8 - 9

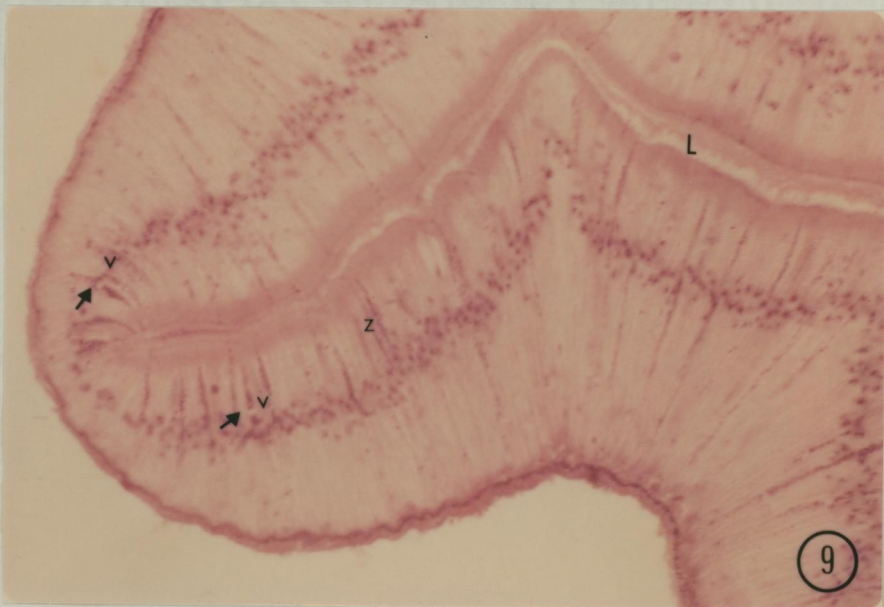
Figure 8. Paraffin section (frontal) of one of the glandular pockets in the pyloric caecum of a *Pisaster* starved for 2 days. Note the long strings of zymogen granules and the scarcity of zymogen vacuoles. The arrow indicates a terminal acinus.

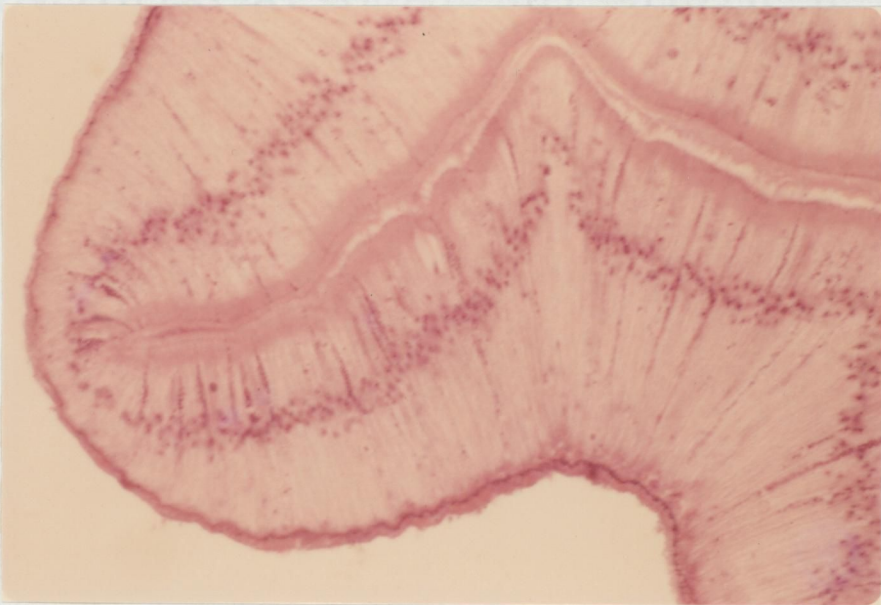
Susa fixative; paraffin embedding; 15 μ , PTAH staining.
x 100.

L - lumen
z - zymogen granules
nz - nuclear zone

Figure 9. Enlarged view of the terminal acini of the glandular pocket indicated in the above figure. x 250.

L - lumen
z - zymogen granules
v - zymogen vacuoles (indicated by arrows)





Figures 10 - 11

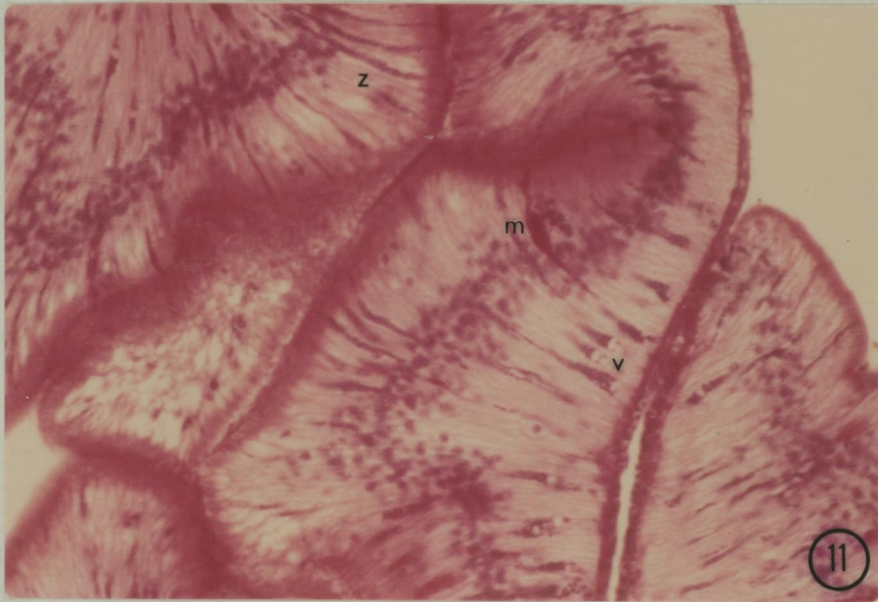
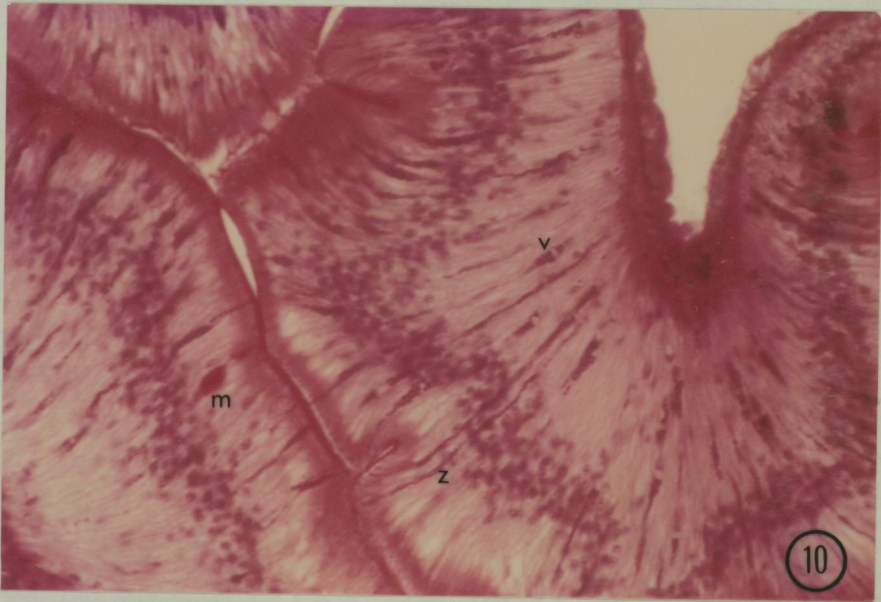
Figure 10. Enlarged view of a portion of a glandular pocket of the pyloric caecum from a *Pisaster* starved for 10 weeks. Note that zymogen granules and a few vacuoles are still present.

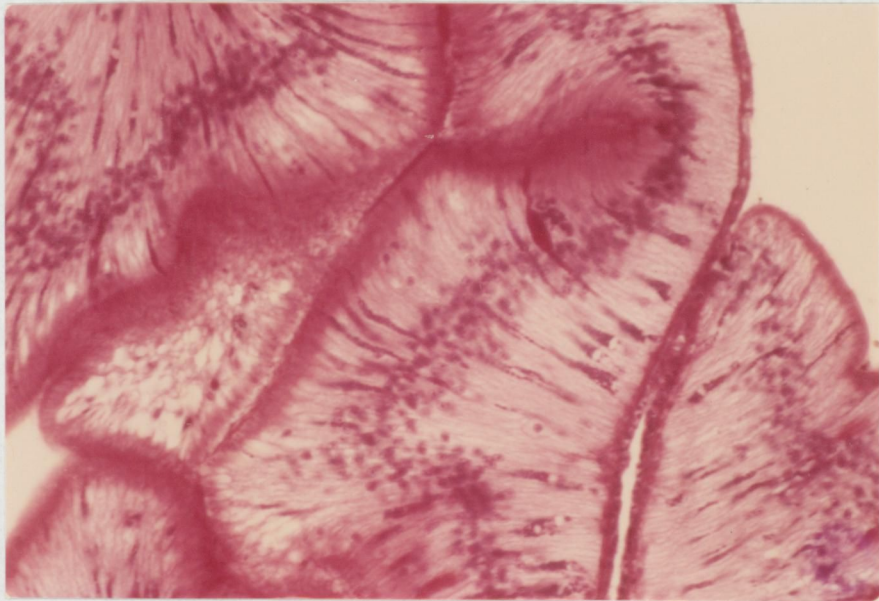
Susa fixative; paraffin embedding; 15 μ ; PTAH. x 250.

z - zymogen granules
v - zymogen vacuoles
m - mucous goblet cell (red)

Figure 11. The same comments as above apply to this figure also.
x 250.

z - zymogen granules
v - zymogen vacuoles
m - mucous goblet cell





starvation. The number of vacuoles is relatively scarce, however. The number of vacuoles present and their size will also be seen to be a function of the nutritional condition of the starfish; when the asteroid is able to feed the number and size of the vacuoles change. However, at no time during the ten weeks did the vacuoles disappear entirely from the digestive epithelium of the starfish.

The storage cells are more numerous in the digestive epithelium than the zymogen cells. The nuclei are elongated and oval in shape and are located in the middle zone of the cells (*i.e.*, area referred to as nuclear zone). The apical ends of the storage cells are provided with a distinct brush border of numerous microvilli, indicative of their role as absorptive cells. Each cell also bears a flagellum. The greenish pigment granules are present in this region. The ground cytoplasm of the storage cells reacts very strongly with PAS reagent except at the apical end where the pigment granules are located (Fig. 12). The PAS reaction is not affected by salivary digestion and is unaltered even after 10 weeks of starvation. Coarse storage granules are localized at the upper middle zone of the cells (Fig. 12). These granules react positively with PAS, PTAH, and Mallory's, negatively with Alcian Blue, and are unaffected by salivary digestion. This staining behaviour thus suggests that the storage granules are a carbohydrate and protein complex serving as a nutritional reserve. When the storage granules are very numerous they may obscure the nuclear zone. The starvation experiment ran from May through July which is during the spawning period for *Pisaster* (most

Figures 12 - 13

Figure 12. Paraffin section (15 μ) stained with PAS from an animal starved for 2 days. The cytoplasm of the storage cells is very PAS positive except along the marginal edge. Note the presence of storage granules in the storage cells.

Susa fixative. x 100.

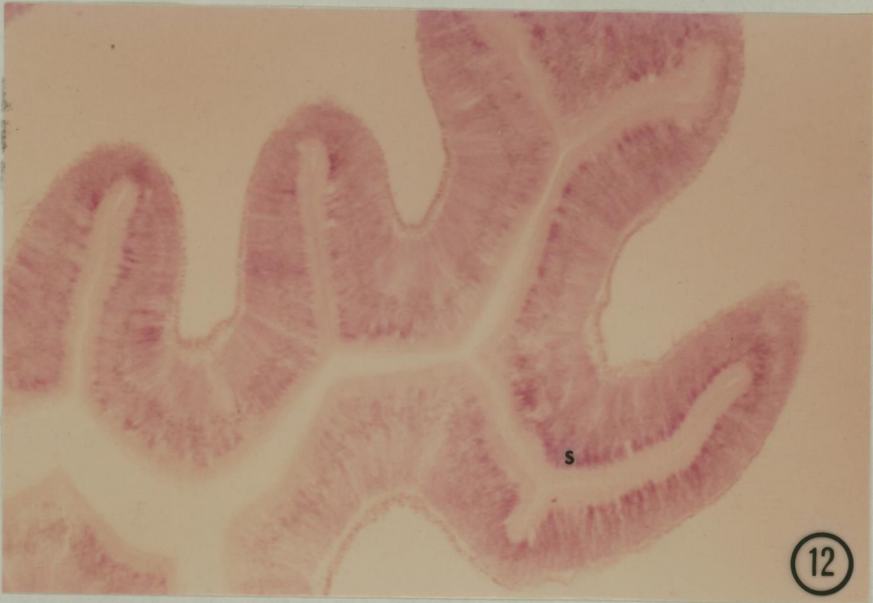
s - storage granules (red)

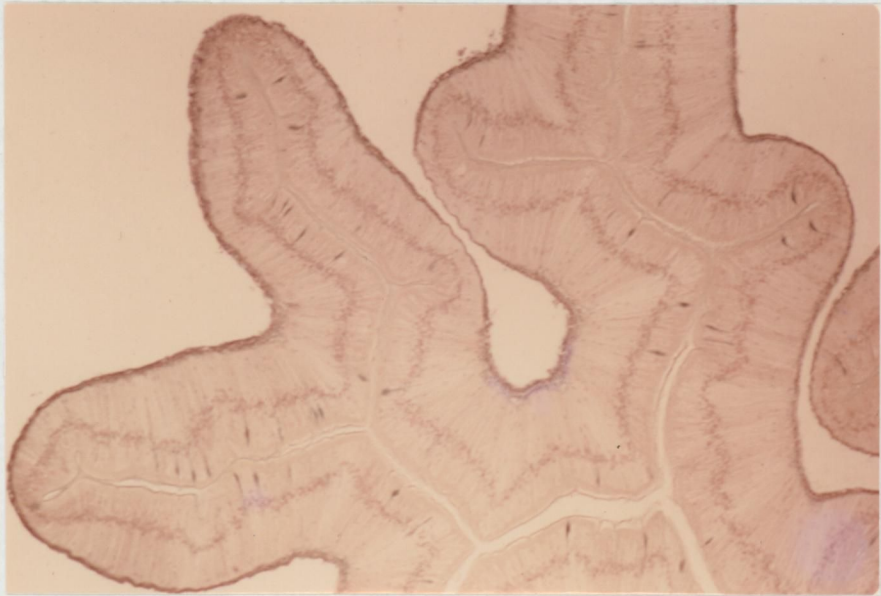
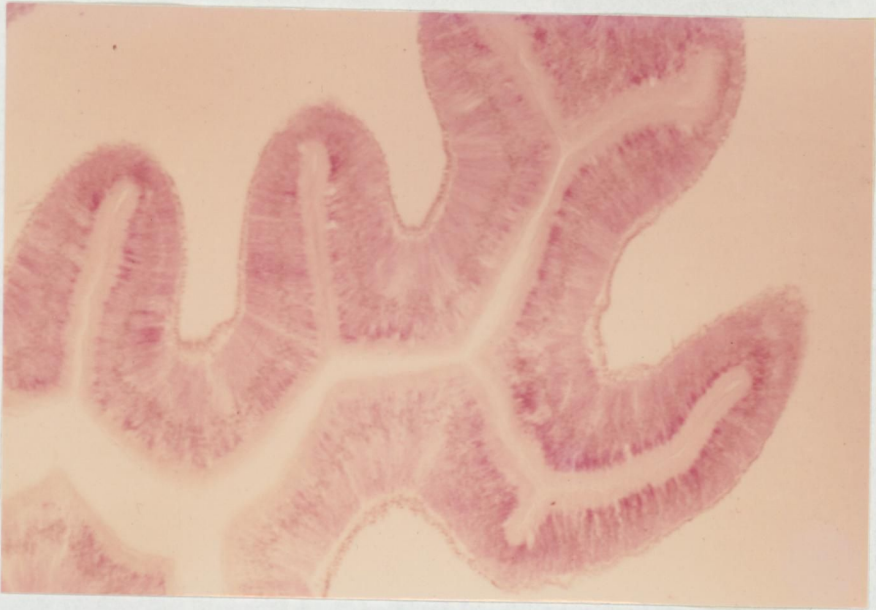
Figure 13. Paraffin section (15 μ) stained with Alcian Blue from an animal starved for 2 days. Note the bluish staining mucous goblet cells.

