THE ULTRASTRUCTURE AND FUNCTION OF THE GUT OF PATELLA VULGATA.

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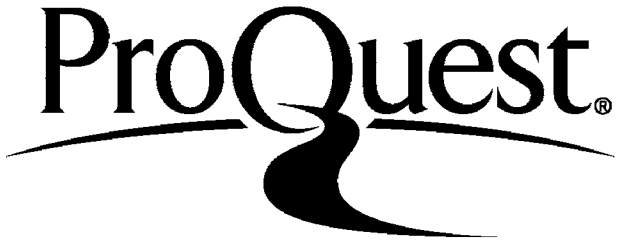

I dedicate this work to my parents, whose co-operation and financial support made it possible.

ABSTRACT.

The limpet gut consists of a long coiled tube lined by a ciliated columnar epithelium, into which open the ducts of the salivary and digestive glands. Six sections can be distinguished: the buccal cavity, oesophagus, stomach, style sac, intestine and rectum. The oesophagus can be subdivided into a dorsal food channel and a series of lateral pouches forming the oesophageal gland. The intestine is divisible into sections designated A, B, C, D and E.

Ultrastructural and histochemical analyses revealed nine cell types, seven of them glandular. A single type of gland cell lining the tubules of the salivary gland produces a viscous secretion that lubricates the radula and entraps particles rasped from the substratum. Mucous cells occur in the buccal cavity, dorsal food channel and rectum; in the rectum, mucus aids defaecation, but elsewhere it entraps loose particles that are consequently transported to the stomach. The only extracellular enzyme, an amylase derived from the gland cells of the oesophageal gland, is mixed with the food in the dorsal food channel.

Ciliated and unciliated columnar cells lining the ducts of the digestive gland, stomach, style sac and anterior intestine, release blebs of cytoplasm into the lumen to consolidate loose particles into a faecal rod that is rotated along the intestine. Clavate gland cells and possibly basal gland cells in the posterior intestine, cover the faecal rod with their secretion to form a durable rod.

The vacuolated digestive cells of the digestive gland, digest food intracellularly releasing undigestible residues in spherules of cytoplasm, these are bound into a liver string by the proteinaceous

secretion of the basophilic cells. Both these cell types and the amylase-secreting cells exhibit phases of activity, but only that of the latter is related to the tidal cycle.

Tritiated D-glucose was absorbed by the oesophagus, intestine and digestive gland by a mechanism inhibited by 2,4-DNP and phloridzin. The mechanisms operating in the oesophagus and posterior intestine were sodium-dependent. Fluid movements from the intestinal lumen to the blood occurred.

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INTRODUCTION

The common British limpet, Patella vulgata Linnaeus, 1758 has a geographical distribution on the European shores from the Northern Mediterranean to the Lofoten Islands of Norway, excluding the east coast of the North Sea, Kattegat and the Baltic Sea. It has an upper limit of distribution between MHWN (mean high water neap tide) and MHWS (mean high water spring tide), the lower limit is in the upper Laminaria zone. This vertical zonation may be affected by local factors such as the degree of shelter, competition from other species and shade. It inhabits any rocky coast with a firm enough substratum for its attachment (Fretter and Graham, 1976).

Due to the abundance of the species it has attracted the interest of biologists as a subject for study. Aristotle was the first to mention the limpet and particularly noted its homing ability. Gibson (1887) reviewed the literature to date which consisted of general descriptions and descriptions of selected organ systems excluding the gut. His account is erroneous in several aspects and his second paper concerning the animal's physiology was not published. Wegmann (1887) produced his paper on the organisation of P.vulgata, abstracts from which formed the majority of Haller's (1894) account. The limpet's power of adhesion, homing behaviour and habits were considered by Aubin (1892), Morgan (1894) and Davis (1895) respectively. Simroth compiled the work of Haller and Amaudraut (1898) to give the account in Bronn (1896-1907). A histological and morphological investigation of the gut was included by Davis and Fleure (1903) in their memoir on Patella.

There have been numerous general ecological studies (Russell, 1907; Orton, 1929; Eslick, 1940; Evans, 1947; Thompson, 1980) and on the reproduction and growth of the limpet (Orton, 1920, 1928a & b; Smith, 1935; Das and Seshappa, 1947; Dodd, 1956; Orton, Southwood and Dodd, 1956; Choquet, 1971). Branch (1981) gives an excellent review of

limpet biology.

P.vulgata is subjected to wetting and drying during each tidal cycle and consequently endures the stressful factors of desiccation and considerable variations in temperature and salinity which determine zonation patterns. During exposure to hot terrestrial conditions, limpets lose water bringing about ionic concentration of the blood (Wolcott, 1973), animals living higher on the shore are more tolerant to water-loss. Tolerance may not be the only factor that determines the upper zonation limit, the limpets must be able to recover this water rapidly once submerged. After losing 28% of their body water, individuals of P.vulgata can recover this in just under 4 hours (Davies, 1969). Orton (1933) reported limpet deaths due to prolonged exposure to hot weather conditions, although limpets higher up the shore survived.

Most limpets feed at fixed periods of the day related to the tidal cycle. Four types of activity rhythms have been recorded for P.vulgata: movement while awash, day or night; movement when submerged, day or night; movement when emersed at low tide only at night and movement during nightime submersion (Branch, 1981). The four patterns reflect differences in the local habitat which reduce the effects of desiccation. The ability of an individual to return to its "home scar" reduces the likelihood of desiccation because the shell can be clamped down tightly thus minimising contact with the air. Scar formation is believed to be by chemical softening of the substratum by the pedal mucus and a carbonic anhydrase from the mantle edge, it can then be scraped away by the radula (Branch, 1981).

The mechanism for homing was considered to be by individuals retracing their outward path from the scar (Fretter and Graham, 1976), but P.vulgata may vary the route home ignoring the mucus trail. Transplanted limpets may follow the trails of conspecifics and settle on their vacated scars, but recognise these as distinct from

their own. Cook, Bamford, Freeman and Teideman (1969) eliminated the mucus trails by scrubbing and altered the topography of the scar; although they reduced the numbers successfully homing, some limpets found their particular scars. This suggests that the mechanism is not simple and the limpets may even "learn" their local rock topography.

Limpet body temperature is always greater than the ambient value and often higher than the substratum; body temperatures of P.vulgata may vary from 5-34°C during emersion at midday so they experience a large range. The cause of thermal death has been attributed to the disruption of intracellular ionic regulation leading to neuro-muscular failure (Branch, 1981).

P.vulgata is an osmoconformer and its only chance of avoiding extreme salinities is to tightly clamp the shell against the substratum and exclude the external medium (Hoyaux, Gilles and Jeuniaux, 1976). This was demonstrated by Arnold (1957) when he dripped hypoosmotic saline onto limpets; he found that this response was elicited by the concentration of sodium and chloride ions in the saline rather than its osmolarity. Chemoreceptors in the cephalic and mantle tentacles perceive salinity. Intracellular ionic concentrations are maintained by minimising the exchange of ions and water with the blood. This is achieved by regulating the cell osmolarity by controlling the intracellular pools of proline and arginine (Florkin and Scheer, 1972).

From this brief account it is clear that the "Patellacea form an extraordinarily successful group of limpets, many enduring severe intertidal conditions" (Fretter and Graham, 1976).

Most metazoans have a digestive system in which digestion may occur intracellularly in the cells lining the digestive tract, extracellularly in the gut lumen or both. The nature of the food determines the differentiation of the gut and the predominance of extracellular or intracellular digestion. Intracellular digestion

occurs in members of groups that are considered to be primitive regarding their phylogenetic position; Porifera, Cnidaria, Platyhelminthes and Xiphosura. The last three examples are macrophagous carnivores. This method of digestion is also suited for processing suspended particulate food and is typical of microphagous feeders such as bivalves, brachiopods, rotifers and cephalochordates. Intracellular digestion requires that: the food exists in a particulate form capable of being endocytosed by a large surface area of absorptive-digestive cells; that feeding is continuous and that only particles of a suitable size are presented to the ingesting surface (Purchon, 1978).

Little is known about the early evolution of the invertebrates during the Pre-Cambrian era. A primitive gut probably only possessed a single cell type which served several roles; absorption, secretion, excretion and the ciliary transport of food through the gut. At some point such "multipurpose digestive cells" could have become specialised in one or two such functions and could be aggregated into glandular areas connected to the gut by ducts. This was correlated with the development of a muscular digestive tract concerned with the transport and sorting of food particles, into which the glands could add their respective secretions. Differentiation of the digestive system together with the appearance of extracellular digestive enzymes enabled new food sources to be exploited, including the elaboration of the carnivorous habit.

The primitive condition of digestion in the Mollusca was microphagous feeding followed by intracellular digestion. Most extant species have a phase of extracellular digestion to break down large pieces of food into a form that can be endocytosed by the digestive cells of the digestive gland and digested intracellularly. Such is the case for P.vulgata which is a generalised feeder grazing on microflora and detritus rasped from the rock face.

The structure and function of the gut as reported by Graham (1932) is summarised below. Food mixed with saliva is passed into the oesophageal food channel which is lined by a ciliated epithelium bearing subepithelial mucous cells which add their secretion to the food mass. The saliva acts to lubricate the radula and lacks enzymes. The only extracellular enzyme in the gut is an amylase produced by gland cells in the oesophageal gland; it has an optimum pH of 6.2 at 30°C and is released from the cells as granules within a cytoplasmic bleb.

The blebs are carried into the dorsal food channel by ciliary currents and mixed with the food-mucus mass to initiate carbohydrate digestion. The mass is worked into a string which passes into the stomach and is deflected into the aperture of the digestive gland duct by a muscular fold of the stomach wall. Food is absorbed only by the absorbing cells of the digestive gland tubules, whilst the secreting cells produce a cement that binds undigested material from the absorbing cells into a string that passes into the intestine. A protease with pH optima of 5.6 and 8.2 at 30°C is present in the gland.

The intestine, divisible into five histologically distinct regions designated A,B,C,D and E is lined by a ciliated epithelium with basal gland cells in the last three sections and clavate gland cells in section D. The secretions of these gland cells bind the faecal rod into a semi-rigid, cemented form which does not disintegrate and foul the mantle cavity once voided.

Graham (1939 and 1949) briefly described the oesophagus and stomach of P.vulgata respectively, relating these regions to those of other selected molluscs, later turning his attention to the functioning of the buccal mass (1964). Pugh (1963) examined the cytology of the salivary and digestive glands. There have been numerous investigations of the enzymes present in the digestive gland. Billet and McGee-Russel

(1955), Marsh and Levvy (1958) and Levvy and McAllan (1963) detected β-glucoronidase, β-fucosidase and β-galactosidase. Cellulase and chitinase activity were reported by Stone and Morton (1958) and Jeuniaux (1963 in Hyman, 1967) respectively. Sumner (1969) localised a non-specific esterase in both cell types lining the tubules, whilst Hass (1979) described a carboxypeptidase. Lipases have not been reported.

The following equation represents the energy budget for a limpet:

$$C=F+U+R+Pg+Pr+Pmuc$$

where C is the energy consumed and F the amount lost in the faeces. Assimilated energy may be partly lost in excretion (U) and as metabolic heat in respiration (R). Pg and Pr represent the energy used in growth and reproduction respectively, whilst a proportion is channelled into secretion of mucus (Pmuc) (Branch, 1981). An energy budget for a population of P.vulgata was produced by Wright and Hartnoll (1981), they calculated that 44.9% of the energy consumed by an individual is assimilated, the remaining 55.1% is lost in the faeces.

Since Graham's histological report there has been a revolution in microscopical technique. The advent of transmission electron microscopy (TEM) has enabled the ultrastructure of the cell to be examined. Cell fractionation followed by differential centrifugation separates the different cell organelles into layers, which can then be used in biochemical tests and examined by TEM to indicate the biochemical functions of identified organelles. Enzyme localisations have revealed a lot about the functions, interrelationships and derivation of lysosomes, the golgi complex and the endoplasmic reticulum (ER). Autoradiographic experiments have demonstrated the routes taken by components of secretory products and which cells are involved in the uptake of certain labelled molecules. Radiotracer techniques demonstrated the ability of tissues to absorb and transport

certain ionic and organic ligands. All of these techniques have allowed correlations between ultrastructure and function and shown that cells performing similar functions have ultrastructural features in common.

The new repertoire of histo- and cytochemical methods enable the reliable and precise demonstration of chemical groups and enzymes. Digestive enzymes can now be detected histologically by using substrate-film methods (Daoust, 1965). A large range of lysosomal enzymes can also be localised allowing certain cellular processes to be followed.

Various modifications of the Periodic acid-Schiff (PAS) method have been described for the localisation of PAS-positive material in ultra-thin resin sections (Thiéry, 1967; Hayat, 1975). The mechanisms of staining are similar to that of the original PAS method in that 1,2-glycol groups (CHOH-CHOH) in carbohydrate-rich inclusions are oxidised by periodic acid (PA) to a dialdehyde (CHO-CHO). These aldehydic groups are then localised by the deposition of metallic silver that leaves an electron-opaque deposit at the reaction site instead of a red colour as in the PAS method.

The most useful staining reactions employ silver methenamine (SM), thiosemicarbazide (TSC) and thiosemihydrazine (TCH). Gomori (1946) introduced SM at the light microscope level to demonstrate glycogen and mucin, later its use in the electron microscope was described by several authors (Marinozzi, 1961; Van Heyningen, 1965; Rambourg, 1967; Rambourg and Leblond, 1967). The aldehydes produced by oxidation reduce the SM to metallic silver which precipitates as granules (3-4nm) on the original sites of the 1,2-glycol groups.

Characteristically, non-specific staining is frequently encountered when using SM because any reducing agent in the tissue will precipitate silver. Such agents may pre-exist in the tissue (aldehyde, ketone and sulphydryl groups, and alpha amino alcohols) or they may be

added during the fixation process. Much concern therefore centers around the choice of fixative for tissues subjected to these staining procedures. Tissue fixed in osmium tetroxide causes a high density of silver granules to mask the outline of the sites on which they are deposited; the specificity of the reaction is also diminished. It is thus advisable to avoid using osmium and use tissue fixed only in glutaraldehyde to reduce any non-specific reactions (Hayat, 1975).

Non-specific staining does occur even when tissues are fixed in glutaraldehyde alone (Burr and Evert, 1973). Rambourg and Leblond (1967) demonstrated that such treatment can produce non-specific staining of nuclei, collagen, ribosomes and certain pigments. Yamane and Davidson (1962) suggested that silver cations react with the purine and pyrimidine bases of nucleic acids, but Smith and Stuart (1971) proposed that the staining of nucleoproteins is due to silver binding to a histone moiety in the protein. Silver methenamine may also oxidise chemical groups in the tissue that reduce silver (Hayat, 1975). Considering the number of factors that may introduce non-specific staining when SM techniques are employed, it is essential to perform a comprehensive series of controls to eliminate false interpretation of the results (Courtoy and Simar, 1974).

The other staining reaction mentioned earlier utilizes the two aldehyde semicarbazones TSC and TCH, which can condense with aldehydes yet still retain their ability to reduce osmium and silver cations by virtue of their hydrazine group. In the original procedures (PATCO and PATO) developed by Seligman, Hanker, Wasserkreig, Dmochowski and Katzoff (1965), osmium deposits indicated the presence of 1,2-glycol groups. The major drawbacks of this method were the cost and unpleasant nature of osmium tetroxide and the coarse staining reaction.

In 1967 Thiéry reviewed the methods for cytochemically staining carbohydrates and introduced a modification of the technique used by Seligman et al. (1965), in which a solution of silver proteinate replaces the osmium vapour. This new method, PA-TSC-SP, is more specific in its reaction, causes little background precipitation and produces a delicate staining of the reactive sites. This last attribute revealed a definite substructure to glycogen particles (Anderson and Personne, 1970). This method is probably the most reliable for the demonstration of glycogen in thin resin sections (Vye and Fischman, 1971).