Susa fixative. x 100.

m - mucous goblet cell

c - connective tissue region (bluish)





of the asteroids were ripe and spawned during the study). Since it is established (Farmanfarmaian *et al.*, 1958; Greenfield *et al.*, 1958) that the nutritional reserves of the pyloric caeca are utilized in the gonads for the development of the gametes, this may account for the scarcity of storage granules found in this study. However, it should be noted that the storage granules were present in some of the samples. The sample taken after eight weeks showed storage granules, which would indicate that starvation, at least for this period of time, does not entirely deplete the nutritional reserves of the pyloric caeca. This agrees with Chia's work on *Leptasterias hexactis* (1969). He found that storage granules were abundant after 10 weeks of starvation. The storage cells seem to contain very little glycogen.

A few mucous goblet cells were dispersed among the other cells of the caeca (Fig. 13). The mucous cells are most numerous in the terminal acini of the caeca. The nuclei are round and basally located. Mucous cells lack both brush border and flagella as do the zymogen cells. The mucous goblet cells stain a vivid sky blue with Alcian Blue which is specific for acid mucopolysaccharides. Alcian Blue also stains the connective tissue region. The mucous cells are seen to be PAS positive sometimes. Sudan Black B was also found to stain the goblet cells at times. This, however, may be due to the fact that the dye decomposes in acid solutions (especially below pH 4), resulting in the staining of nucleoproteins and mucopolysaccharides (Barka and Anderson, 1963).

The four enzymes investigated in this study (alkaline and acid phosphatase, leucine aminopeptidase, and esterase) were found to be present throughout the entire 10 week period of starvation. Though the latter three did not alter significantly in level of activity nor in localization during the course of the experiment, this cannot be said of alkaline phosphatase. This observation will be dealt with in the following section on feeding and possible reasons for this phenomenon will be discussed later on in the thesis. The findings from frozen sections were corroborated by the paraffin sections. Zymogen granules, the products of the zymogen cells, were seen to be present for the entire period of starvation. The zymogen vacuoles, though scarce, were also present during the full 10 weeks. It was also observed that the nutritive stores of the pyloric caeca (the carbohydrate-protein complex of the storage granules and the lipid deposits) were not depleted by 10 weeks of starvation. Thus, it can be seen that in the starved animals there is a slight, but significant change in cellular inclusions in both storage and zymogen cells even after 10 weeks of starvation.

B. Feeding Regime

The tests on the effect(s) of feeding on the enzymes and cellular components of the pyloric caeca of *Pisaster ochraceus* were carried out from June 1970 until February 1971, making an almost complete year's sampling.

Throughout this section reference to Table II will prove useful. Table II gives the localization sites of the four enzymes at progressive time intervals after feeding and indicates the varying level of activity of alkaline phosphatase.

I. Frozen Sections

a) Acid Phosphatase (AcP)

During the entire course of the feeding study, the level of activity remained fairly constant. The red-brown azo dye which marks the site of AcP activity was found outlining the mesothelial region of the caecal tissue in all the samples taken (Figs. 14, 16). The distinct granular staining of the cytoplasm already mentioned in connection with samples from starved animals stained for AcP was also observed in these samples. This granular staining is especially concentrated in the region below the marginal area. Thus, the availability of food seems to have had little effect on the localization or level of activity of AcP in the pyloric caeca of *Pisaster*. This study also represents a monthly sampling of pyloric caeca tissue for almost a year. As the localization and level of activity did not appear to change significantly during the course of this study it seems that there is little seasonal variation in this enzyme within the pyloric caeca of *Pisaster* under what approximate 'normal' conditions.

TABLE II

FEEDING REGIME: Localization of enzymes in the pyloric caeca of *Pisaster ochraceus* at progressive intervals after feeding.

Time After Feeding	LOCALIZATION SITES			
	Aminopeptidase	Esterase	Acid Phosphatase	Alkaline Phosphatase
a) 10 min. (June)	marginal	diffuse	mesothelial *	mesothelial (+++) marginal (+)
b) 10 min. (Aug.)	marginal	diffuse	mesothelial *	mesothelial (+++) marginal (+)
15 min. (July)	marginal	diffuse	*mesothelial	marginal (++)
30 min. (July)	marginal	diffuse	*mesothelial	mesothelial (+) marginal (++)
1 hour (July)	marginal	diffuse	mesothelial	mesothelial (+) marginal (+++)
2 hour (Aug.)	marginal	diffuse	*mesothelial	mesothelial (+++) marginal (+++)
6 hour (Dec.)	marginal	diffuse	*mesothelial	mesothelial (+) marginal (+++)
18 hour (Oct.)	marginal	diffuse	mesothelial	mesothelial (+++) marginal (++)
24 hour (Nov.)	marginal	diffuse	*mesothelial	mesothelial (+++) marginal (+++)
3 day (Dec.)	marginal	diffuse	*mesothelial	mesothelial (+) marginal (+++)
5 day (Dec.)	marginal	diffuse	*mesothelial	mesothelial (+++) marginal (++)
1 week (Jan.)	marginal	diffuse	*mesothelial	mesothelial (+++) marginal (++)
10 day (Feb.)	marginal	diffuse	*mesothelial	mesothelial (+++) marginal (++)

Legend: * = granular staining reaction throughout the cytoplasm.
 +++ = strong positive reaction.
 ++ = moderate positive reaction.
 + = weak positive reaction.

Figures 14 - 15

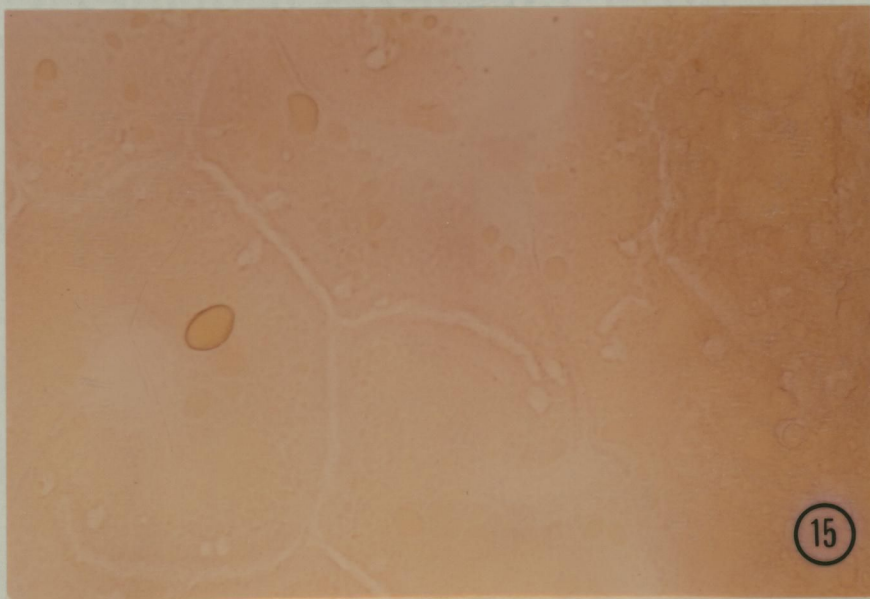
Figure 14. Cryostat section (12 μ) showing acid phosphatase activity. The tissue was taken from a *Pisaster* 1 hour after feeding. The activity is localized along the mesothelial region of the caecum. Distinct granular staining is also noticeable throughout the cytoplasm, especially in the region just below the marginal edge of the duct.

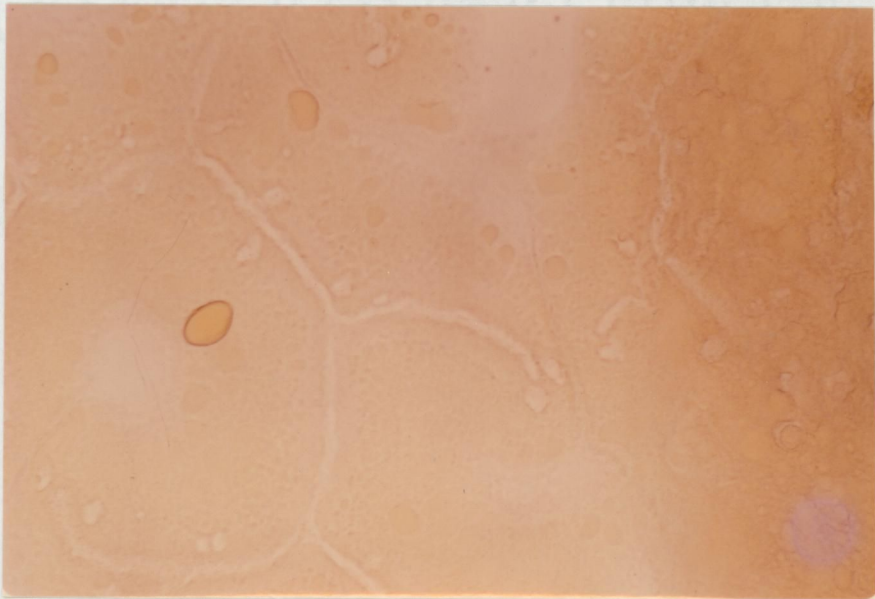
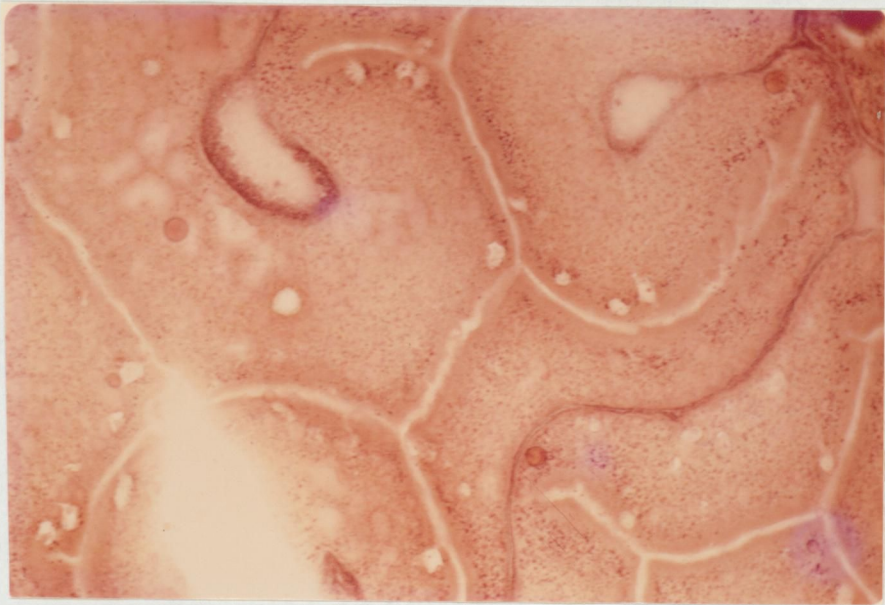
Fresh frozen section; α -naphthyl phosphate; pH 5.0;
Garnet GBC. x 100.

ME - mesothelial activity
G - granular staining of the cytoplasm

Figure 15. Cryostat section (12 μ) from caecal tissue taken 1 hour after feeding. The photomicrograph shows a typical control section for acid phosphatase. Control sections were either incubated without substrate or the incubation medium was boiled to denature the enzyme.

Fresh frozen section; no substrate; pH 5.0; Garnet GBC.
x 100.





Figures 16 - 17

Figure 16. Cryostat section (12 μ) showing acid phosphatase activity 1 week after feeding. The enzyme activity is still localized in the mesothelial region. Note also granular staining throughout cytoplasm.

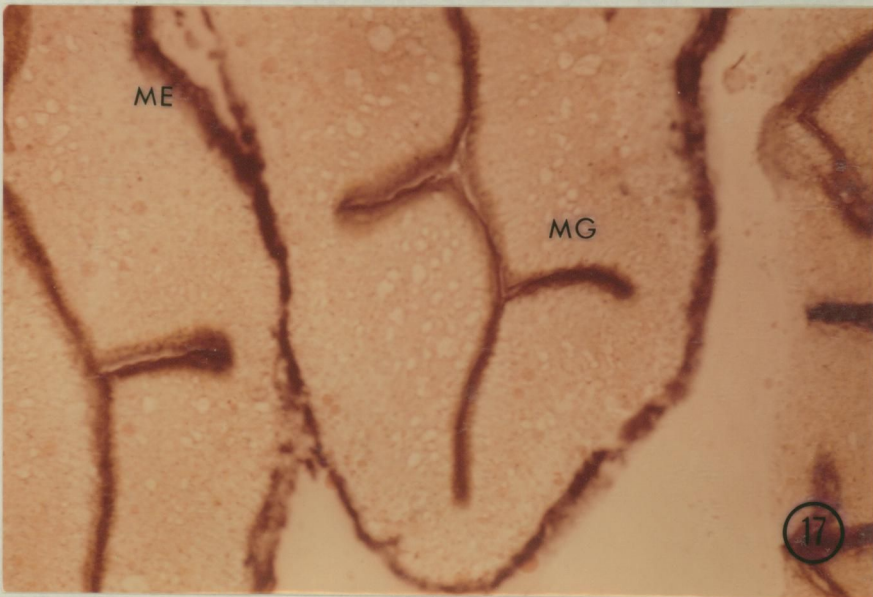
Fresh frozen section; α -naphthyl phosphate; pH 5.0; Garnet GBC. x 100.

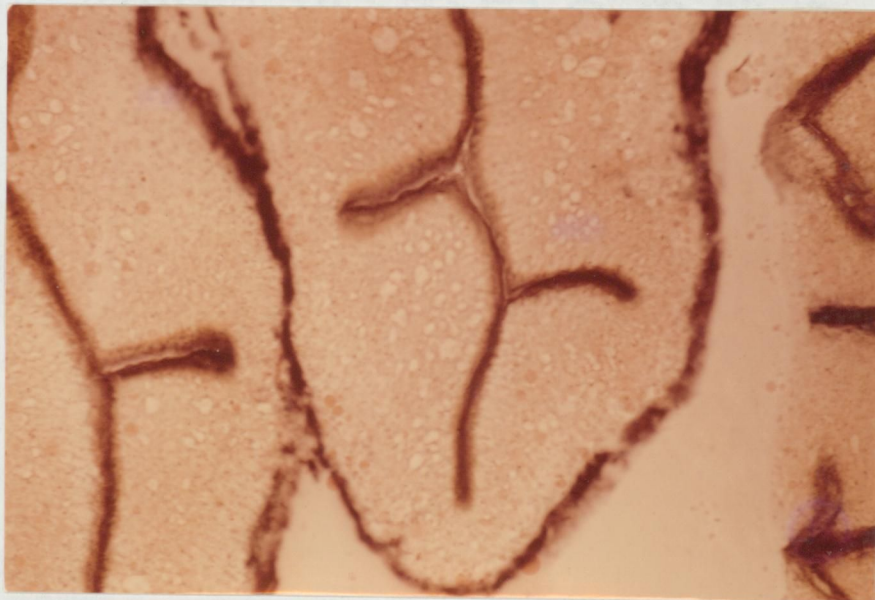
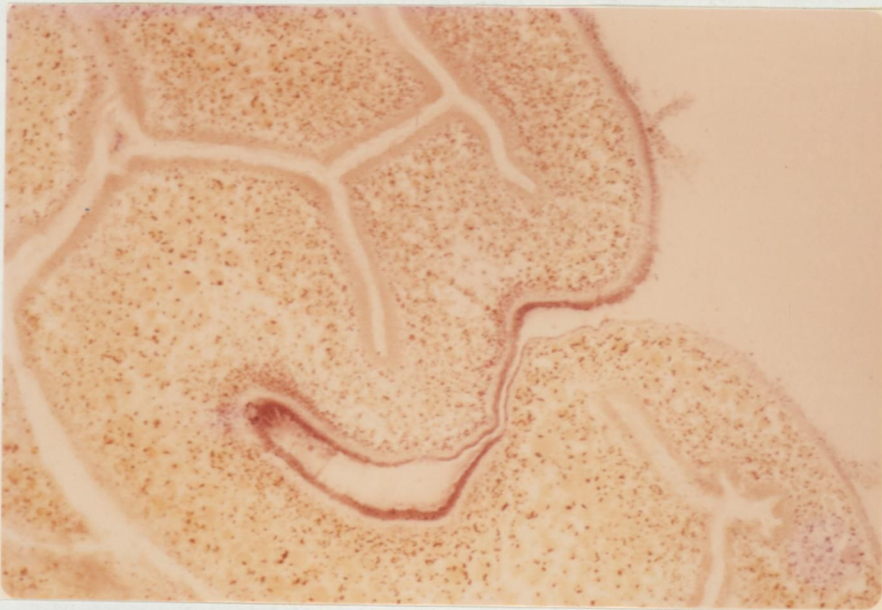
ME - mesothelial activity
L - lumen

Figure 17. Cryostat section (12 μ); alkaline phosphatase activity; 18 hours after feeding. Enzyme activity is nearly equal in intensity in the mesothelial and marginal regions.

Fresh frozen section; α -naphthyl phosphate; pH 9.2; Fast Blue RR. x 100.

ME - mesothelial activity
MG - marginal activity site





b) Alkaline Phosphatase (ALP)

As was seen from the results of the starvation study, ALP activity differs from the other three enzymes tested. This is also true for the results obtained during the course of the feeding study. Not only does the site of localization differ somewhat, but the level of activity also varies. ALP has two sites within the pyloric caecal epithelium at which it can be localized: along the marginal edge of the duct and in the mesothelial area (Fig. 17). Most of the time the enzyme is seen to be present at both of these sites. However, the level of activity at each site fluctuates. Sometimes activity is predominantly marginal with lesser mesothelial activity (Fig. 18), but the opposite situation also occurs; *i.e.*, the activity may be highest in the mesothelial region with lesser degrees of activity observable along the marginal edge (Figs. 20, 21). This fluctuation does not appear to be a daily cycle as samples of tissue from the same starfish were tested each day for a week without a noticeable change in localization (localization occurred at both sites) or in the level of activity. The asteroid was existing under 'normal' conditions with food readily available to it. It seems more probable that the fluctuation is related to the nutritive and metabolic conditions of the animal; *i.e.*, time after feeding (see Discussion). From Table II it can be seen that, shortly after feeding (the 15 minute sample), the activity is mainly concentrated along the marginal edge and from this time until the 2 hour sample this condition persists. From the 2 hour sample until the 3 day sample, the level of activity in the two sites is

Figures 18 - 19

Figure 18. Cryostat section (12 μ); alkaline phosphatase activity; 1 hour after feeding. The greatest intensity of enzyme activity is marginal in localization. To a lesser extent there is some enzyme activity in the mesothelial region.

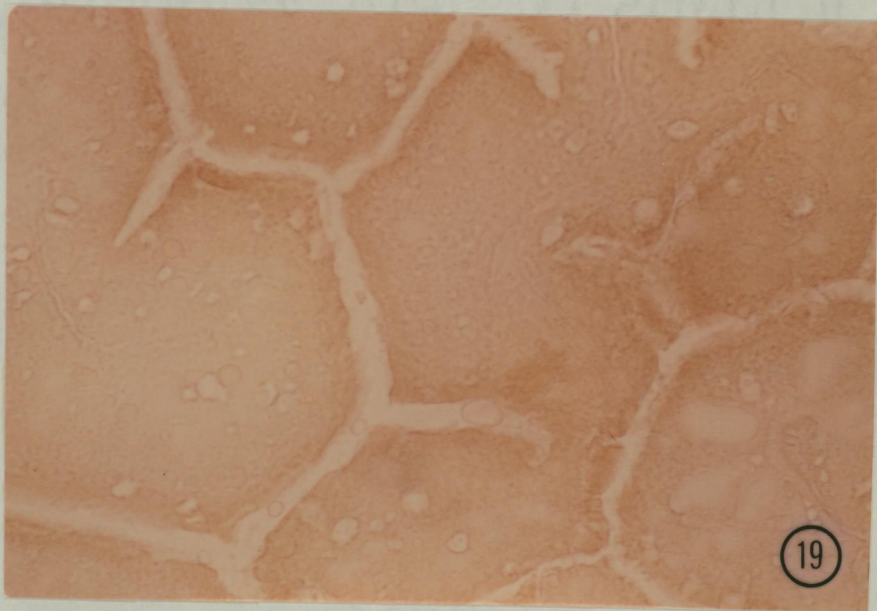
Fresh frozen section; α -naphthyl phosphate; pH 9.2; Fast Blue RR. x 100.

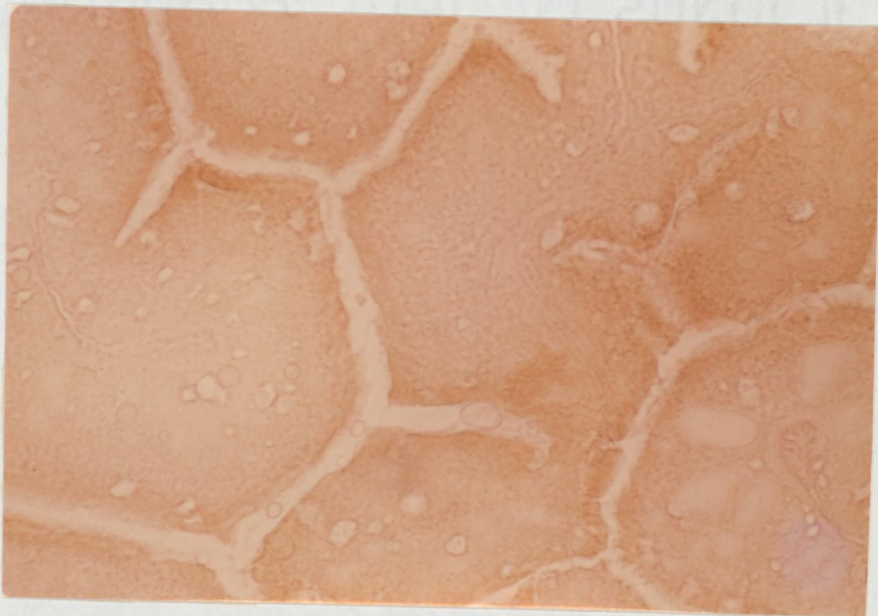
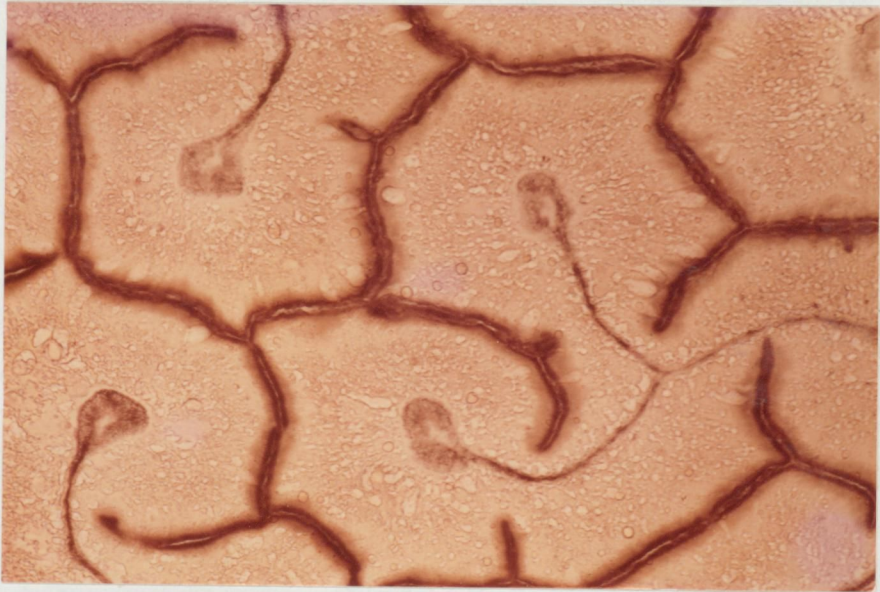
ME - mesothelial activity

MG - marginal activity

Figure 19. Cryostat section (12 μ); alkaline phosphatase control (no substrate in incubation medium); 1 hour after feeding. No activity visible.

Fresh frozen section; no α -naphthyl phosphate; pH 9.2; Fast Blue RR. x 100.





Figures 20 - 21

Figure 20. Cryostat section (12μ); alkaline phosphatase activity; 18 hours after feeding. The greatest enzyme activity is mesothelial in localization. Marginal activity is present to a lesser extent.

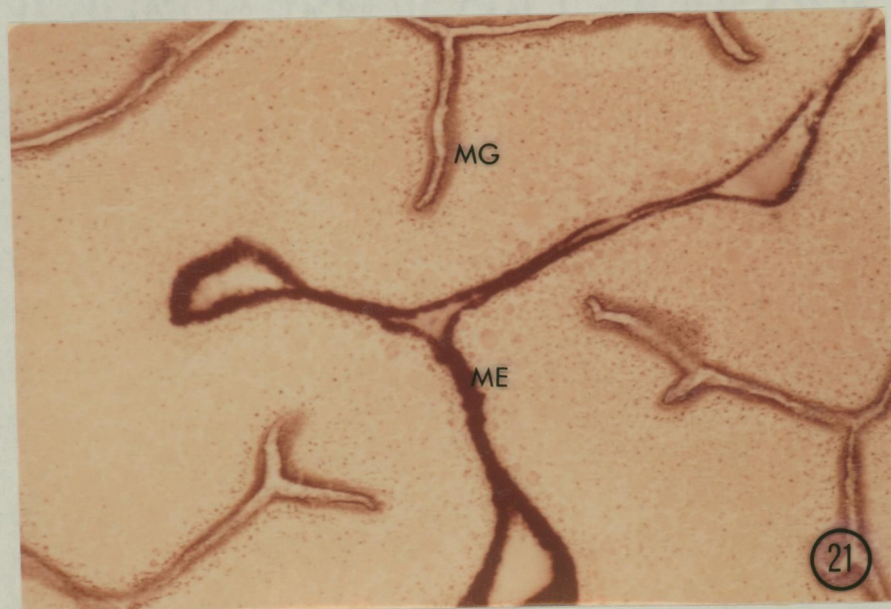
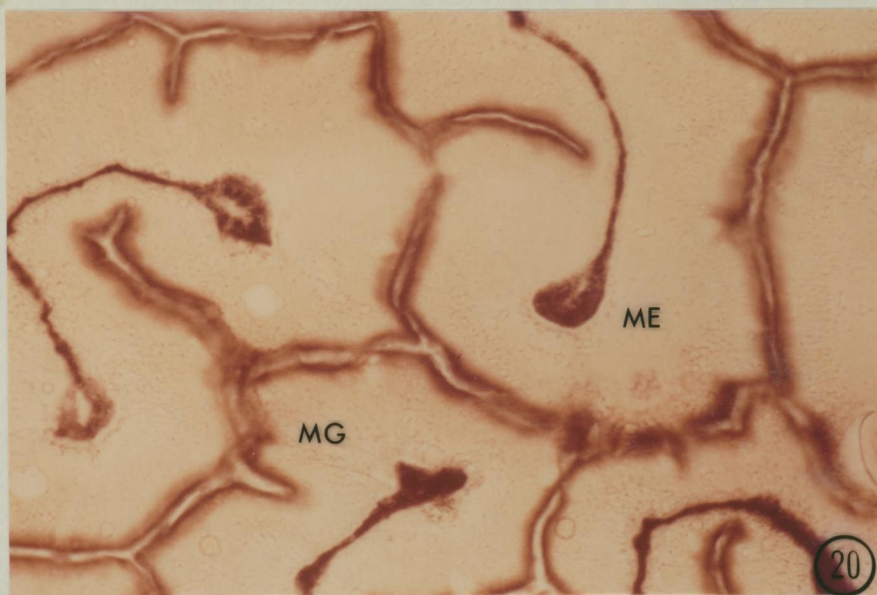
Fresh frozen section; α -naphthyl phosphate; pH 9.2; Fast Blue RR. x 100.