Buma and Roubos (1983) reported the use of a tannic acid-ringer solution to demonstrate at the ultrastructural level, the exocytotic release of secretory products from endocrine, neurohaemal, synaptic and non-synaptic release sites. During incubation of fresh tissue in this solution, exocytosis proceeds but the released material is fixed by the tannic acid before it can diffuse away into the extracellular space which would render it invisible in the TEM. Wagner (1976) used tannic acid fixatives to increase the electron-opacity of the exterior surface of unit membranes. This happens when the tannic acid combines with basic proteins at the cell surface and also subsequently chelates heavy metal cations from the counterstaining solutions, so it can also be used to follow endocytosis.

Numerous general histological studies of the gastropod gut have been published: prosobranchs (Andrews, 1965; Campbell, 1965; Ward, 1966; Lufty and Demian, 1967; Demian and Michelson, 1971; Balaparameswara Rao, 1975); opisthobranchs (Howells, 1936 and 1942; Millot, 1937; Graham, 1938; Fretter, 1939); pulmonates (Carriker and Bilstad, 1946; Ghose, 1963). The gastropod alimentary canal is a simple tube lined by a ciliated columnar epithelium coiled through the visceral mass. Associated with it are three glandular areas; salivary glands opening into the buccal cavity, the digestive gland joining the

stomach and in prosobranchs, an oesophageal gland developed to different extents.

Some authors have concentrated on one or two regions of selected species: salivary, oesophageal and digestive glands (Bhanu, Shyamasundari and Hanumantha Rao, 1981a and b); the stomach and digestive gland have received most attention (Owen, 1955, 1956, and 1958; Sumner, 1965b and 1966b; Palmer, 1979) and the rectum very little (Greenberg and Jegla, 1962). A number of investigations of the intestine were undertaken to demonstrate the existence of endocrine cells (Boquist, Falkmer and Mehrotra, 1971; Fritsch, Van Noorden and Pearse, 1976; Fritsch and Sprang, 1977; Plisetskaya, Kazakov, Soltitskaya and Leibson, 1978). Pacheco and Scorza (1971) and Pacheco (1973) compared the fine structure of the intestinal cells of aestivating and active Pomacea urceus (Müller). Bowen (1970) localised acid phosphatase activity in the cells of the crop and intestine of Arion ater (L.). Giusti (1970) and Judd (1979) examined the style sacs of certain bivalves to determine the cells involved in style secretion; Judd's is the only existing account of the use of the PA-TSC-SP method in a study of the molluscan gut.

The digestive gland has received a great deal of attention reflecting its importance in the digestive process; most authorities agree that it is the main site of absorption (Yonge, 1926b; Graham, 1932; Owen, 1955; 1958; 1970; 1972a and 1973; Fretter and Graham, 1962; Sumner, 1965a, 1966a, b and c; Purchon, 1978). Owen and Sumner were the pioneers of ultrastructural studies of the digestive gland of selected bivalves and pulmonates. McQuiston (1969a), Pal (1971 and 1972) and Walker (1970a) continued the work until Owen (1972a) reviewed the work to date pointing out where future research would be profitable: determining the relation of phasic activity to the tidal cycle and the mechanism by which the tubule epithelium is renewed after each cycle of activity.

These studies have revealed that the digestive gland tubules are lined by at least two mature cell types: one involved in the absorption of food and its subsequent intracellular digestion; the other being a basophilic cell to which various functions have been attributed. The absorbing or digesting cell is characterised by the presence in its cytoplasm of various, diverse, membrane-bound vesicles forming the heterophagic-lysosomal system of the cell. Feeding experiments using coarse particles in suspension demonstrated their accumulation in these vesicles (Graham, 1932 and 1938; Fretter, 1937 and 1939; Owen, 1972a; Nelson and Morton, 1979; Palmer, 1979).

The use of ferritin suspensions has enabled the pinocytotic pathway to be visualised in thin resin sections (Owen, 1970, 1972a and 1973; McLean and Holland, 1973). The terminology used by Owen (1972a) is adopted here. Ferritin is initially concentrated against the luminal surface of the apical membrane which invaginates locally to form "coated" pits and vesicles called pinosomes; pinocytotic activity may be greater if, for instance, pigeon blood is fed instead of ferritin (Owen, 1970). It is then concentrated in another type of apical vesicle (heterophagosome) which lacks enzyme activity. The largest vesicles (heterolysosomes) have acid phosphatase activity and are believed to be the site of intracellular digestion, the soluble products of digestion diffusing into the cytoplasm leaving the indigestible remains in the lysosome which transforms into a residual body. These vesicles are ejected from the cell either in clusters within a cytoplasmic bleb or singly across the apical membrane, depending on the species.

The basophilic cell ultrastructurally resembles the exocrine cell of the mammalian pancreas and has been implicated in either the synthesis of enzymes (McQuiston, 1969a) for extracellular digestion in the tubule lumen or a cement-like secretion (Graham, 1932). Yonge (1926b) considered them to be regenerative cells replacing the

digestive cells. Their exact function is still unknown and further complicated by the discovery of a second type of basophilic cell with the ultrastructural features of an undifferentiated cell (Owen, 1972a). Owen (1970) proposed that this cell type may give rise to digestive cells. McQuiston (1969a) suggested that a group of cells restricted to the blind ends of the tubules replace both cell types.

The digestive gland of marine molluscs may exhibit tide-related activity patterns. Species such as Littorina littorea (L.) and Maoricrypta monoxyla (Lesson) occurring at low tidal positions are submerged during the greater part of the tidal cycle and so interruptions in their feeding patterns are not severe; these animals do not show tide-related activity cycles (Merdsoy and Farley, 1973; Nelson and Morton, 1979). Other species which have interrupted feeding patterns and access to food limited to short periods, tend to have tide-related cycles. At least two patterns of cyclical activity occur: the monophasic and diphasic cycles. The first is of 12 hours duration allowing feeding at the folowing tidal cycle; Cardium edule L., (Morton, 1970), Littorina saxatilis (Olivi) (Boghen and Farley, 1974), Rissoa parva (Da Costa) (Wigham, 1976) and Venerupis decussata (L.) (Mathers, Smith and Colins, 1979) exhibit such activity. In some species in which the physiological processes of digestion are slower there is a diphasic pattern in which half the tubules are digesting food that was ingested during a previous cycle and the other half is ready to absorb food consumed during the subsequent cycle: the two tubule conditions are thus 12 hours out of phase with each other. Chlamys varia L. (Mathers, Smith and Colins, 1979), Lasaea rubra (Montagu) (McQuiston, 1969b) and Pecten maximus L. (Mathers, 1976) all show a diphasic cycle.

The salivary glands have received less attention than the digestive gland. Except for the investigations of Pugh (1963) and Lufty and Demian (1967) on P.vulgata and Marisa cornuarietis (L.)

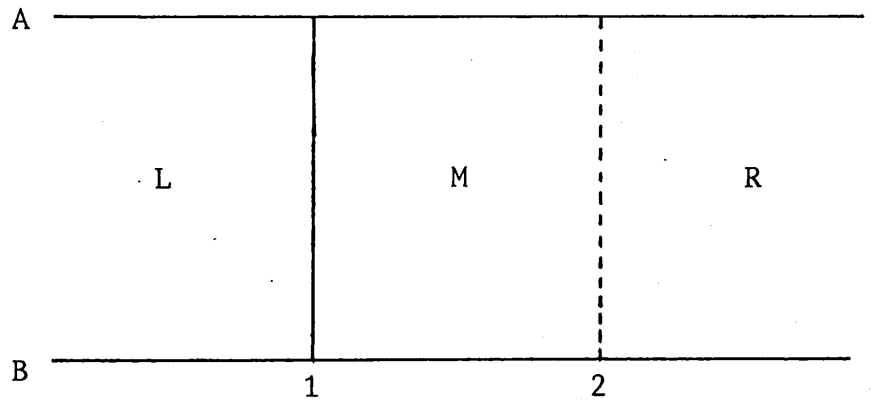
respectively, there are no detailed studies of archaeo- or mesogastropods. Bhanu, Shyamasundari and Hanumantha Rao (1981a) and Schultz (1983) examined the glands of Thais bufo (Lamarck) and two species of Conus respectively. Pulmonates have invoked the most interest. Fine structural investigations of the glands of Lymnaea stagnalis L. were undertaken by Boer, Wendelaar Bonga and van Rooyen (1967) to clarify the earlier conflicting reports of Carriker and Bilstad (1946) and Gabe and Prenant (1948). The salivary glands of the slugs, Agriolimax reticulatus (Müller) and Limax maximus L. were studied by Walker (1970b) and Beltz and Gelperin (1979). Early studies of the pulmonate glands suggested that the salivary cells underwent a secretory cycle, but the recent ultrastructural studies indicate that this is not so. Each "phase" represents a cell type producing a particular type of secretion.

Generally in the Stylommatophora, the salivary glands produce most of the extracellular enzymes (Purchon, 1978), but in the lower prosobranchs their secretion acts as a lubricant for the rasping process of the radula and as an adhesive for food particles (Fretter and Graham, 1962). The ciliated salivary ducts transport the saliva from the tubules to the buccal cavity, but in A.reticulatus and L.maximus the ducts are lined by an epithelium with the features of a transporting epithelium, so they may somehow alter the saliva.

Only in the last 25-30 years has reseach been directed towards the phenomenon of solvent-solute transport across epithelia and this has been concerned mainly with vertebrate tissues. Ultrastructural investigations like those on amphibian skin (Voûte, 1963; Voûte and Ussing, 1968), "salt glands" (Cowan, 1971; Komnick, 1963 and 1964), fish gills (Kessel and Beams, 1962; Threadgold and Houston, 1964), mammalian kidney (Pease, 1955), vertebrate intestine (Trier, 1963), insect midgut (Berridge, 1970; Jones and Zeve, 1968) and insect rectum (Berridge and Gupta, 1967) have revealed that certain cells/epithelia

involved in solvent-solute transport have characteristic ultrastructural features; the association of numerous mitochondria with the increased apical and basal surface areas and the development of lateral intercellular spaces.

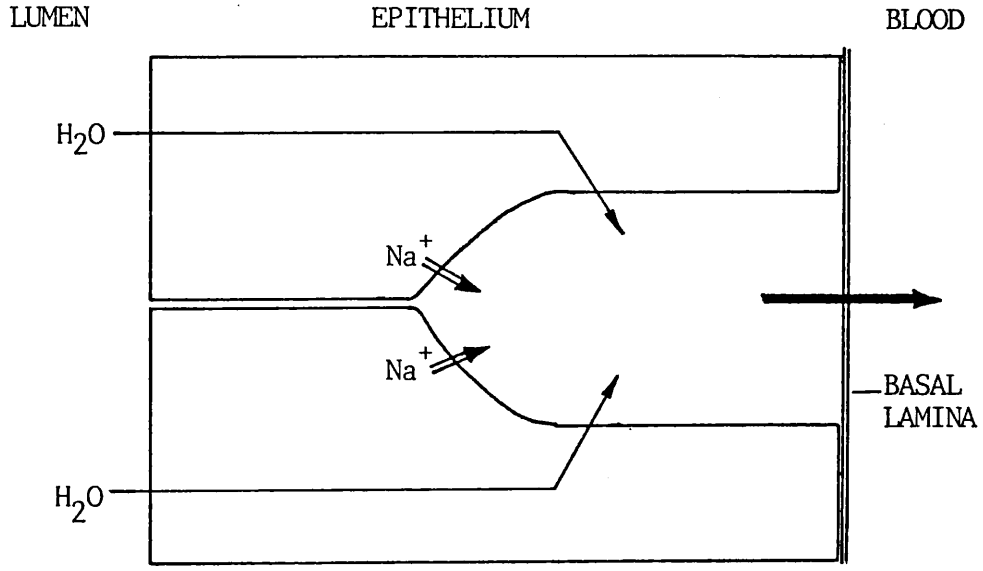
During the early 1960's, Curran and his colleagues (Curran, 1960 and 1965; Curran and Macintosh, 1962; Ogilvie, Macintosh and Curran, 1963) developed their "three-compartment, double-membrane model" to explain intestinal absorption. Consider the diagram below where the walls A and B are inelastic; membrane 1 is permeable to water but not solutes and membrane 2 is permeable to both.



Solutes actively transported across membrane 1 into compartment M, raise the solute concentration there, creating an osmotic flow of water from L into M. This results in an increased hydrostatic pressure in M that forces both solute and solvent through membrane 2 into R.

Kaye, Wheeler, Whitlock and Lane (1966) correlated this model with an ultrastructural and physiological investigation of the fluid transport in rabbit gall bladder. They found that the intercellular space could correspond to compartment M of Curran's model. Solute transport into this space, they proposed, would bring about an osmotic gradient that water moves down, thereby increasing the hydrostatic pressure against the lateral cell membranes and causing fluid flow into the blood (compartment R).

Later, Diamond and his colleagues (Diamond and Bossert, 1967 and 1968; Diamond and Tormey, 1966a and b; Tormey and Diamond, 1967) further refined this model. They suggested that a standing osmotic gradient exists within the intercellular spaces or channels. Solute pumps located at the apical end of the channels make the fluid in that region hyperosmotic to the cytoplasm, water flows into the channel to equalise the osmotic gradient resulting in the formation of an isosmotic absorbate. The walls A and B and compartments M and R in Curran's model above, are equivalent to the lateral cell membranes, intercellular space and blood respectively in the diagram below.



This model has been used to explain absorption, resorption and excretion by "salt glands", vertebrate intestine and kidneys and insect midgut, rectum and malpighian tubules.

Up until the second half of this century the concensus of opinion was that the molluscan intestine was a mere passive conduit concerned with the formation and elaboration of the faecal rod and its transport to the anus (Graham, 1932; Yonge, 1935 and 1937). Unlike the digestive gland, the intestine showed no evidence of absorption. This "classical theory" has only recently been seriously questioned, even though a great deal of evidence has been collated this century to indicate that absorption by the alimentary canal does occur.

Jordan and Lam (1918) reported that soluble substances could diffuse through the intestine of Helix pomatia L. Heidermanns (1924, in Carriker and Bilstad, 1946) showed that the ciliated cells lining the gut of L.stagnalis could absorb carbohydrates and fats. Yonge (1926a) and Graham (1938) both demonstrated the uptake of iron saccharate by the stomach epithelia of Ostrea edulis L. and Aeolidia papillosa (L.) respectively. Fretter (1952 and 1953) using material labelled with ^{131}I, ^{32}P and ^{90}Sr demonstrated the permeability of the intestine of H.pomatia, Helix aspersa L., Agriolimax agrestis (L.) and Arion hortensis (Fer.) to these isotopes and showed that the intestine of Lepidochitona cinereus (L.), Mytilus edulis L. and P.vulgata could absorb ^{90}Sr ions. Lipid absorption by the intestine of H.pomatia was reported by Guardabassi and Ferreri (1953) and Owen (1956) reported that the stomach and anterior midgut of Nucula sulcata Bronn absorbs the soluble products of extracellular digestion.

It was not until the late 1960's that Fretter's radiotracer experiments were pursued by Greer and Lawrence (1967) and Lawrence and Lawrence (1967) who demonstrated, by a series of elegant experiments, the active absorption of sugars and amino acids by the intestine of Cryptochiton stelleri (Middendorff). The anterior intestine absorbs both D-glucose and 3-O-methylglucose using the same carrier system which is reversibly inhibited by anaerobiosis; D-galactose is only absorbed by the posterior section, by a carrier resistant to anaerobiotic inhibition.

This suggests the presence of two carrier systems in the chiton intestine, in the mammalian small intestine these three monosaccharides are transported by the same carrier (Crane, 1960). Water resorption is not coupled to solute transport by the chiton intestine and in this respect too it differs from the vertebrate small intestine. Fretter (1937) reported that the chiton intestine was not

absorptive, but this, explain Lawrence and Lawrence (1967) is because she used substances (iron saccharate, blood corpuscles, olive oil emulsions) not involved in transport systems.