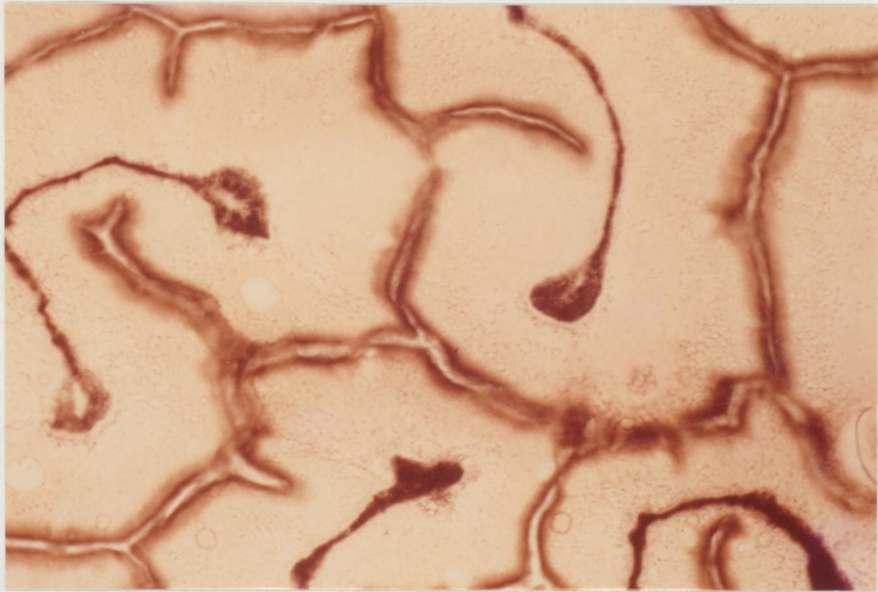
ME - mesothelial activity
MG - marginal activity

Figure 21. Cryostat section (12μ); alkaline phosphatase activity; 1 week after feeding. Activity is predominantly mesothelial.

Fresh frozen section; α -naphthyl phosphate; pH 9.2; Fast Blue RR. x 100.

ME - mesothelial activity
MG - marginal activity





approximately equal. From the 3 day sample until the end of the experiment at 10 days, the activity shifts to be predominantly mesothelial in localization. Thus, it seems that the activity of feeding has some effect on the fluctuation phenomenon observed with ALP. The season of the year, on the other hand, seems to exert little effect on the enzyme.

c) Leucine Aminopeptidase (LAP)

The level of activity for LAP remained approximately stable for the entire set of tests. It has only one site at which it is localized - along the marginal edge of the epithelium (Fig. 22). The site did not vary in any of the samples tested. The time of the year and the time after feeding have little or no effect on the localization or level of activity.

d) Esterases (Es)

As was the case during starvation, esterase activity cannot be as distinctly localized as can the other enzymes in this study. Activity is spread diffusely throughout the tissue (Fig. 24), perhaps being more strongly localized in the region of the nuclear zone and in the upper region of the lipid deposits. The level of activity appears to remain constant throughout the series of tests. It therefore seems, as was the case for leucine aminopeptidase, that time after feeding and season of the year have little effect on esterases in the pyloric caecal epithelium.

Figures 22 - 23

Figure 22. Cryostat section (12 μ); leucine aminopeptidase activity; 1 hour after feeding. Enzyme activity is confined to marginal edge of the duct. Note the lipid droplets in the basal region of the epithelium.

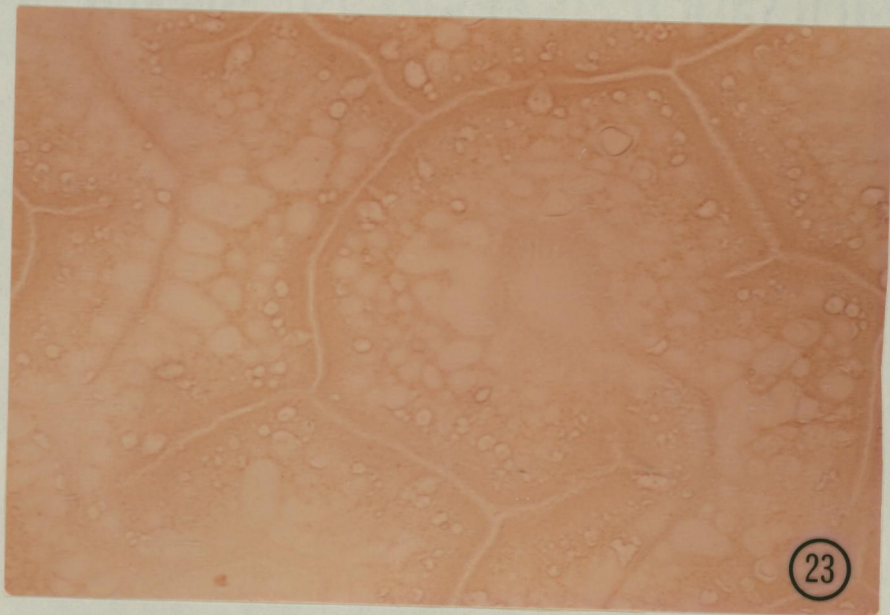
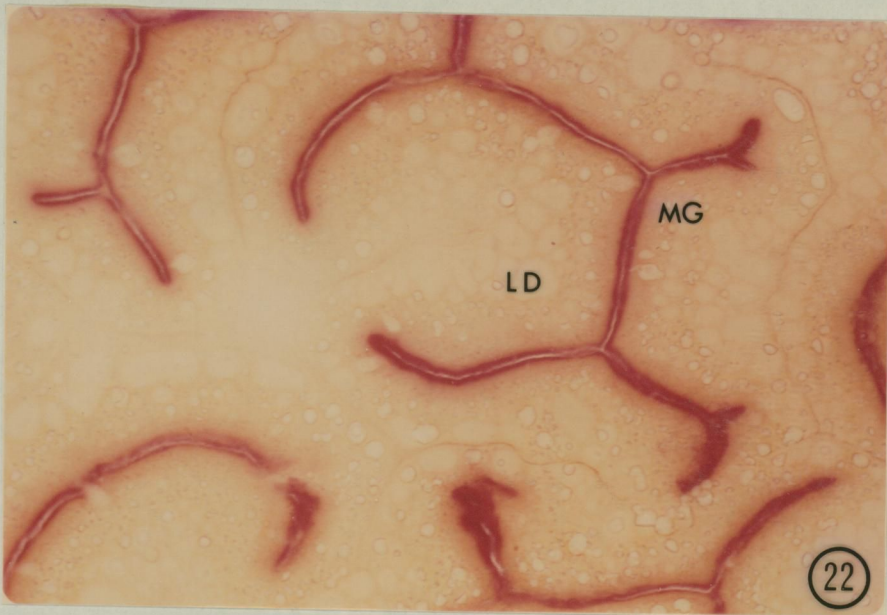
Fresh frozen section; L-leucyl-4-methoxy- β -naphthyl amide HCl; pH 6.5; Fast Blue B. x 100.

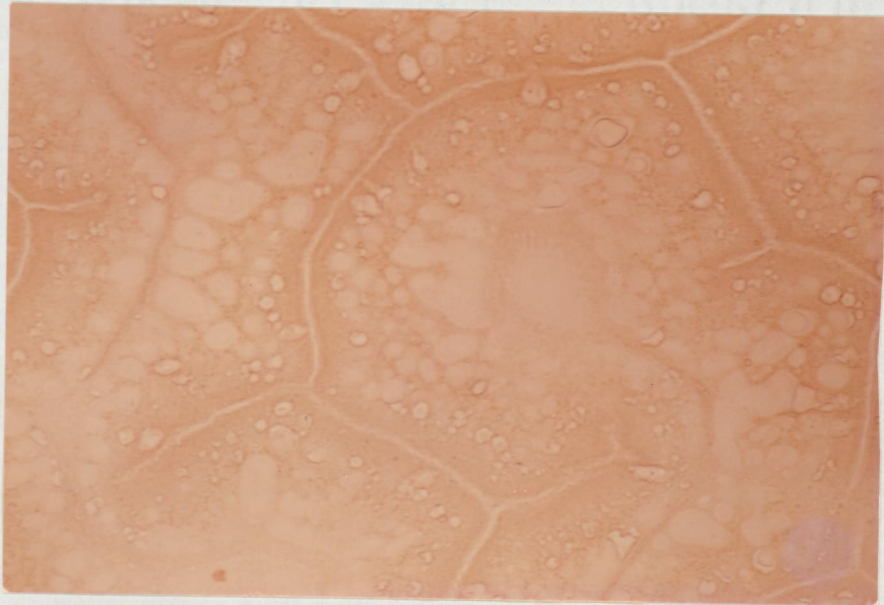
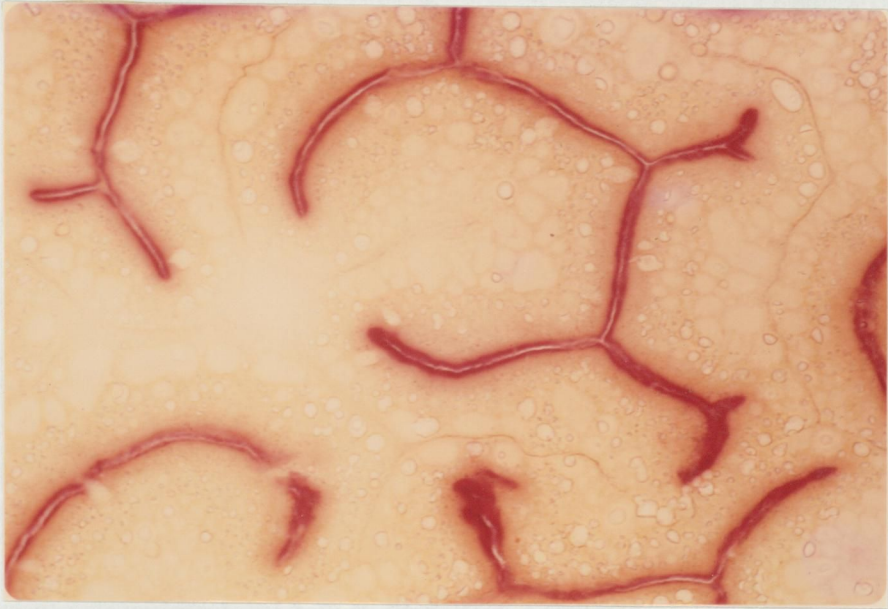
MG - marginal activity

LD - lipid droplets

Figure 23. Cryostat section (12 μ); leucine aminopeptidase control (no substrate in incubation medium); 1 hour after feeding.

Fresh frozen section; no substrate; pH 6.5; Fast Blue B. x 100.





Figures 24 - 25

Figure 24. Cryostat section (12 μ); esterase activity; 24 hours after feeding. Note the diffuse staining reaction that occurs throughout the tissue (activity is perhaps somewhat stronger at the level of the nuclear zone). Note the lipid droplets in the basal region.

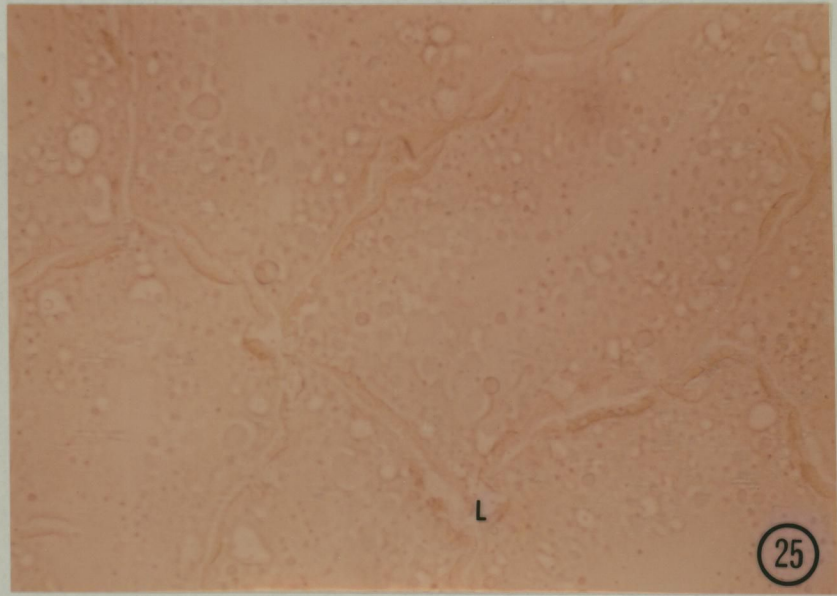
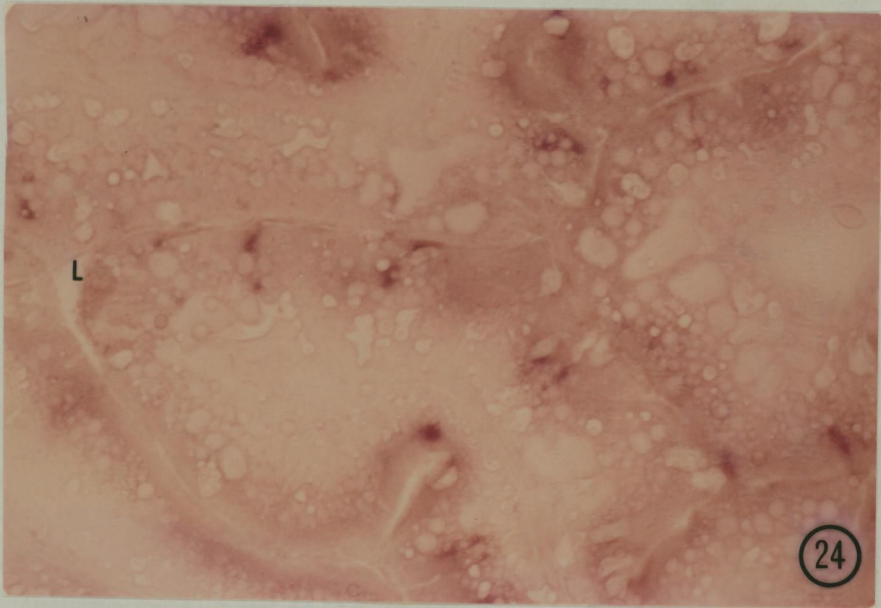
Fresh frozen section; α -naphthyl acetate; pH 7.5; Fast Blue B. x 100.