McLean (1970) reported that the crop (=mid-oesophagus) of Haliotis rufescens Swainson absorbed glucose and phenylalanine and that the intestine is permeable to glucose. These small molecules are retained by the crop cells with only small amounts reaching the blood. After feeding the animals labelled algae, autoradiography demonstrated that the digestive cells of the digestive gland are absorptive. Hanish and Lawrence (1972) reported purine and pyrimidine absorption by the intestine of C.stelleri and Robbins (1975) demonstrated the active uptake of D-glucose and D-galactose by the intestine of this species.

An active, sodium-dependent, carrier-mediated transport system for L-alanine is present in the mid-gut of Mya arenaria (L.); four other amino acids, two hexoses and a fatty acid are also absorbed (Stuart and Bamford, 1976). Forester (1977) working with the intestine of H.pomatia, found that nutrients are absorbed by facilitated diffusion and not by a mechanism like that of the chiton intestine. He suggested that the snail gut is primarily concerned with the resorption of water, an adaptive feature of a terrestrial mollusc. Orive, Berjon and Fernandez Otero (1979 and 1980) reported the active absorption of L-proline by the intestine of H.pomatia and that in Arion empiricorum (Fer.), L-glutamic acid is transported by mediated diffusion in the posterior intestine and by simple diffusion in the anterior section. L-glutamic acid is metabolised by the epithelia of both species.

Considering the advance in techniques since Graham's original histological study, a new investigation of the gut of P.vulgata offered an excellent opportunity to extend his earlier work using ultrastructural, analytical and tracer techniques not available to him in 1932.

MATERIALS AND METHODS

COLLECTION SITE.

Healthy specimens of P.vulgata were identified, using the key given by Fretter and Graham (1976), and collected from a concrete, barnacle-encrusted beach-breaker at Rottingdean, East Sussex. At high tides the breaker is completely submerged and the limpets are inaccessible. Low tides reveal its length and the chalky substratum that (littered with free Laminaria fronds) lies between neighboring breakers and below the stony upper beach. Fucus serratus was common on the breaker.

COLLECTION, TRANSPORT AND MAINTENANCE.

Limpets were always collected at low tide from the mid-section of the breaker, specimens with shells approximately 10-30mm long were selected for microscopy but the largest (40-50mm) ones were used for the radiotracer experiments. Animals that were roughly detached from their scar usually died within a few days, but if the following method was used nearly all survived. With a blunt, stout knife one part of the shell circumference was lifted so that the blade could then be slid against the substratum and under the foot; this reduced the force of attachment allowing gentle removal of the limpet.

A 5L plastic bucket lined with Laminaria fronds and half full with clear sea water was used to transport the limpets to the laboratory aquarium. If the collected limpets were placed upon the algal fronds they usually remained attached, making transfer to the 15L plastic aquarium tank a simple operation. More fronds were packed into the bucket which was then sealed with a tight-fitting lid. The aquarium tank was arranged to maintain a continually advancing 12 hour tidal cycle (Jennings, 1984) upon which was imposed a 12 hour day-night periodicity. Ambient temperature was kept at 10°C. The tidal cycle of the tank was synchronised with that at Rottingdean and as it filled, the water was aerated by compressed air bubbling through

two air stones. The tank was inspected every other day to check the water flow, aeration and to remove any dead limpets and algae.

Three feeding methods were tried. The first involved making films of sodium alginate on glass squares and placing them in the tank for the limpets to graze on. However, these films quickly disintegrated and dissolved. The other two methods involved presenting the animals with smallish algal-covered rocks or fronds of F.serratus, which were more successful.

DISSECTION TECHNIQUES.

Decapitated limpets were dissected by one of two methods, both involving immersion in either sea water, if the tissue was to be kept alive or a suitable fixative. Removal of the ventral portions of the digestive system was started by cutting away the foot of a specimen held ventral-side uppermost, in a block of plasticine. This approach was also employed when undamaged lengths of intestine were required for the in vitro radiotracer experiments. The second method, involving shell removal with a sharp scalpel blade, was used when fixing animals at the collection site or if damage to the peripheral regions was unimportant. Pieces of tissue were cut and manipulated using micro spring-scissors and fine forceps.

PREPARATION OF SECTIONS: 1-WAX.

Portions of tissue excised from the body were routinely fixed for 18-24 hours in Bouin's fluid made up with sea water. The tissue samples were stored in 70% ethanol to remove residual picric acid, then dehydrated in 90% and absolute ethanol for 1 and 2 hours respectively with two changes in each. Chloroform was chosen as the clearing agent because it hardened the tissue less than xylene; tissues were totally immersed overnight. Fresh paraplast wax (M.pt. 56°C) was used for infiltration and embedding. Infiltration was performed, with three changes of wax, at 65°C in an infiltration-embedding oven for 2-3 hours depending on the tissue type and size.

Sections, 6-9μm thick were cut with a Beck rotary microtome and the ribbons mounted on cleaned glass slides smeared with an adhesive mixture of glycerine and albumen. An overnight drying period in a 30°C oven ensured good adhesion of the sections to the slide. Routine staining techniques with Ehrlich's haematoxylin and 1% aqeous eosin, Masson's trichrome, Mallory's triple stain and Heidenhain's iron haematoxylin were performed as outlined by Humason (1979). Sections were mounted in DPX.

2-THICK RESIN.

Tissues prepared for transmission electron microscopy (TEM) as outlined later were cut to give 2μm-thick resin sections. Tissues destined for histochemical work were not fixed in osmium tetroxide. The sections were floated on a droplet of distilled water on a thoroughly cleaned slide and heated gently to evaporate the water slowly and leave the sections stuck to the slide. Resin was etched away from the tissue with a saturated solution of sodium ethoxide to expose it to the staining reagents; five minutes was an optimal etching time. It was frequently necessary to change the lower ethanol solutions of the hydrating series to prevent the alkaline ethoxide solution contaminating the staining reagents and altering their pH. Some of the histochemical treatments caused the sections to detach from the slides and so wax sections were substituted in these tests.

3-FROZEN SECTIONS.

The basic techniques for preparing and cutting frozen sections are described here, but further details concerning fixation and infiltration of tissues are included in the histochemical section of this chapter. Fixed and unfixed pieces of tissue were immersed in a centrally positioned drop of Tissue-Tek II OCT embedding compound (Raymond Lamb) on the microtome chuck. This was allowed a few minutes to infiltrate the tissue before quenching in Arcton 12 (ICI) pre-cooled with liquid nitrogen. Sections were cut using a cryostat

(SLEE) and were manipulated with a small, cold paint brush. A temperature of -20°C proved adequate for the cutting of 8-10μm sections from unfixed-frozen blocks, but a higher temperature of -10°C was satisfactory for sectioning fixed-frozen blocks.

PREPARATION OF TISSUE FOR TEM.

Limpets were dissected under cold (0-4°C), 3.5% glutaraldehyde in 0.1M Sorensen's phosphate buffer (pH 7.2) adjusted to blood osmolarity (976 ± 36mOsm; Cooper-Willis, 1978) with 14% sucrose. The total osmolarity of this primary fixative was 980mOsm. Small (<3mm^2) pieces of opened alimentary canal and glandular tissue (<1mm^3) were excised from the animal and fixed in fresh, cold primary fixative for a total time of 2.5 hours. This was followed by a 45 minute wash in the buffer-sucrose mixture. Post-fixation in a 1% osmium tetroxide solution made up in the same buffer-sucrose mixture was carried out for 45 minutes, followed by another buffer wash of similar duration. Dehydration was accomplished by immersion for 20 minutes in the following ethanol series, 30%, 50%, 70%, 90% and absolute; each solution was changed twice.

Preparation for resin infiltration began with 20 minute soaks in a 50:50 absolute ethanol-propylene oxide reagent followed by propylene oxide alone. Subsequent treatment involved overnight immersion in a 50:50 propylene oxide-resin mixture and a further 24 hours in fresh resin alone; all resin combinations were maintained on a TAAB rotator. Both TAAB and SPURR resins were used as described above, normal hardness mixes proved satisfactory. Tissues were transferred to fresh resin in flat, shallow silicone moulds (Agar Aids) that enabled easy tissue orientation. The resin was polymerised in a 70°C oven in a fume cupboard for 24 hours. All work involving osmium tetroxide, propylene oxide and unpolymerised resin was performed in a fume cupboard.

Ultrathin (gold-silver) sections were cut with LKB-glass knives on

a Huxley Cambridge mkII ultratome, floated onto distilled water and collected on Gilder grids (TAAB Ltd, types 200HH and 200HX) unless stated otherwise in the text. Counterstaining with 50% ethanolic uranyl acetate (Dawes, 1971) and lead citrate (Reynolds, 1963) solutions was performed in plastic petri dishes for 30 and 10 minutes respectively. Before and after cutting the ultrathin sections a few thick resin sections were taken and stained with 1% toluidine blue to correlate the histolgy and ultrastructure of the tissue. Ultrathin sections were examined in one of the following microscopes; a Zeiss EM109 (accelerating voltages of 50 and 80 kV), a Corinth 275 (60kV) or an AEI EM6b (60kV).

PREPARATION OF TISSUES FOR SEM.

Large pieces of opened gut tended to curl up during the processing stages, thus obscuring areas of interest. To counteract this problem the pieces of gut were pinned out flat, luminal surface upper-most, onto squares of soft polypropylene plastic. Specimens could then be handled indirectly by holding the plastic base, avoiding damage to the specimens themselves.

Thin pieces of gut epithelium were fixed and dehydrated as described for TEM, except that the osmication phase was extended to 60 minutes. Larger pieces of oesophagus and rectum were fixed and dehydrated for twice the durations described for TEM. Bulkier pieces of salivary and digestive gland were fixed overnight in primary fixative and osmicated for three hours, the dehydration times were increased by a factor of four. Whole head dissections were fixed for 24 hours in primary fixative, osmicated for four hours and dehydrated over a period of 2 days. Dehydrated specimens were then critical-point dried with liquid carbon dioxide in a Polaron E3000 drier. Once dry they were carefully removed from their bases, mounted on slides with double-sided adhesive tape and then sputter-coated with a gold-palladium layer (50nm thick) in a Polaron 5100 sputter-coater.

Cambridge S4-10 and S100 microscopes were used to examine the specimens.

HISTOCHEMISTRY.

The summarised methods are displayed in table 1, they were performed as outlined in the relevant reference. Some aspects require elaboration. For the carbohydrate and protein demonstrations Bouin's-fixed wax and glutaraldehyde-fixed thick resin sections were used. The alcian blue-alcian yellow method was unsuccessful because staining with AY was negative and so it was replaced with the alcian blue-aldehyde fuchsin method. 1% diastase and 0.05% testicular hyaluronidase solutions were made up in 0.1M Sorensen's phosphate buffer (pH 7.2) and an acetic-acetate buffer (pH 6.0) respectively; both were used at 37°C for 2 hours.

Tissues fixed in Lillie's formal-calcium, embedded in gum sucrose, cut into frozen sections and mounted on "subbed slides" (Humason, 1979), were used together with tissue fixed in Ciaccio's fluid (Lison, 1936) and embedded in wax to demonstrate lipids with sudan black B staining. Spot tests for lipids were made by staining pieces of live tissue with 1% nile blue sulphate in sea water (Andrews, personal communication).

Cryostat sections of tissue fixed in formal-calcium or the TEM primary fixative were used in the naphthol AS-BI phosphate method to demonstrate acid phosphatase. The latter fixative gave better results; fixation was for 8 hours, followed by two 1 hour buffer washes and two days infiltration in cold (0-4°C) gum sucrose. Sections (10μm) were picked up on "subbed slides" and incubated for 1 hour in the incubation medium (pH 5.0) at 37°C. For the demonstration of amylases in frozen sections of oesophagus and digestive gland, it was found that the incubation times had to be extended to 30 minutes. The very weak digestive gland protease was only demonstrated by the gelatin-film method of Kiernan (1981), incubation times of 20 minutes

to 2 hours were tried; an acetic-acetate buffer (pH 5.6) was used to moisten the sections. The 1% trypsin solution in 0.1M Sorensen's buffer (pH 7.2) in the control was used at 37°C for 2 hours. The methods of Adams and Tuqan (1961) and Fratello (1968) were not sensitive enough.

An attempt was made to demonstrate the nerve network in the epithelium of the gut using Liang's sulphurous acid leucofuchsin method. Small pieces of tissue were fixed in a 1% formic acid solution in sea water for 1 hour. Initially the immersion time in the Schiff reagent was 2 hours but this was reduced to 50 minutes to minimise the epithelial staining. The tissues were washed for 40 minutes in distilled water to remove any sulphurous acid. The modification proposed by Owen (1959) was followed.

BIOCHEMICAL ENZYME DEMONSTRATIONS.

The Gomori method for demonstrating lipase activity gave repeated non-specific results and so the biochemical lipid digestion techniques of Agrawal (1963) and George (1952) were employed to demonstrate lipase activity in digestive gland extracts. Extracts were made by blending large chunks of gland with 10ml of distilled water in an homogeniser. Agrawal's method consisted of mixing extract with diluted milk (the enzyme substrate) stained with 0.04% bromothymol blue, 0.1N NaOH was added to turn the indicator blue and a layer of toluene floated on top to prevent bacterial action. Modification of this method was necessary because of the persistent positive results of the controls. Another indicator, 0.04% bromocresol purple was selected so that the NaOH, which might have affected the experiment, could be discarded.

The technique employed by George used an emulsion of olive oil saturated with sudan III as the lipase substrate. To 10 drops of this emulsion were added 5ml of extract, it was mixed thoroughly and a thymol crystal prevented bacterial action. Incubation was carried out

at 37°C for up to 3 days. Control procedures for both series of tests lacked one or more of the constituents or used boiled extract.

CYTOCHEMISTRY.

Table 2 displays the series of cytochemical procedures that were used.

ACID PHOSPHATASE.

Small pieces of tissue were fixed in cold (0-4°C), 3.5% glutaraldehyde in 0.1M Sorensen's phosphate buffer (pH 7.2) adjusted to 980mOsm with 14% sucrose for 2 hours; they were then washed in the buffer-sucrose mixture for 45 minutes. Glandular tissue was quenched in Arcton 12 pre-cooled with liquid nitrogen and 50µm frozen cryostat sections were cut and incubated in a Gomori medium (pH 4.9) (Gomori, 1952) for 1 hour at 37°C (Owen, 1973). Pieces of unfrozen non-glandular tissue were incubated similarly (Bowen, 1970). Following incubation the tissue samples were processed in the normal way for TEM except that uranyl acetate staining was omitted. The enzyme substrate was sodium β-glycerophosphate; control media either lacked this component or had 0.01M sodium fluoride added.

ALKALINE PHOSPHATASE.

Pieces of tissue were processed as described above for acid phosphatase, but they were incubated in a Hugon-Borgers medium (pH 9.0) for 1 hour at 37°C (Hugon and Borgers, 1966).

CARBOHYDRATES.

Carbohydrate staining techniques were performed on silver-gold ultrathin sections collected upon inert metal grids. Osmium tetroxide was omitted from the normal TEM fixation schedule.

A). LOW pH PTA. For the general demonstration of polysaccharides, including mucus, glycogen and glycoproteins, the low pH PTA (dodeca-Tungstophosphoric acid) techniques of Vitellaro Zucarello (1981) and Hayat (1975) were explored. The four staining schedules below were tried on sections mounted on uncoated stainless steel

grids.

i). 1% PTA in 1N HCl (pH 0.1) for 30 minutes.

ii). 2% " " " " " " 20 " .

iii). 4% " " " " " " 15 " .

iv). 1% " in 70% ethanol-1N HCl (pH 0.1) for 30 minutes.

Grids treated with the alcoholic reagent were subsequently rinsed in 30% ethanol, those exposed to aqueous media with distilled water. Uncounterstained sections were examined.

B). PA-SM AND PA-TSC-SP. Demonstration of 1,2-glycol groups in carbohydrates was attempted by using these methods. Different types of formvar-coated stainless steel and nickel grids were chosen to allow differentiation of experimental and control sections. Coated grids were used to prevent damage to the sections caused by surface tension forces due to repeated grid floatation upon the numerous reagents involved.

Hayat (1975) reported that further oxidation with chromic acid (CrA) in the PA-SM reaction increased glycogen staining and minimised the non-specific staining of nuclei and ribosomes. This additional step was incorporated in the staining schedule as outlined below.

i). 1% periodic acid.

ii). 30 minute rinse in distilled water.

iii). 1% CrA for 5 minutes.

iv). Float grids on distilled water overnight.

v). Next appropriate solution.

Blockage of free aldehyde groups was achieved by using a 1% solution of sodium borohydride (SB) in 1% di-sodium hydrogen phosphate for 1 minute at room temperature (Lillie and Pizzolato, 1972). Diastase-labile carbohydrates were removed by treatment with a 1% diastase solution in 0.1M Sorensen's phosphate buffer (pH 7.2) for 2-3 hours at 37°C. Sections were not counterstained. Below is a list of the procedures that were tried.

Experimental: PA-SM, PA-CrA-SM.

Controls: SM, PA-SB-SM, PA-CrA-SB-SM, Diastase-PA-SM, Diastase-PA-CrA-SM

Experimental: PA-TSC-SP.

Controls: TSC-SP, PA-SB-TSC-SP, Diastase-PA-TSC-SP.

TANNIC ACID.

Pieces of tissue were incubated in an aerated Sorensen's phosphate buffered tannic acid solution for 2 hours at 15°C. The incubation reagent was prepared as follows. The mono- and dibasic solutions of the buffer were made up with artificial sea water (ASW; formulation given in the radiotracer section of this chapter); the dibasic one was filtered to remove the precipitate that formed. The two solutions were mixed to give a neutral pH and then tannic acid added to give a 0.5% solution with a final pH of 6.6. Following incubation the tissues were rinsed in ASW and processed in the normal way for TEM examination.

FERRITIN TRACING EXPERIMENTS.

A Cadmium-free ferritin solution in distilled water (50mg ml^{-1}) supplied by Boehringer, was concentrated by evaporation to dryness at room temperature and the addition of a known volume of ASW to yield a stock concentrate (170mg ml^{-1}). This was used undiluted for the *in vivo* feeding experiments in which six limpets were force-fed 10µl aliquots of the stock at t=0 hours. Subsequently one animal was fixed in the normal primary fixative at t=1, 3, 6, 18, 24 and 48 hours. A 1:1 dilution (85mg ml^{-1}) of the stock was employed for the *in vitro* incubation experiments in which pieces of tissue were immersed in the aerated ferritin-ASW solution for 30 minutes at 15°C. All tissues were rinsed in ASW after incubation and processed in the normal way for TEM. Control experiments involved sham-feeding limpets with ASW or incubating the tissue in ASW.

Ultrathin resin sections were stained with an alkaline bismuth sub-nitrate reagent (Ainsworth and Karnovsky, 1972) to enhance the

electron-opacity of the ferritin molecule. Counterstaining with lead citrate provided adequate cytological detail.

pH OF GUT FLUID.

To determine the pH of the gut fluids from the various sections of the alimentary canal, the method adopted by Ward (1966) was employed. Two series of Pehanon indicator papers were used to cover the pH ranges 5.2-6.8 and from 6.3-8.0. Sections of the gut were ligatured *in situ* with cotton thread, excised from the animal, rinsed in ASW, blotted dry and then slit open on the indicator paper to release the luminal contents. Each test was performed three times at different times of the tidal cycle and a mean value calculated for each region of the alimentary canal. Salivary and digestive glands were macerated on the indicator paper. It was realised that the fluid content of the tubules would be contaminated with cellular fluid from mechanically damaged cells and that the pH reading would not be a true measurement of the tubule contents.

CILIARY CURRENTS.

Ciliary activity was studied at different times of the tidal cycle by using both fine and medium colloidal graphite suspensions in seawater, which were pipetted onto the luminal surfaces of the gut wall with a 1ml syringe fitted with a length of narrow gauge latex tubing (Portex gauge 1; 0.055mm diameter). As an opened section of gut did not represent the actual situation in life, the fate of the graphite particles was checked by injecting small quantities of the suspensions into preceeding sections of the gut. After 10-15 minutes the following sections were opened and the distribution of the particles noted. Thin slices of glandular tissue were placed in a drop of seawater on a glass slide and covered with a coverslip. A droplet of the graphite suspension was placed against one edge of the coverslip and could be drawn under it by touching a filter paper against the opposite edge.

PHASIC ACTIVITY.

Two series of tissues were prepared for the investigation of cyclical activity; the first included 24 animals fixed in Bouin's fluid for a histological and histochemical examination. The second series was fixed primarily for an ultrastructural study and involved 12 animals. Every hour a limpet was removed from its "scar" at the collection site, removed from its shell and fixed in the appropriate fixative once the visceral mass had been bisected. Limpets were inaccessible at high tides, so to overcome this problem several recently submerged individuals were placed in a 5L plastic bucket containing rocks and seaweed to simulate high tide.

The second series of tissues was routinely fixed for TEM as described earlier, some pieces being transferred to Bouin's after primary fixation and embedded in wax for further histological and histochemical studies. Mallory's triple stain proved the most useful histological staining technique. The criteria used in examining the digestive gland for phasic activity were compiled from features described in the various phases of activity in the digestive glands of other molluscs.

STARVATION.

Limpets were starved by keeping them for 3 months in a regularly scrubbed, aquarium tank.

RADIOTRACER EXPERIMENTS.

1-IN VITRO EXPERIMENTS.

Partially starved (2 weeks) limpets were used in these experiments because it was almost impossible to dissect and obtain undamaged lengths of intestine from freshly collected animals. Furthermore, the digestive gland was susceptible to mechanical damage and would disintegrate during incubation.

A). GLUCOSE ABSORPTION EXPERIMENTS. These were designed to demonstrate glucose absorption by the gut epithelium. Pieces of

salivary gland, oesophagus, anterior (sections A and B) and posterior (sections C, D and E) intestine and digestive gland weighing between 1-8mg were cut from the animal and transferred to a glass vial containing aerated ASW at 15°C. The ASW contained NaCl (400mMol L^{-1}), KCl (9.8mMol L^{-1}), $CaCl_2.2H_2O$ (10mMol L^{-1}), $MgCl_2.6H_2O$ (53mMol L^{-1}), Na_2SO_4 (28mMol L^{-1}), and $NaHCO_3$ (2.5mMol L^{-1}) at pH 7.2 (Pantin, 1948).

The pieces of tissue were incubated in an aerated 100µMol D-glucose-ASW medium labelled with D-(β-^{3}H) glucose (Amersham International Plc) at a concentration of 0.25µCi ml^{-1} (=0.5µMol L^{-1}) for 30 minutes at 15°C. Each piece of tissue was rinsed with ASW and blotted dry. After weighing , the tissues were transferred to plastic scintillation vials containing 0.1ml of Soluene-350 and left in a 50°C oven to speed tissue digestion. As the tissue contained a large amount of pigment which reduced liquid scintillation counting efficiency by colour quenching, each sampled was bleached with 0.1ml 30% hydrogen peroxide: this took 10-15 minutes. 0.1ml 0.5N HCl was added to each vial to neutralise the Soluene-350 and finally 2ml of the following scintillation reagent was added:

Toluene:Triton X-100 (2:1)

PPO 6.0g L^{-1}

POPOP 0.3g L^{-1}

Samples were counted in an automatic spectrometer (Nuclear Enterprises).

The incubation medium was varied in a number of experiments to determine the effects of inhibitors, sodium concentration, pH and glucose concentration on the absorption mechanism. The metabolic inhibitor 2,4-dinitrophenol (500µMol L^{-1}) and carrier-mediated transport inhibitor, phloridzin (50µMol L^{-1}) were used by adding them separately to the normal incubation medium. The effect of sodium concentration was tested by incubating tissue in media containing 100%

(normal=32‰), 75%, 50%, 25% and 0% of the normal sodium ion concentration. NaCl and Na_2SO_4 were substituted with equimolar LiCl and Li_2SO_4, all other salts were left unaltered. Differences in glucose absorption due to pH were examined by varying the pH of the normal incubation medium with a tris-maleate buffer (Pearse, 1960). Media buffered to pH 6.2, 6.6, 7.0, 7.4, 7.8 and 8.2 were used. Michaelis-Menten kinetic experiments were performed by incubating tissues in normal media with the following glucose concentrations; 500, 250, 100, 50, 25 and 5μMol L^{-1}.

B).GLUCOSE FLUX EXPERIMENTS. A series of *in vitro* experiments was also performed to demonstrate a net efflux of glucose from the gut lumen to the blood space; these were based on the experiments of Lawrence and Lawrence (1967). For their experiments, long lengths of undamaged intestine cleared of all attached tissues were everted over a glass rod. However, the small diameter and thick muscle layers of the posterior intestine of *Patella* made it particularly resistant to eversion; the epithelium always split during such attempts. Similarly the anterior intestine proved difficult to evert due to the extreme delicacy of the epithelium. It was decided not to evert the sections of gut because, firstly, as the posterior intestine could not be everted it seemed wrong to use everted anterior intestine in a comparative series of experiments. Secondly as it was difficult enough to obtain long lengths of undamaged "cleaned" anterior intestine, it was decided that it would be too damaging to the epithelium to be practical.

Lengths of non-everted intestine 10-20mm long were attached to a 50 or 10μl glass capillary tube (depending on the lumen diameter) with Histoacryl tissue cement (Braun, Melsungen AG) and double ligatures (Figure 1). The other end of the intestine was loosely tied with a ligature and then ASW was flushed through it with a syringe. The ASW was removed by flushing through with air and then the intestine was

filled with a 1mMol D-glucose-ASW medium labelled with tritiated-glucose (0.25μCi ml^{-1} =0.5μMol L^{-1}). The open end of the intestine was quickly tied off as the advancing meniscus reached it. Two 10μl samples of the labelled medium were taken from the syringe used to fill the gut; these were the pre-incubational luminal samples. The outer surface of the preparation was washed with unlabelled incubation medium and then incubated in 0.5ml of the medium for 2 hours at 15°C, once two pre-incubational samples had been taken from the external compartment.

Following incubation, as many 10μl luminal samples as possible were taken together with two 10μl samples from the external compartment; these were the post-incubational samples. All samples were added to 2ml of the scintillation reagent in plastic vials and counted in the spectrometer. Each piece of intestine was removed from the capillary tube and processed for counting. The effect of 25μMol phloridzin on glucose fluxes was also examined.

C).CHROMATOGRAPHIC EXPERIMENTS. Strips (15 by 4cm) of Whatman No. 1 chromatography paper were used in conjunction with an n-butanol:acetic acid:water solvent (12:3:5) to investigate the sugars present in the tritiated-glucose stock, the 100μMol D-glucose medium, homogenates of tissues incubated in the labelled medium and the control experiments. The purity of the stock and medium was tested by spotting 5μl aliqouts of the sample at the chromatograph origin, allowing them to dry and then running them for 2 hours. Chromatographs without spots were run consecutively as controls. Aqueous extracts of gut tissue were prepared by homogenising 10mg of tissue with 200μl of distilled water and 5μl aliqouts were spotted as before. To determine the effect that this had on the Rf value of the tritiated-glucose, 5μl of it was placed on top of a dried 5μl sample of the stock solution. Chromatographs were run as before.

Pieces of oesophagus, digestive gland, anterior and posterior

intestine were incubated for 30 minutes in an aerated 100µMol D-glucose-ASW medium labelled with tritiated-glucose (1µCi ml^{-1} =2µMol L^{-1}). The samples were subsequently rinsed in ASW, homogenised and spotted onto duplicate chromatographs and run simultaneously for 2 hours. Tissue incubated in ASW was used as control. One set of the duplicates together with all the other chromatographs described above, were stained with an alkaline silver oxide reagent (Smith, 1969) to visually locate sugars. The other set was processed for scintillation counting. Each chromatograph was cut into 9 transverse strips (10mm) which were placed separately into a scintillation vial containing 2ml of scintillation reagent and counted.

D). NET WATER MOVEMENT. In vitro experiments to determine net water movement across the anterior and posterior intestinal epithelia were based upon the technique of the glucose flux experiments. The only differences were that the lengths of mounted intestine were filled with ASW trace labelled (0.048µCi ml^{-1} =9.6µMol L^{-1}) with (Hydroxy [^{14}C] methyl) inulin (Amersham International Plc) and incubated in aerated ASW for 30 minutes at 15°C.

2-IN VIVO EXPERIMENTS.

Eight limpets were force-fed a labelled 100µMol D-glucose-ASW medium at t=0 hours, after 0.5, 1, 2, 4, 8, 16, 24 and 32 hours these animals were dissected. 10µl blood and gut fluid samples were taken from the efferent pallial vessel and style sac respectively. Pieces of oesophagus, digestive gland and the five sections of the intestine were excised and prepared for scintillation counting as described previously. In an initial experiment, animals were fed 100µl of a 100µMol D-glucose medium labelled with 5µCi ml^{-1} (=10µMol L^{-1}) of tritiated-glucose. In a second experiment, a similar medium labelled with 206µCi ml^{-1} (=412µMol L^{-1}) was used, but only 10µl was administered to each animal. Control animals were

fed unlabelled media.

3-STATISTICAL METHODS.

Differences between the means of two sets of results were tested for significance by a Student's t test. When more than two means were involved a one-way analysis of variance was used. Differences were considered to be significant when t and F values corresponding to $P<0.05$ were obtained.

AUTORADIOGRAPHY.

Pieces of tissue were incubated in an aerated 100µMol D-glucose-ASW medium labelled with 25µCi ml^{-1} (=50µMol L^{-1}) tritiated-glucose for 30 minutes at 15°C. Controls were incubated in an unlabelled medium. After incubation the tissues were rinsed in ASW and blotted dry before quenching in Arcton 12 precooled with liquid nitrogen. Each piece of tissue was stored separately in a polythene sachet immersed in liquid nitrogen until required (Appleton, 1972).

Acid-washed "subbed slides" were coated with a 1:1 dilution of Ilford's nuclear research emulsion L4. The emulsion was prepared with equal volumes of 2% glycerol and the molten emulsion gel as described by Bogoroch (1972). The slides were dipped vertically into the warm (45°C) diluted emulsion, slowly withdrawn and then rested at a steep angle so that the excess could run off. Dried slides were stored in black plastic slide boxes with tight-fitting lids, at 4°C.

Unfixed frozen sections (10µm) were cut at -20°C with normal white light illumination. The coated slides in their boxes were kept at -5°C on a slab of ice; the sections were picked up under Ilford 902 safelight illumination, by touching the slides against them. 10 section-mounted slides were placed in each box; 5 had experimental sections, 3 had control sections and 2 without sections. The boxes were sealed with black tape, placed in strong, black plastic bags which were sealed and left in a freezer (-20°C) for the 4 week exposure period.

After exposure the sections were fixed by immersing the slides in 5% acetic ethanol for 1 minute at 18°C. Following 4 1 minute washes in cold tap water, the emulsion was developed in Kodak D19 for 3 minutes at 18°C and then fixed in a 25% sodium thiosulphate fixer for 3 minutes at 18°C after a brief rinse in tap water. The slides were subsequently washed in a slow stream of cold water for 15 minutes and air dried at room temperature. Sections were stained and mounted according to the procedure described by Appleton (1972).

PHOTOGRAPHIC TECHNIQUE.