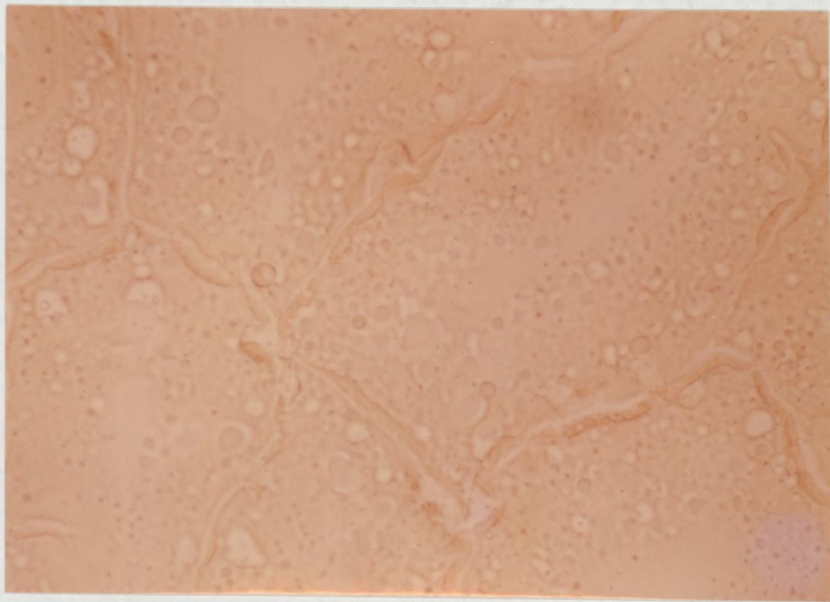
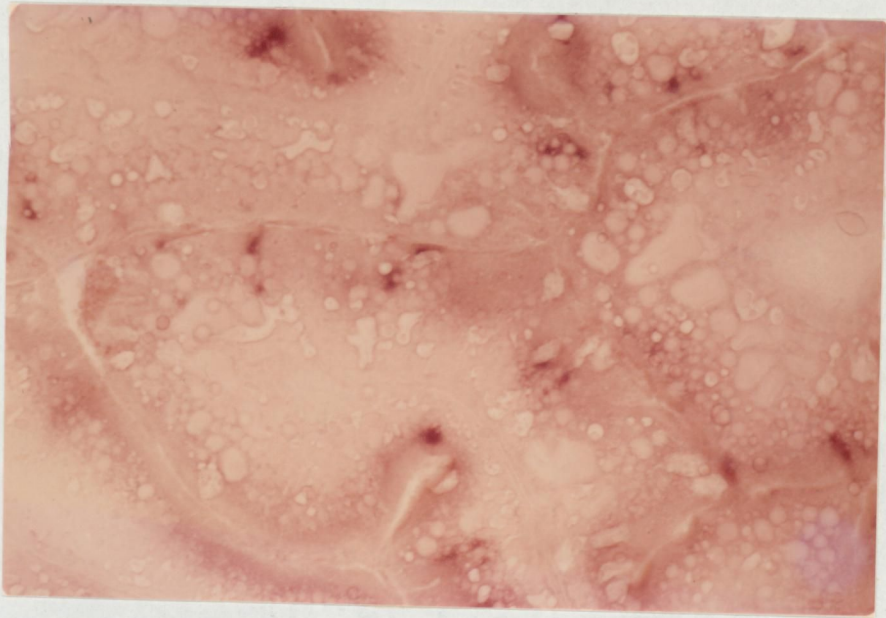
L - lumen

Figure 25. Cryostat section (12 μ); esterase control (no substrate in incubation medium); 24 hours after feeding.

Fresh frozen section; no α -naphthyl acetate, pH 7.5; Fast Blue B. x 100.

L - lumen





e) Lipid Deposits

The lipid deposits of the pyloric caeca, marked by the presence of reddish to purplish-black stained globules, are located basally in the epithelium below the level of the nuclear zone (Figs. 26, 27). Lipid deposits were abundant in all samples, regardless of the time of the year. They never showed marked signs of depletion. There is, however, a marked increase in the amount of lipid over that observed in a starving animal.

II. Paraffin Sections

The zymogen cells with their vacuoles and granules, as previously mentioned, are best demonstrated by the use of PTAH and Mallory's Triple stains. Though the zymogen granules and vacuoles were present in all the samples tested, there appears to be a correlation between the histological appearance of the pyloric caeca and the recentness of feeding but not invariably with the time of the year.

As shown in Table II, the feeding experiment was conducted throughout the various seasons of the year. *Pisaster ochraceus* feeds most intensively in the summer months when food is plentiful, with winter being a period noted for low frequency of feeding (Mauzey, 1966). As part of the experiment was carried out during the fall and winter period of low feeding, a change here would add proof to the hypothesis that histological differences are due to feeding, and not simply to an inherent seasonal change.

Figures 26 - 27

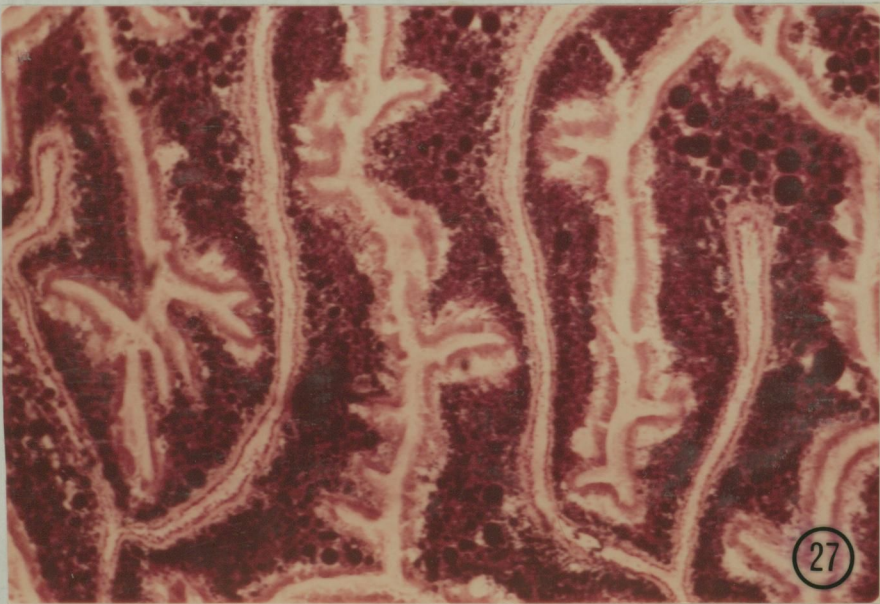
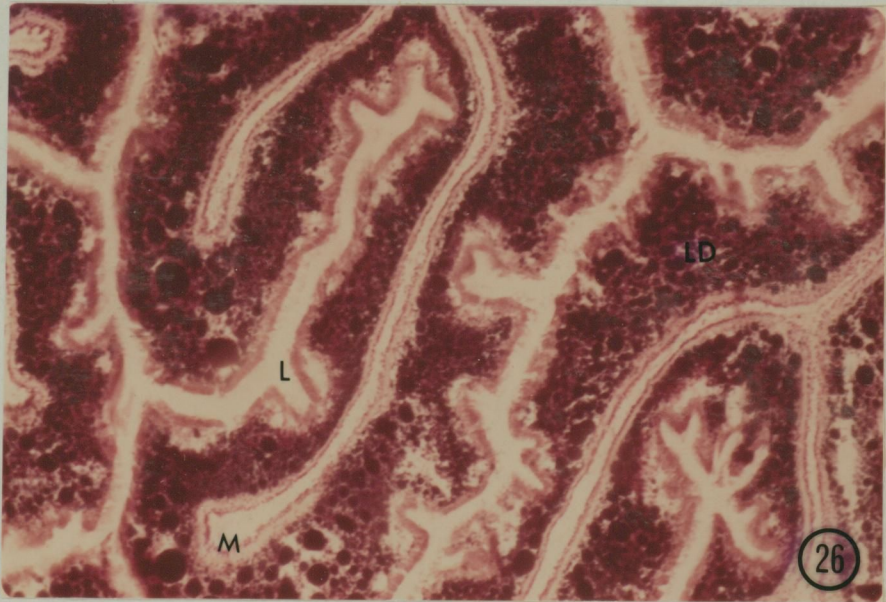
Figure 26. Cryostat section (12μ); Sudan Black B Staining for lipids; 24 hours after feeding. Note the abundance of basally located lipid deposits (purplish-black globules).

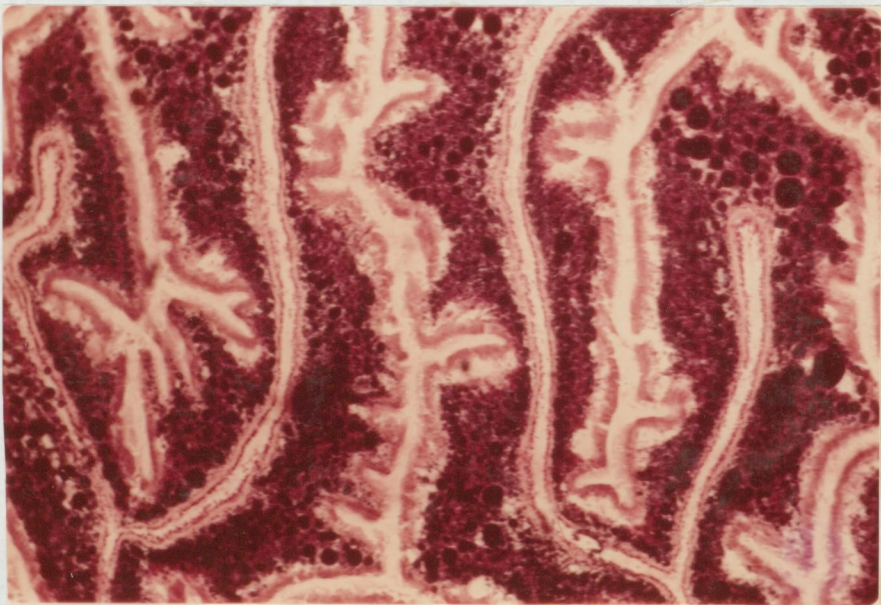
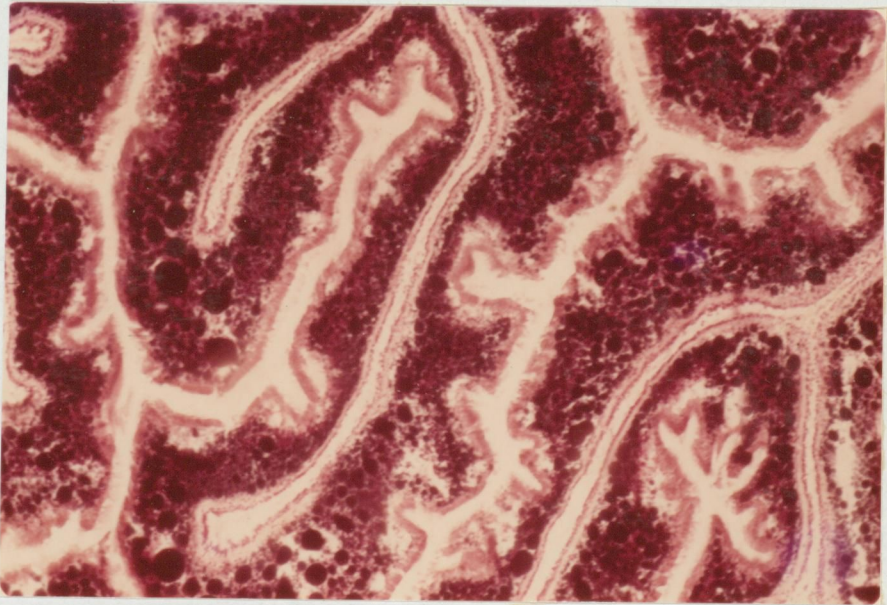
Fresh frozen section; Sudan Black B; carmalum counterstain. Compare with similar section (Fig. 7) in the starvation experiment. x 100.

L - lumen
M - mesothelial region
LD - lipid droplets

Figure 27. Cryostat section (12μ); Sudan Black B staining for lipids; 24 hours after feeding. Another view of the above section, showing the abundant lipid deposits.

Fresh frozen section; Sudan Black B; carmalum counterstain. x 100.





The sections show that the process of feeding leads to a marked increase in the number of zymogen granules and vacuoles over the condition which is typical in the sections examined during the starvation regime. Feeding also seems to cause an increase in the size of the zymogen vacuoles. The long strings of red (Mallory's positive) or purplish-red (PTAH positive) granules begin in the region below the level of the nuclei and pass toward the marginal edge. The vacuole is situated between the granules and the basally located nucleus. Zymogen granules are especially plentiful in the acinus-like termini of the pyloric caeca. Soon after the asteroid commences feeding the number of granules and vacuoles increase markedly. The sample taken 15 minutes after feeding (Figs. 28, 29) is typical of this condition, the epithelium being crowded with long strings of granules and very large vacuoles. The vacuoles decrease in size as the duration since feeding lengthens and the strings of granules decrease in number (Figs. 30, 31). The number of granules appears, however, at all times to be greater than when the animal is being starved.

The duration after feeding seems also to affect the histological appearance of the storage cells. The cytoplasm is intensely PAS positive (especially the basal region) except along the apical edge. The cytoplasm appears to be much more PAS positive than during the starvation experiment (Fig. 32). There may be some zonation of the cytoplasm in storage cells because, when staining with Mallory's, the basal cytoplasmic region sometimes appears blue, the apical region

Figures 28 - 29

Figure 28. Paraffin section (15 μ); PTAH staining; 15 minutes after feeding. Note the presence of numerous long strings of zymogen granules and the abundant large zymogen vacuoles. This section is lacking in storage granules.

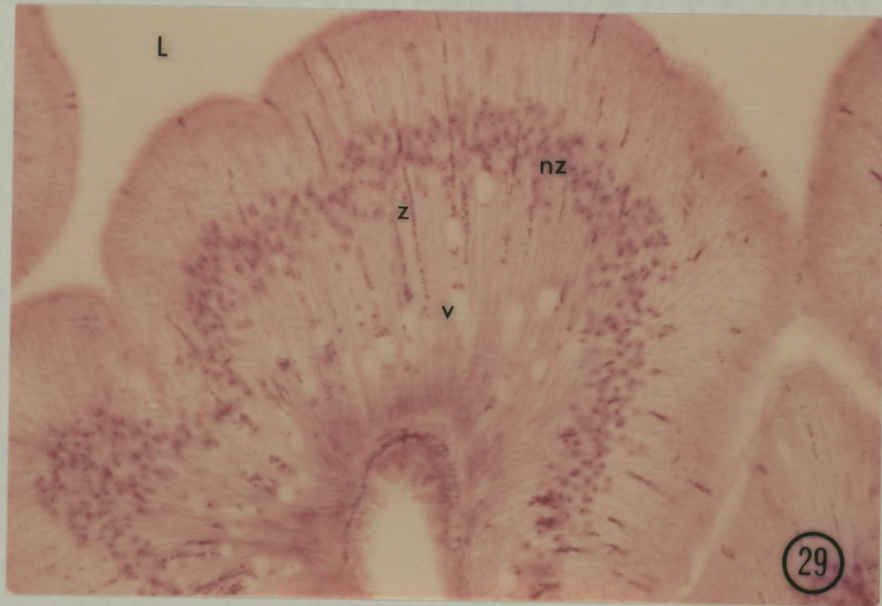
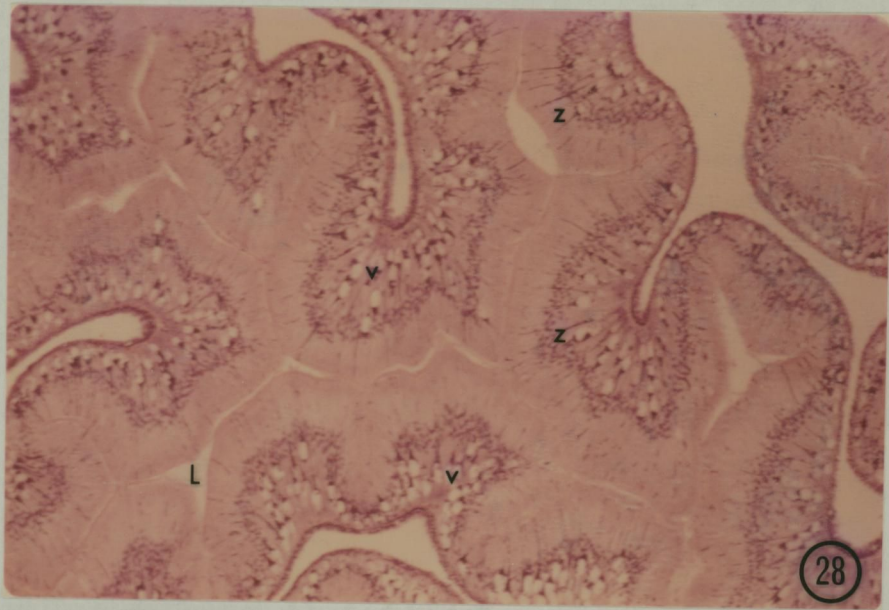
Susa fixation. Compare this with condition in starved animal (Fig. 8). x 100.