All electron micrographs were taken by me on Ilford FP4 film. They were developed and contact prints provided by Mr G.Lawes and Dr K.H. Jennings. Light micrographs were taken on Ilford Pan F film or Kodak technical pan 2417 film using a Zeiss photomicroscope II. All photographs were printed on Ilford Ilfospeed resin coated paper by me.

WORD PROCESSING.

A BBC microcomputer model B fitted with a "VIEW" (A2.1) word processing ROM was used by me to arrange the text. Printing was performed with a JUKI 2200 daisy wheel printer controlled by an Acorn printer driver program.

TABLE 1.

HISTOCHEMICAL TECHNIQUES EMPLOYED.

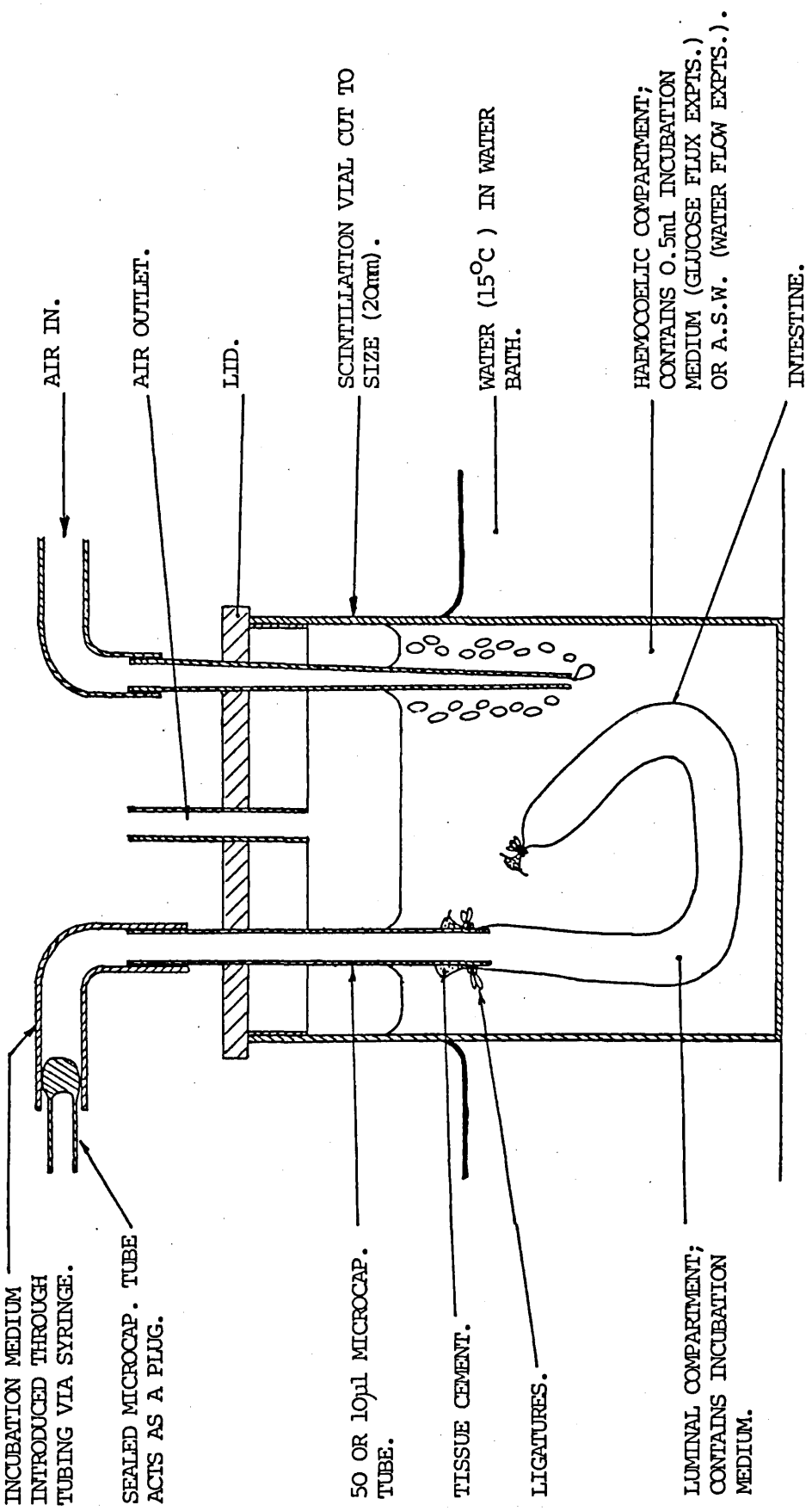
PROCEDURE	DEMONSTRATES	CONTROLS	REFERENCES
CARBOHYDRATES			
P.A.S	1,2-Glycol groups	Schiff, 1% diastase, acetylation, de-acetylation	Culling (1974)
Alcian blue (AB) pH2.5	Sulphated & carboxylated acid mucopolysaccharides (a.m.p)	Active methylation, saponification, 0.05% hyaluronidase	Culling (1974)
AB pH1.0 or Aldehyde fuchsin	Sulphated a.m.p.	As above	Culling (1974)
AB pH2.5-P.A.S	Differentiates between neutral & acid m.p.	-	Mowry (1963)
AB pH0.5-Alcian yellow or AB pH2.5-AF	Differentiates sulphated & carboxylated a.m.p.	Methylation, saponification	Ravetto (1964) Culling (1974)
PROTEINS			
Mercuric bromophenol blue	Protein-bound NH_2 groups	Deamination	Bonhag (1955)
Dinitro-fluoro-benzene (DNFB)	NH_2 & SH groups, tyrosine	Deamination	Danielli (1953)
Diazotization-coupling (DZC)	Tyrosine	Ioditization	Glenner & Lillie (1959)
p-Dimethylamino-benzaldehyde (DMAB)-nitrite	Tryptophan	Alkaline potassium persulphate	Culling (1974)
Dihydroxy-dinaphthyl-disulphide (DDD)	SH groups	Sodium thioglycolate	Lillie (1965)
LIPIDS			
Spot tests on tissue squashes with Nile blue sulphate	Neutral and acidic lipids	-	Pearse (1960) Andrews (Pers. Communication)
Sudan black B	Neutral lipids & phospholipids	-	Pearse (1960)
PIGMENTS			
Schmorl	Melanin, lipofuchsin, SH groups & argentaffin granules	-	Bancroft (1975)
Long Ziehl-Neelsen	Acid fast lipofuchsins	-	Bancroft (1975)
10% Hydrogen peroxide -Nile blue sulphate	Lipofuchsins	-	Pearse (1960)
ENZYMES			
Naphthol AS-BI phosphate	Acid phosphatases	Omit substrate or add NaF to medium	Barka & Anderson (1962)
Starch film-P.A.S	Amylases	1% diastase as positive control & thermal denaturing	Tremblay & Charèst (1968)
Gelatin film method	Proteases	1% trypsin as positive control, thermal denaturing	Kiernan (1981)
Tween 80 method	Lipases	Omit substrate, thermal denaturing	Pearse (1960)
NERVES			
Liang's sulphurous acid leucofuchsin method	Nerves in epithelial mounts	-	Lillie (1965) Owen (1959)

TABLE 2.

CYTOCHEMICAL TECHNIQUES EMPLOYED.

PROCEDURE	DEMONSTRATES	CONTROLS	REFERENCES
Incubation in Gomori medium pH4.9	Acid phosphatases	Omit substrate or add NaF to medium.	Bowen (1970) Owen (1973)
Incubation in Hugon & Borgers medium pH9.0	Alkaline phosphatases	As above	Hugon & Borgers (1966)
Low pH phosphotungstic acid (PTA)	Polysaccharides, mucus glycoproteins and glycogen	Omit PTA from staining reagent	Vitellaro Zuccarello (1981) Hayat (1975)
Periodic acid-silver methenamine (PA-SM)	1,2-Glycol groups	SM alone, sodium borohydride, 1% diastase	Rambourg (1967)
Periodic acid-thiosemicarbazide-silver proteinate (PA-TSC-SP)	1,2-Glycol groups, glycogen especially	TSC-SP alone, sodium borohydride 1% diastase	Anderson & Personne (1970)

FIGURE 1. INCUBATION APPARATUS USED IN THE IN VITRO RADIOTRACER EXPERIMENTS.



RESULTS.

THE CILIATED AND UNCILIATED COLUMNAR EPITHELIUM.

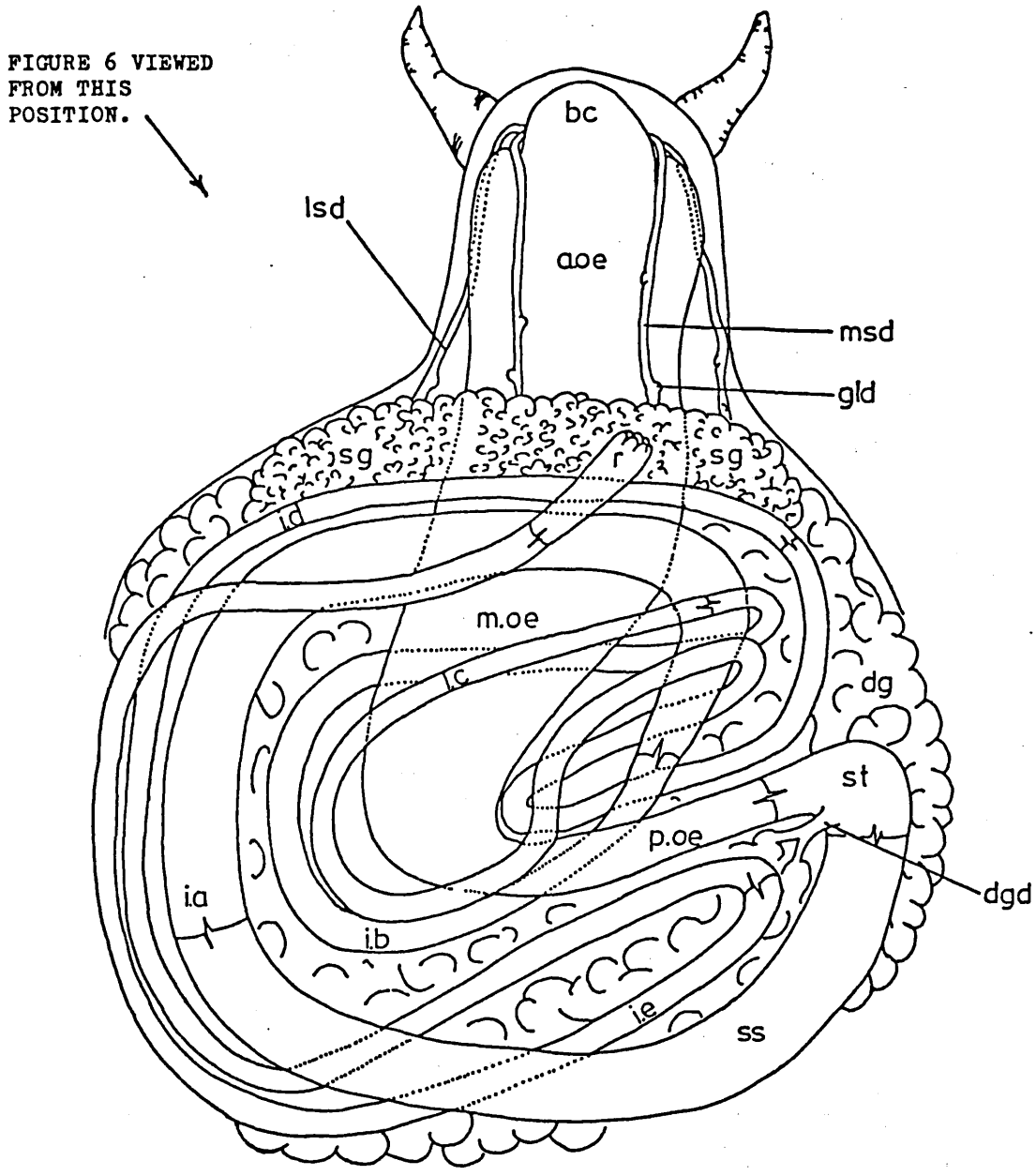
The fundamental form of the gut of Patella is that of a very long, coiled tube lined by a ciliated columnar epithelium; certain regions are characterised by the presence of one or more glandular cell types. Into the gut open the ducts of the salivary and digestive glands. Figures 2 and 6 display the complex course of the gut, the positions of the salivary and digestive glands and their ducts.

On the basis of anatomy, histology and function the gut is divisible into six sections: the buccal cavity, oesophagus, stomach, style sac, intestine and rectum. The style sac is indistinct and merges with the intestine which it resembles in many ways. This terminology, based upon that used by Fretter and Graham (1962), varies from that originally used by Graham (1932) in which the terms "fore-gut" and "hind-gut" correspond to the buccal cavity and oesophagus and the rectum respectively, whilst "mid-gut" encompasses the stomach, style sac and the five distinct sections of the intestine (A, B, C, D and E).

Table 3 presents the function and distribution of the nine different cell types identified and described later in this chapter. The cells responsible for the secretion of the chitinous jaw, the radula and odontophore cartilages are not described. Underlying the columnar epithelium is a layer of connective tissue and below this muscle fibres which ensheath the gut. The ciliated columnar cell is the most abundant and widespread cell type of the digestive system, it is absent only from the digestive gland tubules. Gland cells may be incorporated into this epithelium as they are in the buccal cavity, oesophagus and posterior intestine (sections C, D and E), rectum and salivary glands. In the stomach, style sac, anterior intestine (A and B) and ducts of the digestive gland, an unciliated columnar cell type is present which sheds apical blebs of cytoplasm into the gut lumen to

FIGURE 2.

A DIAGRAMMATIC REPRESENTATION OF THE COURSE OF THE ALIMENTARY CANAL OF PATELLA VULGATA.



ANIMAL REMOVED FROM SHELL AND VIEWED FROM DORSAL ASPECT.

NOTATION:

aoe....Anterior oesophagus.
bc.....Buccal cavity.
dg.....Digestive gland.
dgd....Digestive gland duct.
gld....Glandular lobule of duct.
i......Intestinal sections A,B,C,D,E.
lsd....Lateral salivary duct.
moe....Mid oesophagus.
msd....Median salivary duct.
poe....Posterior oesophagus.
r......Rectum.
sg.....Salivary glands.
ss.....Style sac.
st.....Stomach.

TABLE 3.

REGIONAL VARIATIONS OF pH, HISTOLOGY AND FUNCTION OF THE GUT.

	REGION OF GUT		pH OF LUMINAL CONTENTS	CELL TYPES PRESENT	FUNCTION
	BUCCAL CAVITY		-	CC MG	COLLECTION OF FOOD BY RADULA.
OESOPHAGUS	DORSAL FOOD CHANNEL		6.0	CC MG	1.MIXING FOOD WITH AMYLASE AND MUCUS. 2.TRANSPORT OF FOOD TO STOMACH. 3.ABSORPTION.
OESOPHAGUS	OESOPHAGEAL GLAND		6.0	CC AS	1.PRODUCTION OF AMYLASE ENZYME. 2.ABSORPTION.
	STOMACH		6.4	UCC CC	1.COLLECTION OF LOOSE PARTICLES AND TRANSPORT INTO THE STYLE SAC. 2.DIRECTION OF FLUIDS INTO DIG. GLND.
	STYLE SAC		6.4	UCC CC	1.FORMATION OF THE FAECAL ROD. 2.COLLECTION OF PARTICLES ONTO ROD. 3.ABSORPTION. 4.WATER RESORPTION.
INTESTINE	A		6.4	UCC CC	1.FORMATION OF THE FAECAL ROD. 2.COLLECTION OF PARTICLES ONTO ROD. 3.ABSORPTION. 4.WATER RESORPTION.
INTESTINE	B		6.4	UCC CC	1.COMPACTION OF THE FAECAL ROD. 2.ABSORPTION. 3.WATER RESORPTION.
INTESTINE	C		6.4	CC BG	1.COMPACTION OF THE FAECAL ROD. 2.ABSORPTION. 3.WATER RESORPTION.
INTESTINE	D		7.0	CC BG CG	1.SECRETION OF CEMENT LAYER ONTO FAECAL ROD. 2.ABSORPTION. 3.WATER RESORPTION.
INTESTINE	E		7.2	CC BG	1.COMPACTION OF FAECAL ROD. 2.ABSORPTION. 3.WATER RESORPTION.
	RECTUM		-	CC UCC MG	ELIMINATION OF THE FAECAL ROD.
SALIVARY GLAND	DUCTS		-	CC SC	TRANSPORT OF SALIVA TO BUCCAL CAVITY.
SALIVARY GLAND	TUBULES		6.1*	CC SC	PRODUCTION OF SALIVA LACKING EXTRACELLULAR ENZYMES. SALIVA LUBRICATES RADULA.
DIGESTIVE GLAND	DUCTS		-	CC UCC	1.TRANSPORT OF MATERIAL INTO AND OUT OF GLAND. 2.CEMENTATION OF FAECAL MATERIAL INTO LIVER STRING. 3.ABSORPTION OF LIPIDS, POSSIBLY WATER TOO.
DIGESTIVE GLAND	TUBULES		5.3*	BC DC	ABSORPTION OF FOOD .