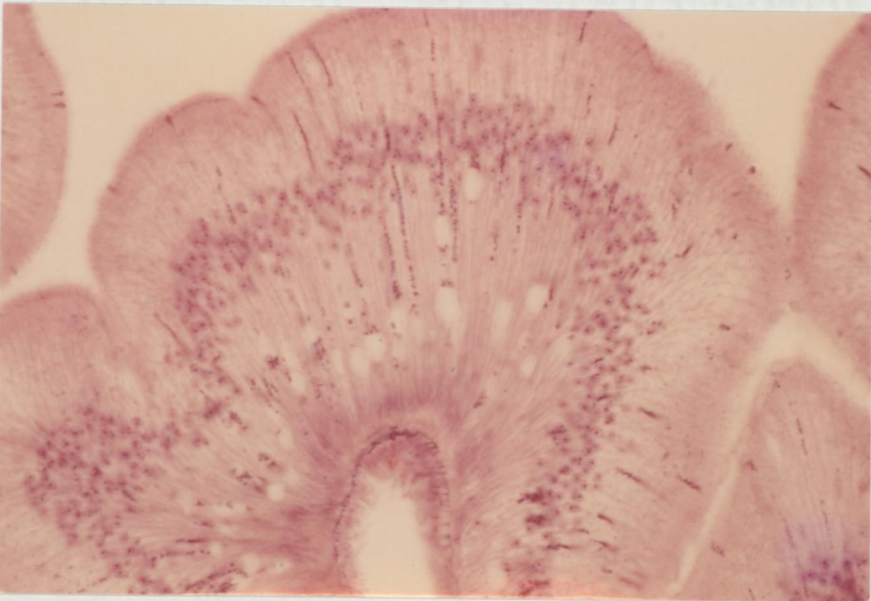
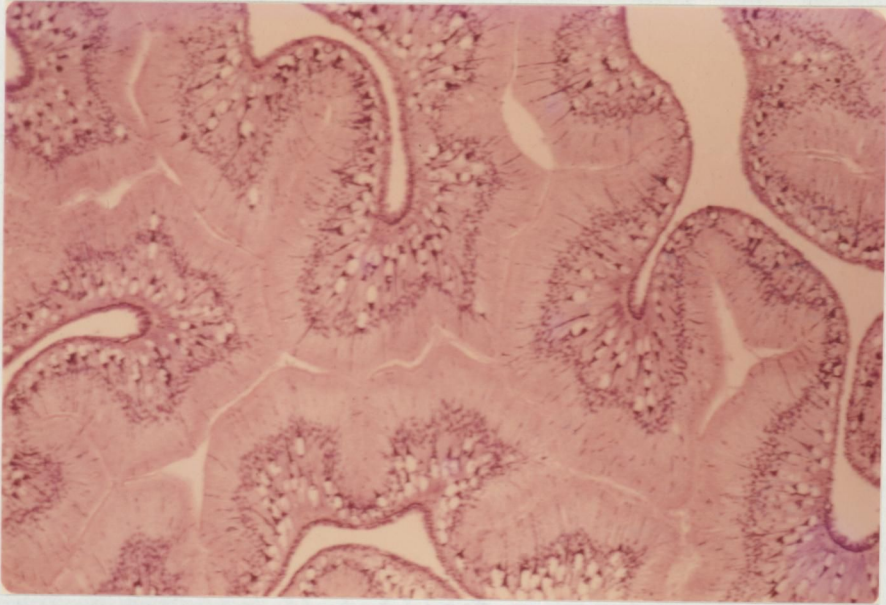
z - zymogen granules
v - zymogen vacuoles
L - lumen

Figure 29. Paraffin section (15 μ); PTAH staining; 15 minutes after feeding. Enlarged view of a section similar to the one above. Note large zymogen vacuoles and strings of zymogen granules.

Susa fixative. x 250.

~~z - zymogen granules~~
v - zymogen vacuoles
L - lumen
nz - nuclear zone





Figures 30 - 31

Figure 30. Paraffin section (15μ); PTAH staining; 1 hour after feeding. The vacuoles are smaller than in the 15 minute sample. Note the storage granules above the level of the nuclear zone.

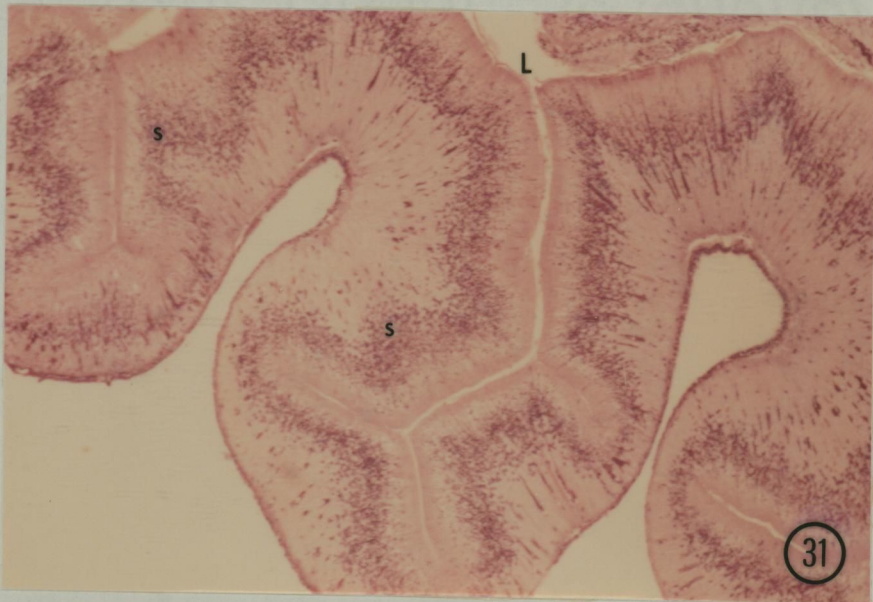
Susa fixation. x 100.

L - lumen
z - zymogen vacuoles
s - storage granules (reddish)

Figure 31. Paraffin section (7μ); PTAH staining; 10 days after feeding. By this time the zymogen vacuoles are very small and few in number and the strings of zymogen granules have also decreased in number. Note the abundant storage granules.

Susa fixation. x 100.

L - lumen
s - storage granules





Figures 32 - 33

Figure 32. Paraffin section (15 μ); PAS staining; 15 minutes after feeding. The cytoplasm of the storage cells is very PAS positive; compare with Figure 12. Note the lipid droplets in the basal regions of the epithelial tissue.

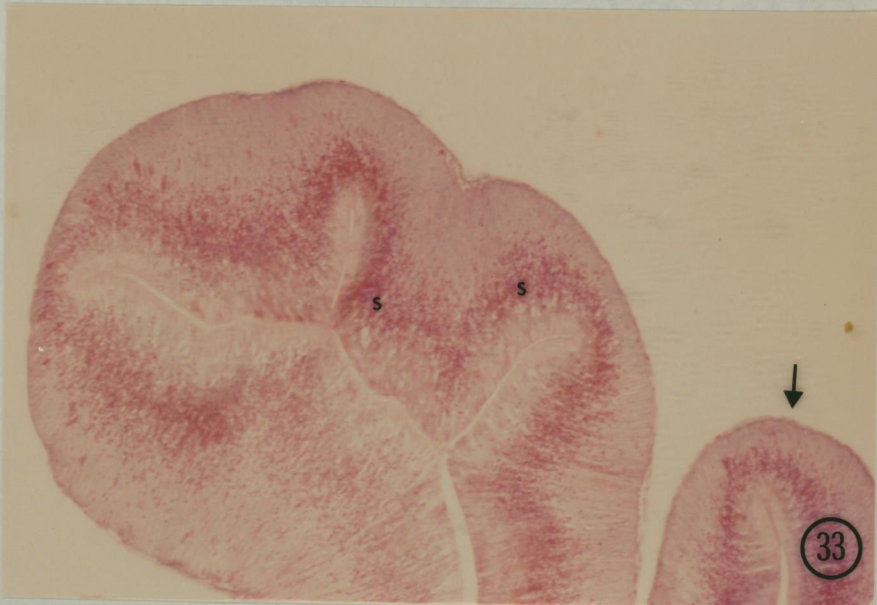
Susa fixation. x 100.

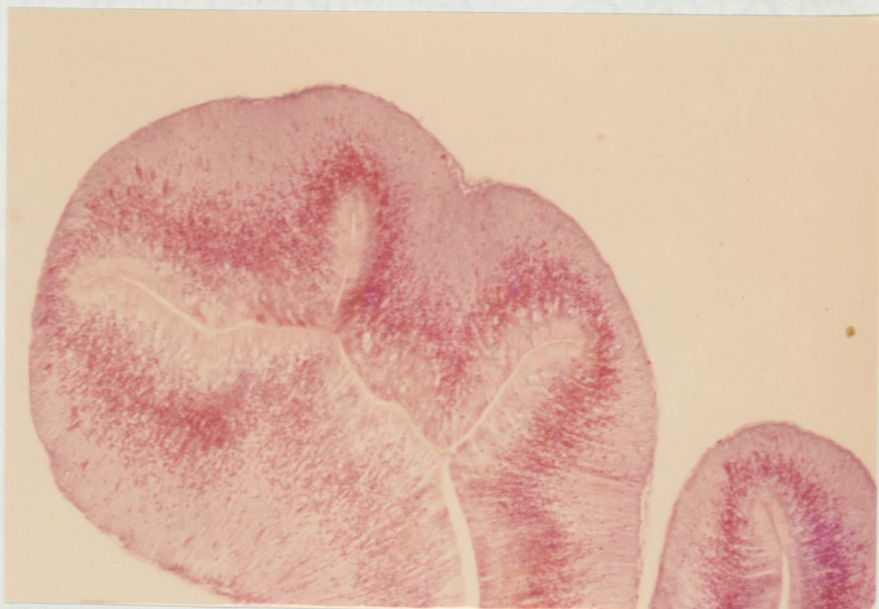
LD - lipid droplets
L - lumen

Figure 33. Paraffin section (7 μ); Mallory's Triple Stain; 10 days after feeding. Note the abundant red-staining storage granules which are so plentiful that they obscure the nuclear zone.

Susa fixation. x 100.

s - storage granules





yellowish. Mauzey (1966) also noted this but could not supply a reason for its occurrence. There were also changes in the storage granules (probably a protein-carbohydrate complex; PAS positive, PTAH positive, stains with Mallory's; Alcian Blue negative and stable to salivary digestion). The number of granules increase after feeding, peaking in this experiment after 24 hours, at which time the granules were so plentiful as to obscure the nuclear zone. The storage granules remained plentiful in the epithelium from this time until the end of the experiment (Figs. 33, 34). The basal regions of the storage cells become extremely vacuolated shortly after feeding (Fig. 32). This condition was also noted by Mauzey (1966). The vacuoles are noticeable in sections stained with any of the substances used during this study. It seems reasonable to assume that these vacuoles mark the position of the lipid deposits that react positively with Sudan Black B in the frozen sections and which are removed during the processing of paraffin sections. Sudan Black B applied to paraffin sections, as mentioned during the starvation experiment, does however stain the brush border and some mucous goblet cells.

Though the mucous goblet cells do not seem to be significantly affected by the length of time after feeding they are very abundant within the epithelium, more abundant than in starved animals. From staining with Alcian Blue, it can be seen that many of these cells are releasing mucus into the lumen of the duct (Fig. 35). Perhaps the secretion of mucus has a function to perform during feeding.

Figures 34 - 35

Figure 34. Paraffin section (7 μ); Mallory's Triple Stain; 10 days after feeding. Enlarged view of section in Figure 33 indicated with an arrow. Note the abundant red storage granules in the storage cells.

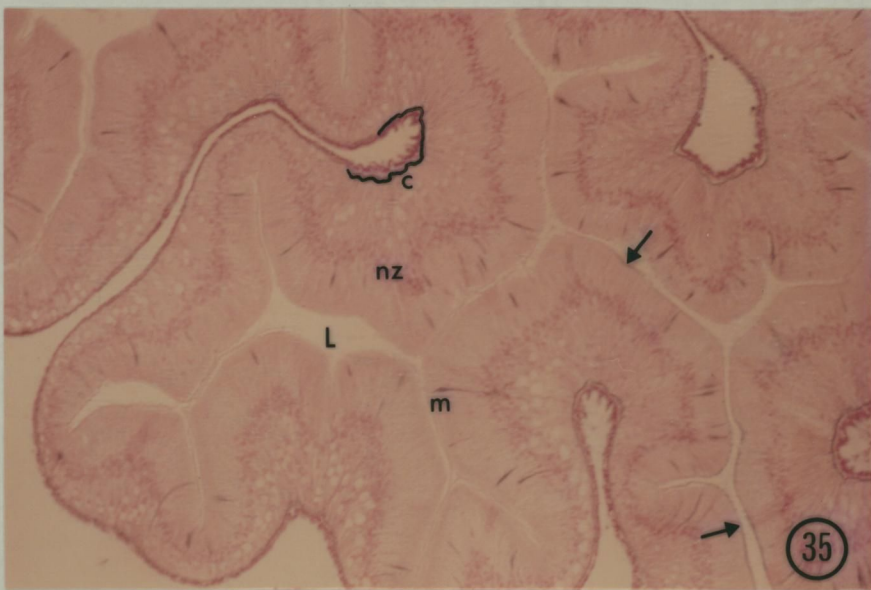
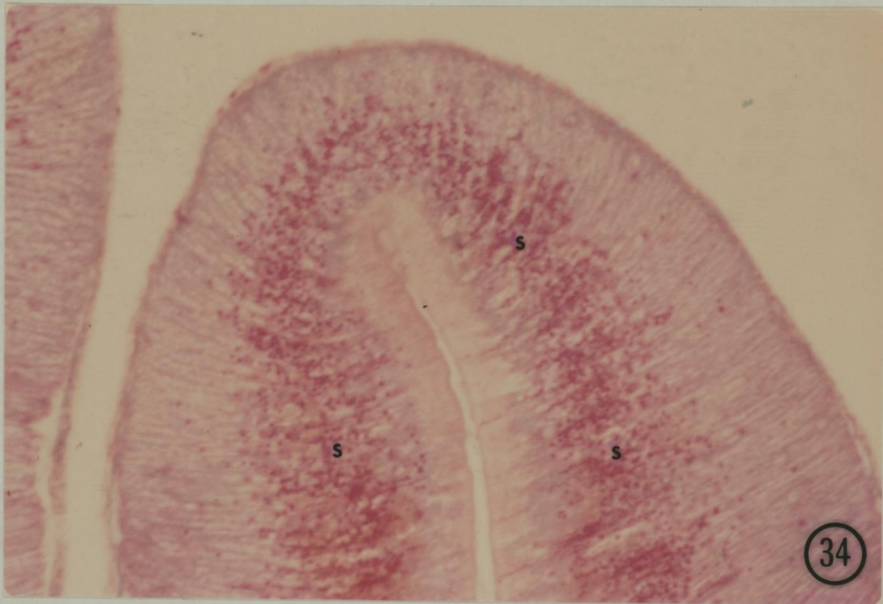
Susa fixation. x 250.