NOTATION:

AS.....AMYLASE-SECRETING CELL.
BC.....BASOPHILIC CELL.
BG.....BASAL GLAND CELL.
CC.....CILIATED COLUMNAR CELL.
CG.....CLAVATE GLAND CELL.
DC.....DIGESTIVE CELL.
MG.....MUCOUS GLAND CELL.
SC.....SALIVARY SECRETORY CELL.
UCC.....UNCILIATED COLUMNAR CELL.
*.....HOMOGENISED GLAND USED.

form a white secretion.

In this section is described the basic histology and histochemistry of the columnar epithelium from the different regions of the gut and an ultrastructural and cytochemical representation of a typical ciliated columnar cell (fig 3, plate 1A, tables 5 & 6). There are some variations in ciliated cells of different regions as shown in table 4.

Epithelial height varies greatly, the cells lining the floor of the intestinal groove and areas adjacent to the intestinal typhlosoles may be as short as 6μm, whereas those forming the typhlosoles and the two stomach folds F1 and F3 reach heights of 90-100μm. The crests of the oesophageal dorsal and ventral folds are formed of cells much taller than those elsewhere in the oesophagus. Short (9-12μm) rectal cells, possessing cilia 16μm long, are restricted to the dorsal rectal channel, but the ventral channel is lined by taller (15-25μm) cells with cilia only 5-8μm long.

ULTRASTRUCTURE.

The numerous cilia projecting from the apical cell surface are not associated into cirri or undulating membranes (plate 1A) and their length is not correlated to the muscularity of the gut. The cilium (170nm in diameter) is bounded by a continuation of the apical plasma membrane (9nm thick) that encloses the typical motile axoneme (9+2 arrangement of microtubule doublets) in a moderately electron-opaque amorphous matrix. As is characteristic of molluscan cilia, an electron-opaque transverse basal plate delineates the proximal end of the axoneme below which, located in the apical cytoplasm, is a basal body (150nm by 300nm) (plate 1B). At its axoneme end the basal body abuts against the basal plate, but is separated from it by a narrow lucent band; the cytoplasmic end is attached to a single rootlet that penetrates deeply into the cytoplasm. Depending on the plane of section, rootlets penetrate without discontinuities at least 6μm.

The rootlets are approximately 90nm in diameter at their origin,

FIGURE 3. A DIAGRAMMATIC REPRESENTATION OF A CILIATED COLUMNAR CELL.

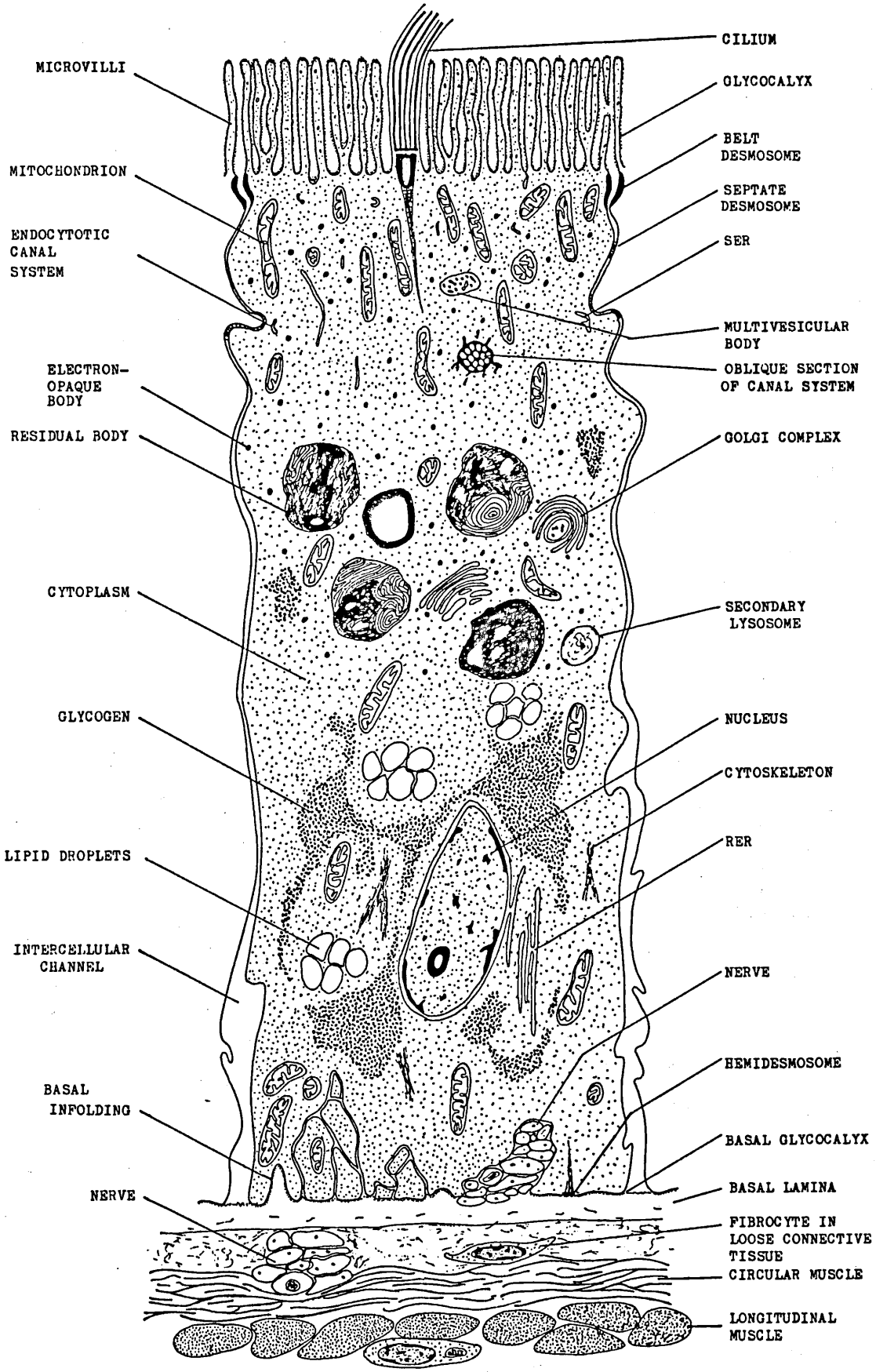


TABLE 4.

A CHART COMPARING SOME OF THE REGIONAL VARIATIONS OF THE COLUMNAR EPITHELIUM.

REGION OF DIGESTIVE SYSTEM.

	OESOPHAGUS DORSAL CHANNEL	OESOPHAGUS LATERAL POUCHES	STOMACH, STYLE SAC, INTESTINE A	DIGESTIVE GLAND DUCT	INTESTINE B	INTESTINE C	INTESTINE D	INTESTINE E	RECTUM
CELL HEIGHT (μm)	15-90. Tallest on fold summit.	20-25.	50-100. Tallest on F1 & F3. 55-65 on typhlosoles. 6 in trough of GI.	20-60. Folds of various height.	40-50.		55-60.	35-40.	Short 9-12. Tall 15-25.
SUBEPITHELIAL MUSCLE	Surrounded by thick (12μm) layers.		Stomach valve is very muscular. Inner circ. & long. outer layers (7μm).	Mainly circ. muscle (7μm).	Very muscular; same arrangement of circ. & long. layers as in stomach, but thicker.				
SECRETORY BLEBS	Very rare.		The cells associated with the secretory regions produce most blebs. Common.		Rare. Glandular cells occur in these regions though.				
CILIARY LENGTH (μm)	20 on summits, 10 elsewhere.	5	10 on typhlosoles.	7	5		10	7	Short 15-17. Long 5-8.
ELECTRON-OPAQUE BODIES	Rare.	Common in the apical half of the cells.							Rare.
RESIDUAL BODIES	Rare, small and dense.		Common. Very heterogeneous in form, content and staining reactions.						Rare, small.
EVIDENCE OF CYTOSKELETON	No.		Yes.	No.	Yes.				No.
MITOCHONDRIA	Present in the apical cytoplasm of cells from all regions.		Common in the basal regions.		Also basal.				
INTERCELLULAR CHANNELS	No.		Yes. Degree of development varies.						No.
NERVES	Sub-epithelial, small.		Small. Sub-epithelial, epithelial & in basal lamina.	As for stomach. small & large.	Sub-epithelial & epithelial. Large & small. Epithelial nerves closely associated with basal gland cells of sections C,D & E.				
AMOEBOCYTES	Commonly seen free in blood space of pouches.		Small spherical cells incorporated in the basal regions of epithelium could be regenerative cells or amoebocytes.	No.					No.

they taper to 75nm and retain this diameter along most of their length and then disappear. Each rootlet displays a striated appearance of alternating electron-opaque and lucent transverse bands (10-15nm wide) with a periodicity of approximately 40nm. A narrow transverse line bisects the lucent band, but this is only visible at very high magnifications. Associated with the rootlets are invariably one or two mitochondria and in some instances their cristae are aligned opposite to the dark bands of the rootlets (plate 1C).

Apparent in the nuclear and basal regions of these cells are strands of cytoskeleton (plate 1D), of which only short lengths (3-4μm) of microfilament bundles up to 200nm in diameter are visible in sections of the stomach, style sac and intestinal cells. Individual filaments could not be distinquished making measurements impossible. Single bundles cannot be followed in their entirety from the basal regions where they frequently associate with hemidesmosomes (plate 2A) into the apical cytoplasm where finer branches occur. There is no evidence to suggest that the ciliary rootlets attach to the cytoskeleton.

Neighboring cells are joined by junctional complexes composed of two components; a belt desmosome (zonula adhaerens) below which are septate desmosomes (plate 2B). The belt desmosome arises where the adjacent apical plasma membranes meet and extends for a distance of 300nm with a straight or wavy path. The opposing plasma membranes are separated by an intercellular gap (25-30nm wide) containing a moderately electron-opaque granular material. On the cytoplasmic face of the membranes is an electron-opaque filamentous component which diminishes as the intensely stained membranes converge reducing the intercellular gap to 18-20nm; here the belt desmosome ends. The septate desmosome consists of transverse, electron-opaque bars (septa) spaced 15nm apart, that extend across the intercellular space from one membrane to the other. Septate desmosomes are restricted to the apical

third of the cell, so that the remaining lateral membrane is not connected to its neighbour.

The lateral membranes of ciliated cells from the oesophagus, rectum, salivary gland tubules are closely juxtaposed along their length and frequently interdigitate. However, in the remaining sections of gut, the lateral intercellular space dilates forming channels that extend from the basal lamina up to the apical third of the epithelium (plates 1A, 27A, 28A, 38B, 41C & 45A). The cells bear basal infoldings that are connected to the basal lamina by hemidesmosomes, characterised by their electron-opacity and association with projections of the cytoskeleton. Although their ultrastructure was never perfectly preserved, an electron-opaque plaque (90-100nm wide) separated from the basal plasma membrane by a 10nm wide lucent zone could be distinquished (plate 2A). Anchoring filaments radiating into the subjacent basal lamina were not visible.

The large basal and apical surface areas of the cells together with the presence of the intercellular channels and the numerous apical and basal mitochondria suggests that the epithelium is involved with the transport of solutes from the gut lumen. A conspicuous feature (indicative of absorption) of the columnar cells is the presence of a well developed brush border formed of regular, distinct microvilli 2.5-3.0μm long and 70-90nm in diameter; each is separated from its neighbour by a gap of 40-60nm (plates 1A, 14A & B, 27A, 28A, 36B, 37B, 38B, 41C, 45A, 47A & B). A single stem may bifurcate giving rise to two microvilli. Only the cells lining the ducts and tubules of the salivary gland lack this feature (plate 10B).

The plasma membrane limiting each microvillus is similar to the rest of the apical membrane, it stains intensely and a fine attenuate glycocalyx coats the luminal surface. Lying against the cytoplasmic face of the membrane is a material that is more electron-opaque than the surrounding cytoplasm, this may be associated with membrane-bound

carrier proteins or it may be a fixation artefact due to clumping. At random points along their length and frequently at the very tip, the microvilli stain electron-opaque and also contain minute (15-30nm) lucent vesicles. Both mitochondria and a central core of filaments are absent from the microvilli.

The apical plasma membrane commonly invaginates inwards forming "uncoated" pinocytotic depressions with luminal accumulations of a moderately electron-opaque, amorphous material (plate 2D). Evidence to suggest that pinocytotic vesicles are formed from these areas was not found, but short, tubular invaginations common in the apical regions are associated with them. They vary in shape from rod-like "uncoated" profiles (20-25nm diameter) to "U" shapes; all are bound by an intensely stained unit membrane and contain a moderately electron-opaque amorphous matrix. Very rarely the profiles contact the apical plasma membrane (plate 2E) and are believed to be derived from it by invagination of the membrane and the material associated with its luminal surface. It is suggested that they form an endocytotic canal system that ramifies through the apical cytoplasm, which in section, appears as the diversely shaped profiles described above. It may have a vesicular appearance in oblique section (plate 4A) and could be confused with multivesicular bodies.

The endocytotic canal system associates with and connects to numerous, small (100-170nm), spherical electron-opaque bodies which are most abundant in the apical halves of the cells from the stomach, style sac and intestine (plates 1A & 2F). They are membrane-bound, "uncoated" vesicles with an homogeneous, granular matrix; fine microfilaments sometimes radiate from the cytoplasmic face of the membrane. The connection of these bodies with the canal system is presumed to be transient and of short duration because of the low incidence of such observations. The electron-opaque bodies are derived from the distal margins of the golgi cisternae. Both <u>cis</u> and <u>trans</u>

cisternae accumulate a material similar in appearance and staining reactions to that of the electron-opaque body matrix (plate 3A). Sequestration of this material in the cisternal margins produce swellings that become abstricted to form free bodies that are paler than those encountered in the apical cytoplasm. The origin from the golgi suggests either a lysosomal, secretory or storage function.

There is typically a large population of secondary and post-lysosomes in the mid region of the stomach, style sac and intestinal columnar cells (plates 1A, 36B, 37B, 38B,41C & 45A). Secondary lysosomes range in size from 300-600nm, they are usually moderately electron-opaque or lucent, lack all components typical of post-lysosomes (residual bodies) and are bound by a single unit membrane (plate 7D). The residual bodies are large, membrane-bound and heterogeneous in structure, staining reaction and content (plates 3B & C). The smaller ones (600-900nm) are generally more electron-opaque than the larger ones (1-2μm), this difference is due to the nature and density of the contents. In sections they reveal a combination of three components; there are very dense granular areas, lucent vacuolated regions and moderately electron-opaque whorls of membranous stacks. The smaller residual bodies which are common in the ciliated cells of the oesophagus, rectum and ducts of the salivary and digestive glands contain a high proportion of granular material. The larger bodies are composed mainly of myelin figures.

In the apical third of the cells, multivesicular bodies sometimes occur, these membrane-bound, oval bodies ranging in size from 250-600nm resemble a lucent vesicle containing smaller vesicles (40-50nm) and profiles of the endocytotic canal system (plate 3D). It is suggested that they originate from the golgi complex because of the following evidence. The golgi profiles that appear in sections as a concentric whorl of cisternae , very frequently surround a central, lucent, vesiculated area which may be isolated from the cytoplasm by

one, two or three cisternae that lack acid phosphatase activity, although this enzyme is present in the other golgi cisternae (plate 7E).

The small, inconspicuous golgi complex is restricted to the mid region of the cells and is typically composed of five or six cisternae separated from each other by a gap of 10-20nm. Frequently several cup-shaped profiles or concentric whorls are present in the same cell (plates 3A & 7C). Near to the cis face are cisternal and vesicular elements of transitional ER. The cisternal lumen of the golgi is narrow (10-20nm wide) but is commonly dilated along its distal margins. A moderately electron-opaque, filamentous material fills the cisternal lumen, but where accumulations occur the opacity increases greatly and the contents then appear finely granular (plate 3A). The content of those cisternae producing distal accumulations of material is denser than others, suggesting that this material is being concentrated within their lumina. Small lucent vesicles (30-60nm) are abundant in the golgi region and are believed to be derived from it. On ultrastructural evidence they resemble primary lysosomes.

Endoplasmic reticulum (ER) is sparse in both the ciliated and unciliated columnar cells and is only conspicuous at high magnifications (plates 1A & 3A). It consists of the rough (RER) and smooth (SER) varieties that exist as an anastomosing system of tubules; only around the nucleus are small parallel stacks of RER present. The cisternal lumen contains a filamentous or amorphous material which has an electron-opacity similar to that of the cytoplasm. The apparent low level of RER activity indicates that the cells are not involved with the synthesis of a proteinaceous secretion.

Graham (1932) reported that the ciliated cells of intestine sections A and B produced spherical "secretion droplets". In fact all ciliated cells regardless of their distribution can do this, but the

unciliated cells are much more active. Masses of apical cytoplasm dilate into the gut lumen and are pinched off from the cell to produce free membrane-bound blebs of cytoplasm; this apocrine secretion corresponds to Graham's "secretion droplets". During this process, cilia remain attached to the cell but the microvilli disappear. The characteristic lack of any cytoplasmic organelles from the blebs and the loss of the brush border suggests that the blebs are formed from the expansion of the microvilli themselves.

If the microvilli lost their structural integrity and their membranes distended away from the cell, a mass of microvillar cytoplasm bounded by the microvillar membrane would be formed (plate 4B); this would be facilitated by the lack of central core filaments in the microvilli. A free bleb would be formed when this mass became pinched off, to leave the cell with a flat luminal border (plates 4C & D). The membrane junctions remain intact which further indicates that only the dilated microvilli are lost.

Mitochondria are distributed throughout the entire cell but are especially concentrated in the apical regions of all ciliated and unciliated cells (plates 1A, 14A & B, 36B, 37B, 38B, 41C, 45A, 47A & B). The cells lining the stomach, style sac and intestinal sections A and B also have numerous basally situated mitochondria. Localization of large numbers of mitochondria suggests the presence of active processes such as ciliary beating, endocytosis and solute transport. Typical septate cristae are embedded in a moderately electron-opaque, amorphous matrix that lacks particulate material. They did not show signs of fixation or osmotic artefacts.

Displaced to the basal third of the cell by the residual bodies, is the large (6-8μm by 2-3μm), oval nucleus (plates 1A & D). The nucleoplasm has an opacity similar to that of the cytoplasm and contains dense areas of heterochromatin which are conspicuous with optical microscopy. These areas are scattered in the nucleoplasm and

also against the inner membrane of the nuclear envelope, the outer membrane of which is sparsely studded with ribosomes and continuous with the RER. An oval (2μm by 1μm) nucleolus may occur.

Separating the basal plasma membrane from the underlying basal lamina is a lucent layer of glycocalyx 20nm thick . The basal lamina separates the cell from the sub-epithelial connective tissue, it stains homogeneously, moderately electron-opaque and appears amorphous with normal counterstaining (plates 1A & 5A). It varies considerably in thickness throughout the gut, ranging from 70nm in the oesophageal gland and digestive gland duct to 150-400nm in the intestine. The basal lamina may project finger-like processes up to 2μm tall into the basal regions of the intestinal epithelium affording mechanical support for these tall cells (plate 5A). Usually the epithelium is closely applied to the basal lamina, but the epithelium lining the oesophageal gland contacts the lamina only rarely; hemidesmosomes being scarce.

The sub-epithelial connective tissue is composed of a loose collagenous matrix embedded in which are fibrocytes, putative amoebocytes and abundant nerve fibres. Separating this matrix from the ubiquitous blood space are two muscle layers; an inner circular layer and an outer layer of longitudinal fibres. The muscular development of each region of the gut is variable; the oesophagus, intestine and rectum are very muscular (plates 13A, 36B, 37B, 38B, 41C, 45A, 47A & B), but the salivary and digestive gland tubules have poorly developed muscles (plates 12B & 28C).

The spindle-shaped fibrocytes possess a very large nucleus that occupies the greater part of the cell, large clumps of heterochromatin are scattered in the nucleoplasm and against the inner face of the nuclear envelope. Little RER is present, a golgi complex was not observed and mitochondria are scarce. Possible amoebocytes were found associated with the intestinal connective tissue and epithelium only

on very rare instances, but the basal lamina was not damaged in their vicinity. They are small (3-5μm diameter) round cells, the nucleus is large, irregular in shape and contains large amounts of heterochromatin. The granular cytoplasm contains few mitochondria, a small golgi complex and very little ER. There is no evidence to suggest endocytosis, a few electron-opaque bodies are the only inclusions present.

The nerve fibres have a very characteristic ultrastructure, at low magnifications they appear lucent and empty. Higher magnifications reveal mitochondria, neurofilaments, lucent vesicles (50-100nm) and larger electron-opaque ones (80-120nm). The majority of the neurofilaments run longitudinally along the length of individual axons (plate 5C). Small nerve fibres intrude into the epithelium of the stomach, style sac, intestine and rectum and it is these that correspond to the "intra-epithelial canals" described by Graham (1932) in Patella. The neurilemma has intimate contact with the basal plasma membranes of the columnar cells, but the synaptic connections or release of neurosecretory material was not observed. Nerve size increases in the last three sections of intestine where basal gland cells occur and it is common to find these cells resting on a nerve fibre running through the epithelium.

CYTO- AND HISTOCHEMISTRY.

1-GENERAL.

Specific staining of a chemical group or enzyme is that when an organelle/inclusion appears more intensely stained after experimental treatment compared to that of the control treatment. An organelle/inclusion is defined as being non-specifically stained when it retains a similar intensity after control and experimental treatments.

The results obtained from the low pH PTA, PA-SM and PA-TSC-SP methods were variable. The PTA method required considerable modification of procedure before satisfactory results were obtained.

Incubation of ultrathin resin sections for 30 minutes or longer caused a great deal of damage due to the acidic nature of the staining reagent. Imperfections in the sections, such as knife marks, acted as focal points for acid attack and these regions were very weak in the electron beam and tended to disintegrate or fold up. Successful results came from the 15 minute incubation in the 4% PTA solution. The sections stained with the ethanol-based reagent tended to stain the tissue very intensely and non-specifcally. Treatment with the control reagents left the sections electron-lucent.

The PA-TSC-SP technique produced consistent results in ciliated columnar cells from all regions of the gut. Artefactual silver deposits did occur but they were easily identified as such; they are dense, spherical bodies (6nm) that usually occur in small clusters. Resolution was very good with this and the PTA method.

PA-SM staining was consistently non-specific. Following the treatment, the cytoplasm and organelles appeared coarsely granular and heavily stained thus obscuring fine detail. The use of chromic acid failed to increase the specificity. Sections treated with sodium borohydride reacted much less intensely with the SM stain, indicating that the non-specific reaction was due to pre-existing aldehyde groups in the sections. The nuclei and residual bodies remained heavily stained suggesting the presence of other reducing groups.

The enzyme demonstrated by the Gomori incubations at pH 4.9 was sodium β-glycerophosphatase, there was no activity recorded at pH 9.0 with the Hugon-Borgers medium. It should be realised that the actual enzymes present in the cells may have different pH optima to those chosen for the incubation media. So there may be an alkaline phosphatase at pH 10 that is being overlooked. If an organelle has an ultrastructure suggestive of a lysosome, yet fails to react to the incubations, this should not rule out the possibility that it is lysosomal. One should test for other common lysosomal enzymes

(esterases, cathepsins, pyrophosphatases) to confirm the result.

2-CARBOHYDRATES.

Summarised results are presented in table 5. The blebs of cytoplasm do not react with any cyto- or histochemical method; staining with Mallory and Heidenhain's haematoxylin reveals a thread-like material, but ultrastructurally they contain only ground cytoplasm. The brush border contains carboxylated and sulphated acid mucopolysaccharides as well as 1,2-glycol groups present in neutral mucopolysaccharides. The brush border of the typhlosole cells from the style sac and anterior intestine is alcianophobic and that of the cells from the posterior intestine fails to react with the PAS method. The apical glycocalyx reacts with the low pH PTA stain (plate 6A) (except that of the cells from the oesophageal gland and intestinal sections D and E), but not with the PA-SM or PA-TSC-SP methods which it should have done regarding its PAS reactivity.

Residual bodies also contain PAS-positive material, they are not stained with PA-TSC-SP treatment, but are stained non-specifically by all PA-SM methods. The residual bodies of the cells from the oesophageal food channel sometimes contain intensely stained areas following PTA treatment (plate 6B). The endocytotic canal system, electron-opaque bodies and multivesicular bodies are all non-specifically stained by all PA-SM methods, but remain unstained with the PA-TSC-SP method. The material within the lumen of the canal system stains with PTA (plate 6C) except that in the cells from the oesophageal gland and intestinal sections D and E, indicating that this material is chemically similar to the apical membrane glycocalyx. The majority of the electron-opaque bodies do not stain with PTA, but those in the cells of the intestinal sections A and B do (plate 6D). The matrix of the multivesicular body also displays an affinity for PTA (plate 6E).

PA-TSC-SP-positive β-glycogen deposits occur mainly in the basal

TABLE 5.

SUMMARISED CARBOHYDRATE HISTOCHEMISTRY: COLUMNAR EPITHELIAL CELLS.

PROCEDURE		SITES EXAMINED								
		SECRETORY BLEBS	MICROVILLI	ENDOCYTOTIC CANAL SYSTEM	ELECTRON-OPAQUE BODIES	MULTI-VESICULAR BODIES	RESIDUAL BODIES	GOLGI COMPLEX	GLYCOGEN	BASAL LAMINA
SCHIFF		–	–				–		–	–
P.A.S		–	+[P]				+		+	–
ACETYL/P.A.S		–	–				–		–	–
DIASTASE/ P.A.S		–	+				+		+	–
P.A./SB/S.		–	–				–		–	–
AB pH 2.5		–	+				–		–	–
M/AB pH 2.5		–	–				–		–	–
M/S/AB pH2.5		–	+[R]				–		–	–
HY/AB pH 2.5		–	+				–		–	–
AB pH 1.0		–	+[W]				–		–	–
M/AB pH 1.0		–	–				–		–	–
M/S/AB pH1.0		–	–				–		–	–
HY/AB pH 1.0		–	+[R]				–		–	–
AF		–	–				–		–	–
M/AF		–	–				–		–	–
M/S/AF		–	–				–		–	–
AB pH2.5/AF		–	+/–				–		–	–
M/AB pH 2.5/ AF		–	–				–		–	–
M/S/AB pH2.5 /AF		–	–				–		–	–
LOW pH PTA		–	+[M]	+[M]	+[M]	+[M]	+[M]	–	–	+[M]
PA-SM		–	–	–	–	–	–	–	–	–
PA-TSC-SP		–	–	–	–	–	–	–	+	–

NOTATION:

P.A.S.....Periodic acid-Schiff.
ACETYL....Acetylation.
SB.....Sodium borohydride.
AB.....Alcian blue.
M.....Methylation.
S.....Saponification.
HY.....Hyaluronidase.
AF.....Aldehyde fuchsin.
PTA.....Phosphotungstic acid.
PA-SM.....Periodic acid-silver methenamine.
PA-TSC-SP....Periodic acid-thiosemicarbazide-silver proteinate.
R.....Reduced reaction.
W.....Weak reaction.
M.....See text for variations.

two thirds of the cell (plate 7A). After diastase digestion it is absent and it does not stain with the PTA or PA-SM methods. The fine granular structure of individual particles (30-40nm diameter) can be determined (plate 6F). PAS-positive, diastase-resistant material occurs in a similar position in the cell. The cells lining the oesophageal food channel, stomach, style sac and anterior three intestinal sections contain larger deposits of glycogen than the cells forming the oesophageal gland, typhlosoles and posterior two intestinal sections. It is significant that the latter cells lack a PAS-reactive brush border.

The basal glycocalyx and weakly staining filaments associated with the basal lamina are apparent after staining with PTA (plate 7B). They are 14-18nm in diameter and possibly constitute an interlacing network that holds the basal lamina and connective tissue together in the stomach, style sac and intestine where they are most obvious.

3-PROTEINS.

Summarised results are presented in table 6. The only notable reactions are those of the apical cytoplasm and the granules (3-5μm) in some oesophageal nerves. Abundance of organelles in the apical cytoplasm is responsible for the weak results with the DNFB and diazotization-coupling methods, demonstrating amino groups and tyrosine respectively.

4-LIPIDS.

Frozen sections of intestine and digestive gland ducts reveal sudanophilic droplets (1-2μm) in the basal regions of the cells. Residual bodies also stain with sudan black B (table 6).

5-PIGMENTS.

Summarised results are presented in table 6. Residual bodies contain lipofuscins except in the cells lining intestinal section A.

6-ENZYMES.

Summarised results are presented in table 6. After incubation in

TABLE 6.

SUMMARISED HISTOCHEMICAL RESULTS OF THE COLUMNAR EPITHELIAL CELLS.

PROCEDURE		SITES EXAMINED: SECRETORY BLEBS	MICROVILLI	APICAL CYTOPLASM	ENDOCYTOTIC CANAL SYSTEM	ELECTRON-OPAQUE BODIES	MULTIVESICULAR BODIES	SECONDARY LYSOSOMES	RESIDUAL BODIES	GOLGI COMPLEX	LIPID DROPLETS	GRANULES IN NERVES.
PROTEINS	BROMOPHENOL BLUE	–	–	–					–	–	–	–
	DNFB	–	–	+W					–	–	–	+
	DE-NH_2/DNFB	–	–	+R					–	–	–	+
	DIAZOTIZ'TN-COUPLING	–	–	+W					–	–	–	+
	IODITIZATION/ DIAZ-COUPLING	–	–	–					–	–	–	–
	DMAB-NITRITE	–	–	–					–	–	–	–
	DDD	–	–	–					–	–	–	–
	THIOGLYCOLATE /DDD	–	–	–					–	–	–	–
	SUDAN BLACK B	–	–	–					+	–		–
PIGMENTS	SCHMORL	–	–	–					+*	–	–	–
	LONG ZIEHL-NEELSEN	–	–	–					+*	–	–	–
	H_2O_2-NILE BLUE SULPHATE	–	–	–					–	–	–	–
ENZYMES	NAPHTHOL AS-BI PHOSPHATE	–	+*	+*							–	–
	GOMORI MEDIUM pH4.9	–	–	–	–	–	–	+	–	+	–	–
	HUGON-BORGERS MEDIUM pH9.0	–	–	–	–	–	–	–	–	–	–	–

NOTATION:

AS FOR TABLE 5.
$DE-NH_2$.....Deamination.

the naphthol AS-BI phosphate reagents the mid-region of the cells (where the golgi, lysosomes and residual bodies are situated) gives a weak positive result that is absent from control sections. Residual bodies from intestinal section D cells give a weak positive result that is partially inhibited by sodium fluoride. The brush border of the cells lining the digestive gland duct and intestinal section A and the apical cytoplasm of the cells lining the oesophageal food channel give positive results.