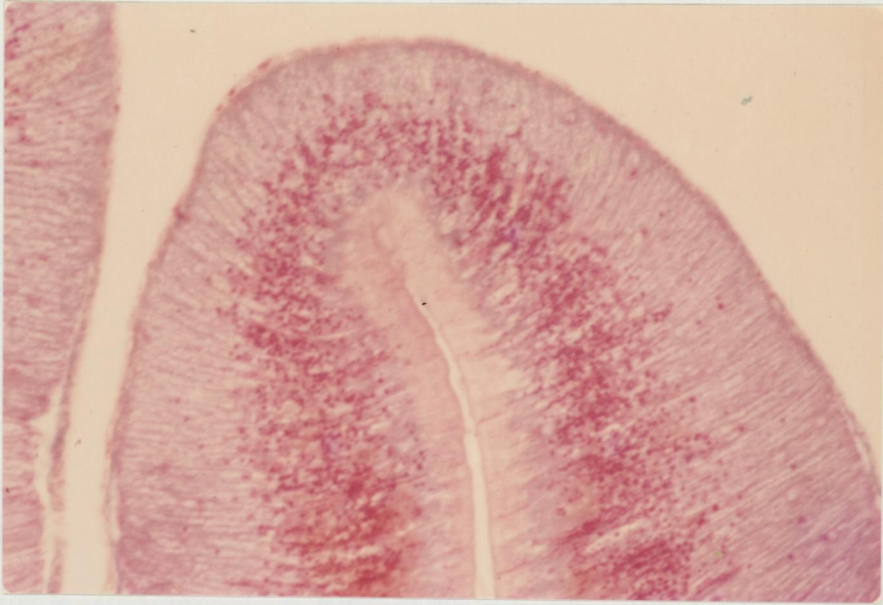
s - storage granules

Figure 35. Paraffin section (15 μ); Alcian Blue staining; 15 minutes after feeding. Note the abundant blue-staining mucous goblet cells. The arrows indicate where mucus has been extruded into the lumen. Compare with Figure 13.

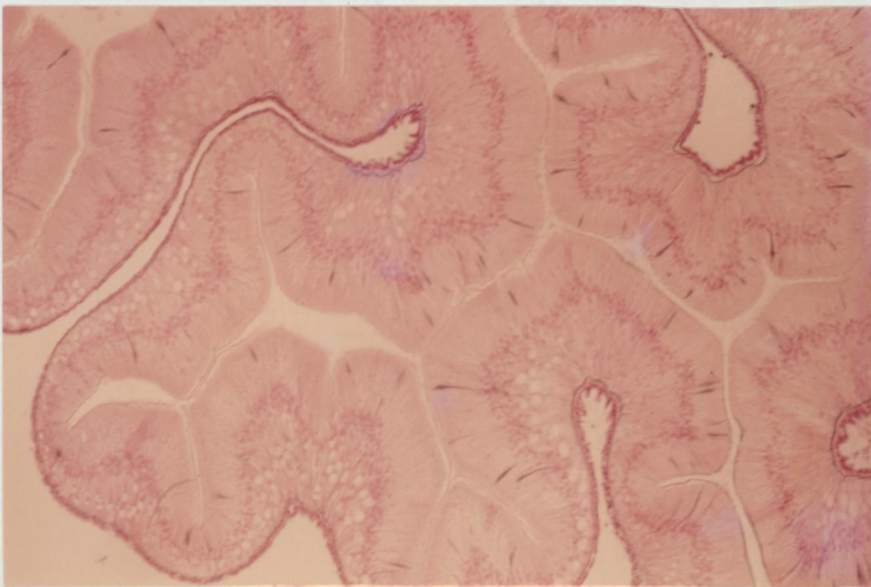
Susa fixation. x 100.

L - lumen
m - mucous goblet cell
c - connective tissue region
nz - nuclear zone





SUPERFINE



Thus it would seem that the feeding process, more specifically the duration after feeding, has a direct effect on the histological appearance of the digestive epithelium of the pyloric caeca of *Pisaster*. Certain cellular inclusions - the zymogen granules and vacuoles, the storage granules, and the lipid deposits - are affected by the asteroid feeding. The zymogen granules increase in numbers as do the vacuoles and the storage granules. The zymogen vacuoles also grow larger in size following feeding. The cytoplasm of the storage cells becomes extremely vacuolated by the presence of lipid deposits. The increase in storage granules and lipid is indicative of the pyloric caeca's role in absorption and nutrient storage. When food is available, these reserves are stored in the pyloric caeca until they are needed (*e.g.*, reserves are transferred to the gonads to aid in the development of the gametes).

It is impossible to say from just subjective examination of the frozen sections whether there is an increase in the level of activity of the enzymes in feeding asteroids as compared to the starved animals. Such an increase would seem logical, especially since there is a marked increase in zymogen granules, but a quantitative assay is necessary to verify this assumption. However, three of the enzymes (acid phosphatase, leucine aminopeptidase, and esterase) did not appear significantly to alter either their level of activity or their localization at progressive intervals after feeding. This cannot be said of alkaline phosphatase. Alkaline phosphatase is localized at two sites (marginal edge and mesothelial region) and fluctuates in its

level of activity between these two sites. The feeding process, in particular the time since feeding, seems to influence alkaline phosphatase activity. This will be related to its function(s) in the Discussion. The time of the year apparently exerts little influence on the enzymes, but the duration of time after feeding does, at least in the case of alkaline phosphatase.

Chapter V

DISCUSSION

That the pyloric caeca of a starfish possess a variety of enzymes is not a new discovery. Over the years researchers (Frédéricq, 1878; Chapeaux, 1893; Stone, 1897; Van der Heyde, 1922; Sawano, 1936; Yokoe and Yasumasu, 1964; Araki and Giese, 1970; Camacho *et al.*, 1970; and Sasaki, 1971), using tissue extracts on various substrates, have compiled data on the abundance and variety of enzyme types in the pyloric caeca of various starfish. Their findings have led several of them to conclude that the pyloric caeca are comparable to the vertebrate pancreas in wealth and variety of enzymes. This is, however, the first time that histochemical techniques have been applied to the study of the enzymes *in situ* in the pyloric caeca and that the effects of starvation, feeding, and season of the year have upon these enzymes has been investigated for any asteroid. The correlation of enzyme tests on frozen tissue sections with the examination of cellular details in paraffin sections gives a clear overall picture of what is transpiring at several levels of organization.

The histological changes taking place among the cellular inclusions of the caecal epithelium reflect a relationship to the nutritional state of the animal but not to the season of the year. That is, the histological differences in the pyloric caeca of animals collected at various periods of the year are due to feeding differences, and not simply to an inherent seasonal change. This conclusion was also reached

by Mauzey (1967). In an asteroid that is unable to feed (because of lack of food in its natural surroundings or from forced experimental starvation), zymogen granules are few in number, zymogen vacuoles are scarce and small in size, and there are few storage granules relative to the condition of the epithelium when an animal is feeding. However, the zymogen granules and vacuoles and the storage granules never disappear entirely from the epithelium even after the 10 weeks of starvation. Likewise the lipid deposits are abundant within the basal region of the epithelium and show no signs of depletion after 10 weeks. These findings concur with those of Chia (1969) for *Leptasterias hexactis* where there is little or no change in storage and zymogen cells even after weeks of starvation. The cells that Chan and Fontaine (1971) called zymogen cells and characterized by being PTAH positive but without an accompanying vacuole were most likely identical to the zymogen cells of Anderson (1953) but from an animal that had not fed recently. The vacuoles would therefore be scarce within the epithelium and missing from certain strings of zymogen granules. When feeding occurs, the strings of granules become abundant as do their concomitant vacuoles. In this condition the granules are PTAH positive and have a vacuole (Figs. 28, 29), satisfying Anderson's description of a zymogen cell (= secretory cell). The zymogen vacuoles also increase in size. The function(s) of the zymogen vacuole is unknown, though some have been postulated. The most satisfactory explanation so far is that given by Chia (1969) who reasoned that, since the secretory granules most often take a position between the vacuole and the apical surface,

that any change of the shape, size, or position of the vacuoles would inevitably be connected with the movement of the secretory granules. Therefore, he thought that the vacuoles might operate as vehicles to transport the zymogen granules into the lumen of the pyloric caecum. His theory seems to be strengthened by the observation made during the course of this study that, as a result of feeding, the zymogen vacuoles do increase in size as well as number. The vacuoles could most likely hasten the movement of the granules (and hence the enzymes if the granules are assumed to be the site of enzyme storage) towards the lumen to fulfill their various functions. The presence of food within the gut presumably stimulates this cycle. Accompanying the process of feeding, the nutrient stores of the caeca (*i.e.*, the basal lipid deposits and storage granules) also become plentiful within the epithelium.

The enzyme histochemistry carried out during this research also led to some interesting findings. The enzymes were found to be present in the epithelium under all conditions (even after 10 weeks of starvation) and during all seasons of the year (even being present throughout the winter when the asteroid's feeding frequency is at its lowest). Though *Pisaster* has abundant stores in its pyloric caeca and can survive for many months without feeding and granted that the starfish cannot 'know' when its next meal will occur, this continuous production of enzymes even under conditions of food deprivation appears to be a most 'wasteful' system of enzyme synthesis. A supply and demand (enzyme induction caused by a definite stimulus) would seem more

'beneficial', at least from a consideration of energy utilization. However, this is true only if the enzymes are considered to fulfill a single function; *i.e.*, hydrolysis for digestive purposes. It may be that these enzymes (especially acid and alkaline phosphatase) serve other purposes than hydrolysis and that these other functions are perhaps of more importance in the metabolism of *Pisaster*. This will be discussed further on.

Also of fundamental concern is the fact that the majority of enzymes, if not all, exist in multiple molecular forms (isozymes). Therefore, consideration should be given to the functional significance and underlying causes of isozymic variation. It is reasonable to suppose that genetic duplication would confer a selective advantage on its possessor in that mutation would be less likely to deactivate, for example, two equivalent loci as opposed to a single locus. If mutation were to result in allelic variation, a second type of selective advantage would be conferred, that of widening the adaptive range of an organism. Lindsay (1970) speculates that isozymes could have arisen this way. From a functional point of view, it seems probable that isozymes afford a metabolic advantage in some cases and that the differences in tissue localization reflect different metabolic needs. If these ideas of selective advantage are not valid, it is difficult to envision how the occurrence of cell and tissue-specific isozymes has become such a wide-spread phenomenon.

The concept of isozymes and how diverse sites of localization may reflect metabolic needs and hence enzyme function is of great importance, especially when discussing acid and alkaline phosphatase. Numerous studies on a diversity of organisms from bacteria to men are available for the isozymes of acid phosphatase (Doré and Cousineau, 1967; Bowen, 1968; Maggi and Carbonell, 1969; Beadle and Gahan, 1969) and alkaline phosphatase (Robinson *et al.*, 1965; Robson and Harris, 1965; Schlesinger and Anderson, 1968; Susman *et al.*, 1968; Lyons *et al.*, 1970; Lindsay, 1970). It would seem probable that the phosphatases of the pyloric caeca also exist as isozymes, with different localization sites indicating differing metabolic needs. Isozymes of acid phosphatase in addition to showing different localization sites also respond differently to histochemical reactions. It has been found that some types of acid phosphatase will react to the β -glycerophosphate method of Gomori while others will not; but they in turn can be identified by their reaction with a naphthol-AS substrate. Some forms are inhibited by sodium fluoride, others are unaffected (Bowen, 1968; Maggi *et al.*, 1969). It is possible to separate these isozymes electrophoretically (Beadle *et al.*, 1969).

Acid phosphatase has received a great deal of attention, mainly as a result of de Duve's work on lysosomes (1959, 1963). Lysosomes are membrane-bound vesicles of acid hydrolases which, according to de Duve (1959), fulfill the following functions: (1) metabolism, (2) regulation, (3) intracellular digestion, and (4) autolysis. Lysosomes occur as four biologically and morphologically distinct but inter-related functional forms; namely

- (1) zymogen-like granules containing newly synthesized enzymes;
- (2) pinocytotic vacuoles;
- (3) residual bodies; and
- (4) the autolytic vacuole resulting from cellular autophagy.

Lysosomes are known to contain approximately twelve enzymes, including acid phosphatase. Perhaps the zymogen granules of the pyloric caeca represent a type of lysosome, the enzymes being released when the membrane bursts upon reaching the lumen. This could be elucidated by combining enzyme histochemistry at the electron microscope level with Strauss' horseradish peroxidase technique for lysosomes (Strauss, 1963, 1966, 1968, 1969). From some preliminary work at the light microscope level, it appears that horseradish peroxidase when injected into the ray of *Pisaster* is absorbed across the peritoneal membrane of the caeca; within the tissue it is enclosed within vacuoles that have acid phosphatase activity associated with them.

Though acid phosphatase is best known functionally for its hydrolysis of the phosphate-ester bond in an acid medium and in connection with the lysosome concept, some recent work suggests that another important function may concern the relationship between acid phosphatase and active transport. Southward and Southward (1966) in discussing the enzyme histochemistry of the pogonophoran *Siboglinum atlanticum* stated that, though there is no direct proof that the phosphatases are involved in active transport, they are frequently found at the sites of presumed active transport. Jennings and Van der Lande (1967), in their histochemical studies on digestion in leeches, concluded that acid phosphatase was concerned with the absorption of material from the gut lumen.

Functionally acid phosphatase, therefore, seems linked to the process of endocytosis (phagocytosis, pinocytosis, *etc.*) and active transport and is known to be produced in response to the uptake of particles (Ricketts, 1971). This relationship between particle uptake and active transport probably explains the localization of the enzyme. From Ferguson's work on nutrient transport in starfish, it is known that transport is accomplished as part of a continual flux of substances between the coelomic fluid and the various tissues of the body and that most of this transport occurs across the peritoneum and the connective tissue of the pyloric caecum (Ferguson, 1963 b, 1964 a, b). Acid phosphatase localized in the mesothelial region of the caecum is probably related to the transportation of nutrients in this area. Likewise, its occasional localization along the marginal edge may be indicative of nutrient absorption or active transport in this region of the gut tissue. Bargmann and Behrens (1967) in electron micrographs of the digestive epithelium of *Asterias rubens* noted an intense vesiculation of the cytoplasm and the occurrence of many pinocytotic invaginations at the base of the brush border, indicating the high absorption activity of the epithelial cells. These same researchers also noted the presence of numerous lysosomes in the upper regions of these epithelial cells. It should also be noted that the granular staining of the cytoplasm with the acid phosphatase reaction is concentrated in this region in the present study. It is also probable that the two localization sites indicate two isozyme types of the enzyme.

Recent studies indicate that LAP may be linked functionally with the acid phosphatases (Sylvén, 1970). Aminopeptidase is an exopeptidase widely distributed in animal tissues, plants, and microorganisms and is known to exist in several forms. Leucine aminopeptidase was originally described as a metal peptidase with a broad specificity for leucyl and other compounds, activated by Mn^{2+} and Mg^{2+} ions and inactivated by EDTA and citrate (Pearse, 1972). It has not been conclusively established that leucine aminopeptidase is a single entity and this has caused considerable dispute about the specificity of the histochemical method. When substrates such as leucyl naphthylamides were first used it was generally believed that the enzyme chiefly responsible for their hydrolysis was leucine aminopeptidase (LAP). Subsequently, a number of enzymes were described, having no LAP activity, which could hydrolyze a variety of amino acid naphthylamides. The hydrolysis of leucine naphthylamide (LNA) could be due to, among others, cathepsin B which thus may participate in the staining reaction. Cathepsin B is an intracellular acid peptidase and is known to occur in lysosomes (Pearse, 1972). Because of dispute surrounding the specificity of the histochemical method, Pearse prefers to use the term leucine naphthylamidase (LNAase) in place of the older name, leucine aminopeptidase (LAP), for the enzyme(s) hydrolyzing LNA. In this discussion LAP and LNA are treated as being synonymous.

Leucine aminopeptidase is found to be localized along the marginal edge and is always present irrespective of the nutritive state of the asteroid. This continuous production of leucine aminopeptidase, regardless of the organism's nutritive condition, has also been demonstrated in the snail, *Helix pomatia* (Rosenbaum and Ditzion, 1963), and in nine species of leeches (Jennings and Van der Lande, 1967). In planarians, aminopeptidase activity was found to parallel that of acid phosphatase. The researchers felt that when aminopeptidase activity was present in the parenchyma of starved animals that this activity was associated with the 'self-digestion' phenomenon related to its nutritional state (Rosenbaum and Rolón, 1960). Sylvén (1970) has made a detailed study of the 'leucine aminopeptidase' reaction. He considered that the ability to visualize enzymically active subcellular sites with the reaction at the light microscope level was considerable, provided that these enzymes were not scattered throughout the cytoplasm. Thus, it appears possible to localize distinctly the final reaction product (FRP) to lysosomes and autophagosomes. The 'aminopeptidase' reaction was found to parallel closely the acid phosphatase reaction of Gomori. Sylvén noted that the micro-crystalline FRP was insoluble and had a marked diffusion hindrance in tissue sections. He concluded that if the primary reaction product (PRP) is liberated and trapped in compartments containing an excess of the dye Fast Blue B (*i.e.*, in lysosomes and autophagosomes), then the rate of coupling seems rapid enough to ensure correct subcellular localization.

These results suggest that leucine aminopeptidase may, in some instances at least, be localized within lysosomes. The lysosomal activity of aminopeptidase (and, of course, acid phosphatase) would be more important to the metabolic state of starved asteroids, where it perhaps performs a necessary and significant function. In addition to the possible role of aminopeptidase in intracellular digestion, its primary function is most likely as an exopeptidase in extracellular (luminal) digestion.

Alkaline phosphatase is an ubiquitous enzyme found in many tissues of a wide range of organisms, existing as isozymes which in many cases exhibit cell and tissue specificity (Lindsay, 1970). Differences in localization most likely reflect different metabolic needs. Alkaline phosphatase is so named on the basis of its ability to catalyze the hydrolysis of organic phosphate esters. The velocity of the reaction, as is obvious from the name, is optimal at alkaline pH. Alkaline phosphatase has also been reported to possess catalytic properties. It has been shown to catalyze the phosphorylation of glucose, producing glucose-6-phosphate. Alkaline phosphatase can also phosphorylate other sugars (to a lesser extent than glucose), amino acids, and some commonly used buffers (Lindsay, 1970). This enzyme can also catalyze phosphate transfer without the intervention of water. These reactions were shown to be more specific than simple hydrolysis.

Though the biological significance for the presence or lack of alkaline phosphatase needs to be clarified, the discovery of these non-hydrolytic properties has produced some interesting suggestions as to

its role within tissues. It has been pointed out that high alkaline phosphatase activity reflects high metabolic activity (Kouvalainen, 1971), though the significance of this fact remains unknown. One theory as to the function of alkaline phosphatase is based upon its ability to phosphorylate. It is believed that as a result of this property alkaline phosphatase is involved in the transport of phosphorylated molecules through cell membranes (Posen, 1967). This is thus a form of enzyme-catalyzed active transport across a cell membrane. This theory would account for the finding that alkaline phosphatases are frequently present at the sites of presumed active transport. This theory can also explain, perhaps, the localization and fluctuation in enzyme activity observed for alkaline phosphatase in the pyloric caeca of *Pisaster*.

The function of the enzyme is, of course, closely related to the function of the organ. The pyloric caeca are known to be not only the major digestive organ, but also the major site of absorption and nutrient storage (Anderson, 1966; Greenfield *et al.*, 1958; Allen and Giese, 1966). Therefore of great importance when discussing the functions of the pyloric caeca is Ferguson's work on nutrient transport in starfish (Ferguson, 1963 b, 1964 a, b, 1968). Ferguson used ^{14}C -labelled nutrients to determine the probable routes of nutrient transport. Autoradiographs revealed dense concentrations of radioactivity in the pyloric caeca with significant amounts present in the parietal peritoneum and adjacent to the connective tissue layer. He also injected labelled substances (^{14}C -glucose, glycine, and algal

protein hydrolysate) into the perivisceral coelom and observed that these substances were readily absorbed by the body tissues, especially the pyloric caeca and to a lesser extent the coelomocytes. This study indicates that the coelomic fluid is the most important medium of transport, with transport being accomplished as part of a continual flux of metabolites between the coelomic fluid and the various tissues of the body. The coelomocytes apparently take up nutrients to fulfill their own metabolic requirements but do not play a significant part in nutrient transport.

It seems that alkaline phosphatase, through its ability to bring about phosphorylation of molecules and hence their transport across membranes, is bound irrevocably to the functions that the pyloric caeca fulfill in nutrient absorption, storage, and transport. Jennings and Van der Lande (1967), in their study of digestion in leeches, also considered alkaline phosphatase to be concerned with the absorption of material from across the gut lumen. Alkaline phosphatase was found to be consistently present within the gasterodermis of these animals. In *Pisaster*, when alkaline phosphatase is seen to be predominantly localized along the mesothelial edge (during starvation the highest level of alkaline phosphatase activity is mesothelial in localization), the enzyme is most likely involved in the transport of nutrients out of the relatively nutrient-rich pyloric caeca and into the coelomic fluid for distribution to the rest of the body tissues. During starvation, the body tissues would, of course, be in need of nourishment. It is suggested that alkaline phosphatase would catalyze the phosphorylation

of these substances and assist in their transport across the peritoneal membrane. Transport could also occur in the opposite direction, *i.e.*, into the caeca. Predominant marginal localization of the enzyme would indicate that it is functioning in this instance in the absorption of at least some of the products of extracellular digestion. The enzyme may help in the transport of phosphorylated digestive products from the lumen into the digestive epithelium for storage.

A progression of functions is postulated in the feeding animal. Shortly after feeding, alkaline phosphatase activity is mainly marginal (absorption of nutrients), shifting to a period when activity is nearly equal between the two localization sites (both absorption and nutrient transport are occurring) and, finally, activity become predominantly mesothelial in localization suggesting that mainly nutrient transport is occurring. The two different sites of activity may represent different isozymes, each fulfilling a distinct and different metabolic need. This correlates with the observations that the nutritive state of the animal, rather than the season of the year, affects the localization and activity level of alkaline phosphatase. The supposed functioning of alkaline phosphatase also helps to explain why the enzyme is observed to be present at all times even under differing conditions since the flow of nutrients is a continuous process.

It has been mentioned that coelomocytes, though not major vehicles in nutrient transport, do take up some material for their own maintenance. It would seem reasonable to expect alkaline phosphatase to be present in these cells to assist in their active nutrient uptake. Indeed, Lindsay

(1970) in his work on the isozymes of alkaline phosphatase in *Strongylocentrotus purpuratus* was able to localize enzyme activity within these cells. He found that the large gut of the echinoid had no intrinsic alkaline phosphatase, the activity here being due to the presence of coelomocytes. In the pyloric caeca of *Pisaster*, though the enzyme is intrinsic to the tissue, there also is evidence that some enzyme activity is found within the coelomocytes where it presumably functions in nutrient uptake (Fig. 36).

Esterases hydrolyze the carboxylic acid esters of alcohols, phenols, and naphthols. The majority are both hydrolytic and synthetic in action (Pearse, 1972). If a simple ester such as methylbutyrate or naphthyl acetate is the substrate concerned, the enzyme is classed as a non-specific esterase of which there are several types; carboxyl esterases, arylesterases, and acetylersterases. If the substrate is the ester of a higher fatty acid with glycerol (or some other alcohol) the enzyme concerned is a lipase. However, there is some degree of overlap between the two because esterases also act on fatty acid esters. Broadly speaking, the esters of short-chained fatty acids ($C_2 - C_4$) are acted upon by esterases while long-chained fatty acids ($C_8 -$ upwards) are acted on by lipases. The full list of esterase types now histochemically identifiable, according to Pearse (1972) are:

- i) A-esterase (carboxylesterase), E.C. 3.1.1.1;
- ii) B-esterase (arylesterase), E.C. 3.1.1.2;
- iii) C-esterase (acetylersterase), E.C. 3.1.1.6;
- iv) Acetylcholinesterase (AChE; true cholinesterase), E.C. 3.1.1.7;
- v) Cholinesterase (ChE; non-specific, pseudocholinesterase), E.C. 3.1.1.8.

Figure 36

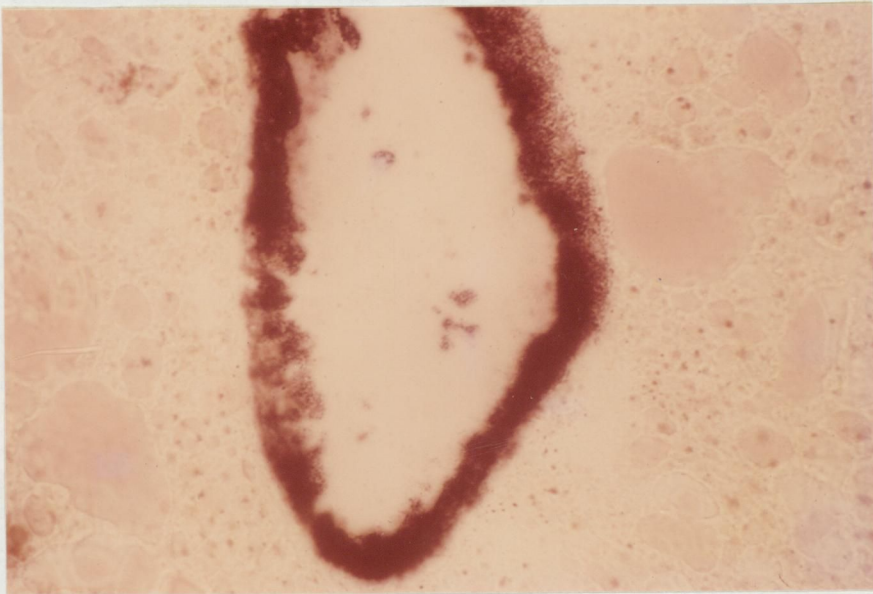
Figure 36. Cryostat section (12 μ); alkaline phosphatase activity; 18 hours after feeding. Note the heavy black staining of the mesothelial region indicating alkaline phosphatase activity. The arrows indicate clumps of coelomocytes that are staining positively for alkaline phosphatase activity.

Fresh frozen section; α -naphthyl phosphate; pH 9.2; Fast Blue RR. x 250.

ME - mesothelial activity

LD - lipid droplets





Different esterase enzymes are distinguished from each other by substrate specificity, pH optima, inhibitors and activators, *etc.* In the field of esterase histochemistry, the use of inhibitors and activators is of utmost importance in separating the different enzyme types. For instance, the distinction between the true lipases and the non-specific esterases is based on the observation that lipases are activated by low concentrations of the salts of bile acids, especially sodium taurocholate whereas non-specific esterases are inactivated. The non-specific esterases are sub-divided into A, B, and C types on the basis of inhibitor and activator studies using the organophosphate compounds, DFP (diisopropylfluorophosphate) and E600 (diethyl p-nitrophenyl phosphate), and sulphhydryl-blocking agents (*e.g.* p-mercuribenzoate). The cholinesterases are separated from the other two groups of esterases by their ability to hydrolyze esters of choline and their inhibition by eserine; AChE is distinguished from ChE by the use of specific inhibitors (*e.g.* DFP).

It has long been recognized that within the four main esterase groups (carboxylesterase, arylesterase, acetyesterase, cholinesterase) there are multiple forms of the enzymes (iso-enzymes). These individual isozymes may act on the same substrate with the same pH range. They can be separated by electrophoretic and chromatographic techniques.

Most of the substrates used for the histochemical demonstration of esterases are hydrolyzed by a number of different enzymes. The α -naphthyl acetate method for esterase using the diazonium salt Fast Blue B as utilized in this research is not only hydrolyzed by esterase, but by

lipase, AChE, and ChE as well. To differentiate clearly which enzymes are present in the pyloric caecum of *Pisaster*, an electrophoretic study coupled with the use of inhibitors, activators, and different substrates would be needed.

The metabolic function of the simple esterases are not known (Pearse, 1972). However, the non-specific esterase activity demonstrated within the cytoplasm of the caecal epithelial cells is most likely confined to the storage cells where it fulfills its hydrolytic capacity on the storage products of the organ. The abundance of lipid deposits within the epithelium strongly suggests the presence of lipase activity within the caecum; however, conflicting evidence (Chapeaux, 1893; Stone, 1897; Van der Heyde, 1922; Sawano, 1936; Das *et al.*, 1972) exists concerning this assumption.

Esterase activity was observed at all times during this study. As nutrient transport is a continuous process and as the esterases no doubt function in the hydrolysis of nutrient stores in the pyloric caecum, the consistent presence of the esterase complex within the epithelium is not remarkable but merely reflects its function within the pyloric caeca.

From this study it can be seen that enzymatic functioning as visualized at the cellular and tissue levels reflects functioning at the organ level in the pyloric caecum of *Pisaster*. For a long time mystery has surrounded the pyloric caecum as to its probable functional significance. Earlier researchers concluded that the pyloric caecum was analogous to the vertebrate pancreas. It seems more likely,

however, that the pyloric caecum is a complex organ, fulfilling a variety of functions in the metabolism of the starfish and having no vertebrate equivalent.

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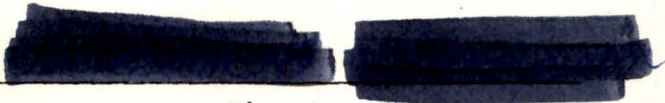
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