Tissues incubated in the Gomori medium give specific deposits over the golgi complex and primary and secondary lysosomes (plates 7C, D & E). Trans golgi cisternae contain the heaviest deposits, the cisternae have a beaded appearance and their distal margins are swollen with reactive material. Reaction deposits lie against the luminal face of the bounding membrane of the secondary lysosomes and to a lesser extent free in their lumen, indicating that the enzyme is membrane-bound.

ABBREVIATIONS FOR PLATES 1-7.

am...Apical membrane.

bb...Basal body.

bd...Belt desmosome.

bg...Basal glycocalyx.

bgc..Basal gland cell.

bl...Basal lamina.

bm...Basal membrane.

c....Cilia.

cb...Cytoplasmic bleb.

cc...Ciliated columnar cell.

cm...Circular muscle.

cr...Ciliary rootlet.

ct...Connective tissue.

cy...Cytoskeleton.

dv...Dense neurosecretory vesicle.

ecs..Endocytotic canal system.

eob..Electron-opaque body.

f....Fibril.

fc...Fibrocyte.

fct..Fibrils in connective tissue.

g....Golgi complex.

gd...Glycogen deposits.

gl...Gut lumen.

gy...Apical glycocalyx.

hc...Heterochromatin.

hd...Hemidesmosome.

hl...Heterolysosome.

is...Intercellular space.

jc...Junctional complex.

lm...Longitudinal muscle.

lv...Lucent neurovesicle.

m....Mitochondrion.

mv...Microvilli.

mvb..Multivesicular body.

n....Nucleus.

nt...Neurotubule.

nv...Nerve.

oecs.Oblique section of ecs.

pd...Pinocytotic depression.

pl...Primary lysosome.

rb...Residual body.

rer..Rough ER.

sd...Septate desmosome.

ter..Transitional ER.

v....Unspecified vesicle.

LEGENDS TO PLATES 1-4.

THE CILIATED COLUMNAR CELL.

PLATE 1.

A. The ciliated epithelium of intestine section E. 7K.

B. Apical region of cell lining the intestinal groove. 9K.

C. A ciliary rootlet and associated mitochondria. 61K.

D. Nuclear region of cell. 9K.

Scale bars: A,B,D=2μm; C=250nm.

PLATE 2.

A. Cytoskeleton joining a hemidesmosome. 153K.

B. A junctional complex between two cells. 52K.

C. Microvilli and the apical membrane. 112K.

D. Pinocytosis. 70K.

E. Endocytotic canal system connected to apical membrane. 213K.

F. Canal system connected to an electron-opaque body. 126K.

Scale bars: A,C,E,F=100nm; B,D=250nm.

PLATE 3.

A. A typical golgi complex. 37K.

B. Residual bodies. 37K.

C. A residual body with membranous whorls. 85K.

D. A multivesicular body. 180K.

Scale bars: A,B=500nm; C=250nm; D=100nm.

PLATE 4.

A. An oblique section through the endocytotic canal system. 144K.

B. Production of blebs; arrow indicates constriction of neck. 7.5K.

C. Release of blebs. 5.25K.

D. Apices of cells once blebs have been shed. 11K.

Scale bars: A=200nm; B,C,D=2μm.

LEGENDS TO PLATES 5-7.

THE CILIATED COLUMNAR CELL.

PLATE 5.

A. Basal region of intestinal epithelium showing projections of the basal lamina. 11K.

B. A regenerative/amoebocytic cell in the intestinal epithelium. 21K.

C. A typical nerve lying in the intestinal epithelium. 30K.

Scale bars: A,B=1μm; C=300nm.

PLATE 6 (PTA staining A-E).

A. Glycocalyx of microvilli (T.S.). 130K.

B. Residual bodies. 19K.

C. Endocytotic canal system. 130K.

D. Electron-opaque bodies. 19K.

E. A multivesicular body. 77K.

F. Glycogen stained by the PA-TSC-SP method. 130K.

Scale bars: A,C,D,F=100nm; B=1μm; E=200nm.

PLATE 7.

A. Glycogen in ciliated cells stained by the PA-TSC-SP method. 4.7K.

B. Basal glycocalyx stained by the PTA method. 77K.

C-E acid phosphatase localisation.

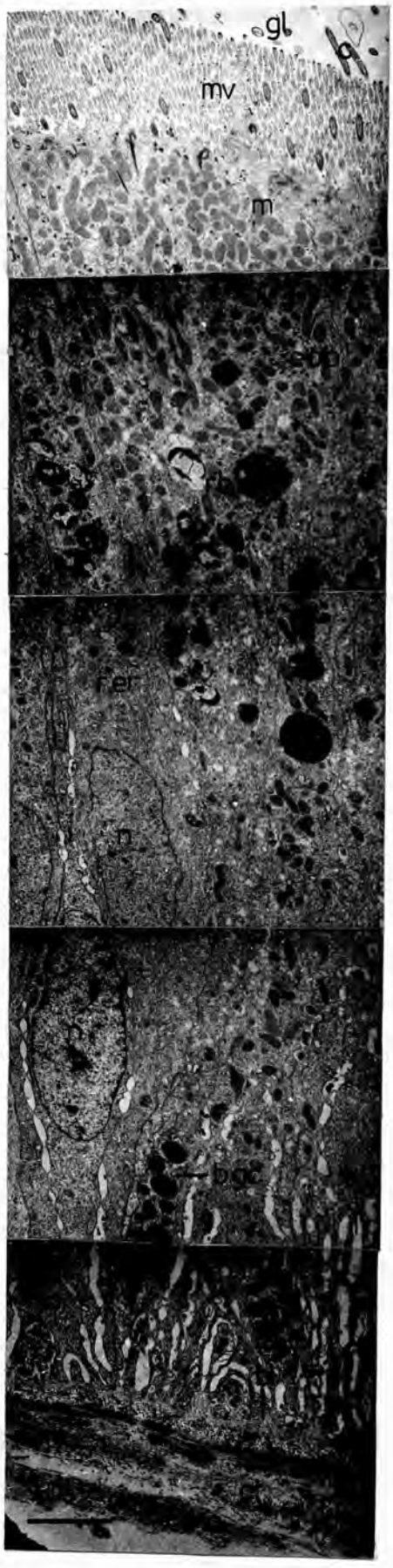
C. Golgi complex. 50K.

D. Golgi complex forming a multivesicular body. 53K.

E. A heterolysosome and golgi complex. 130K.

Scale bars: A=4μm; B,C,D=200nm; E=100nm.

PLATE 1



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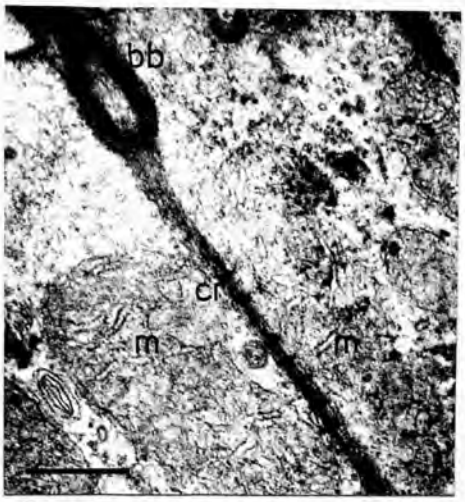

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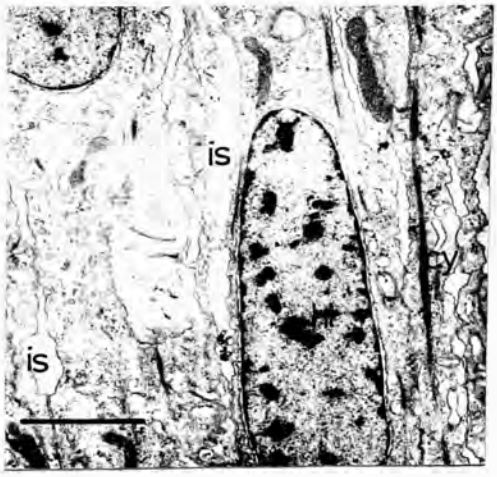

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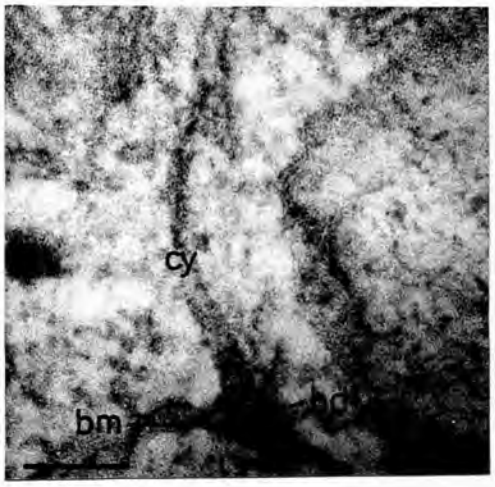
PLATE 2



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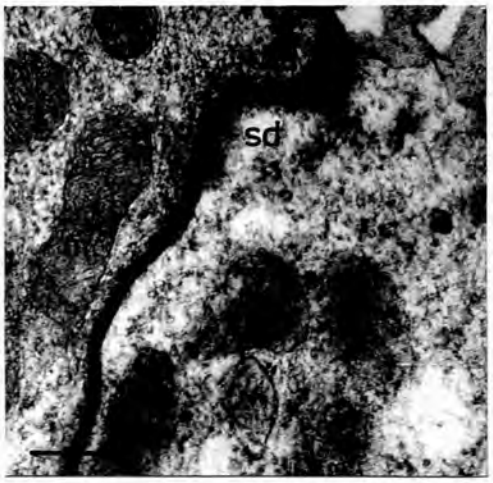

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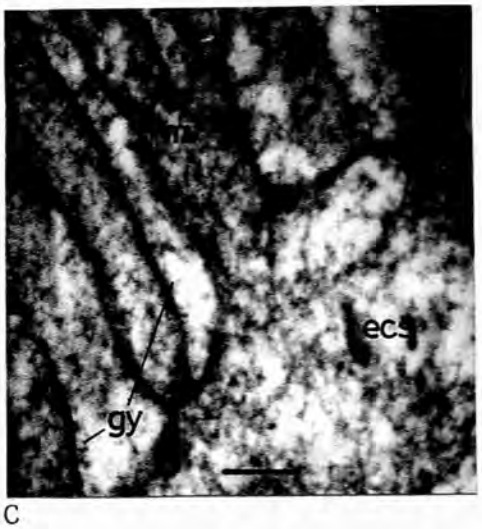

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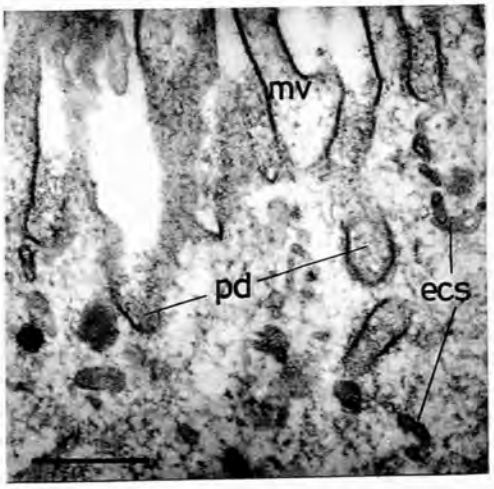

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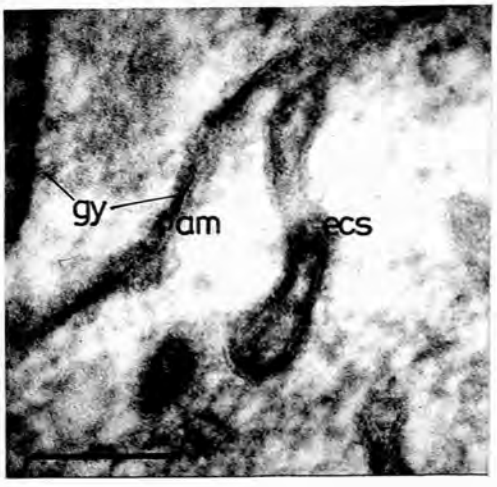

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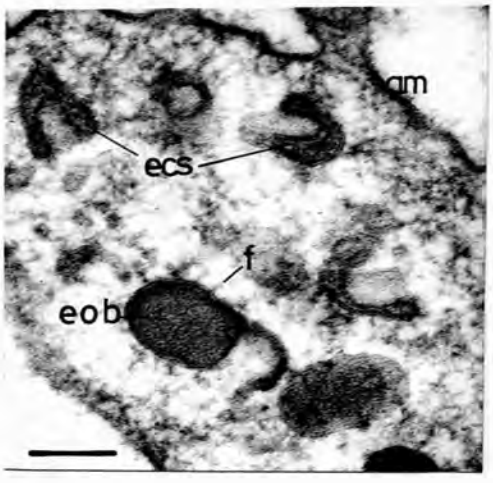

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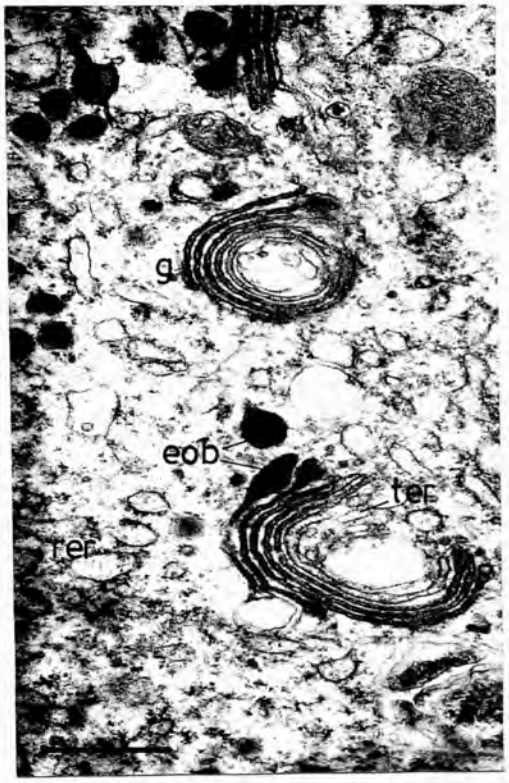
PLATE 3



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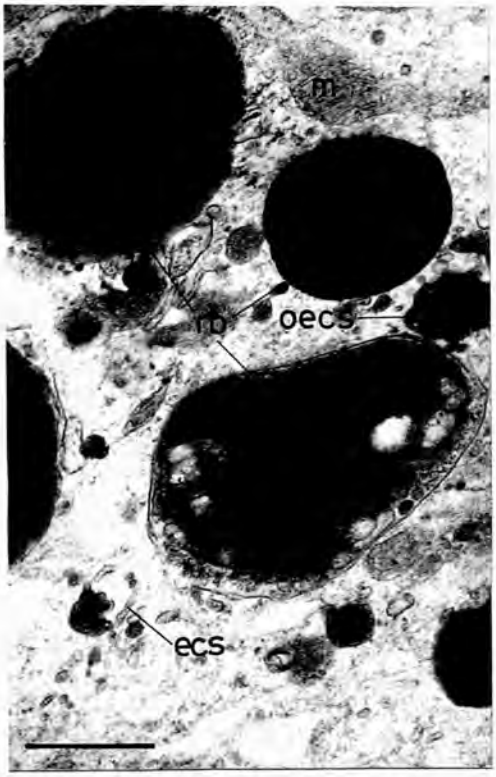

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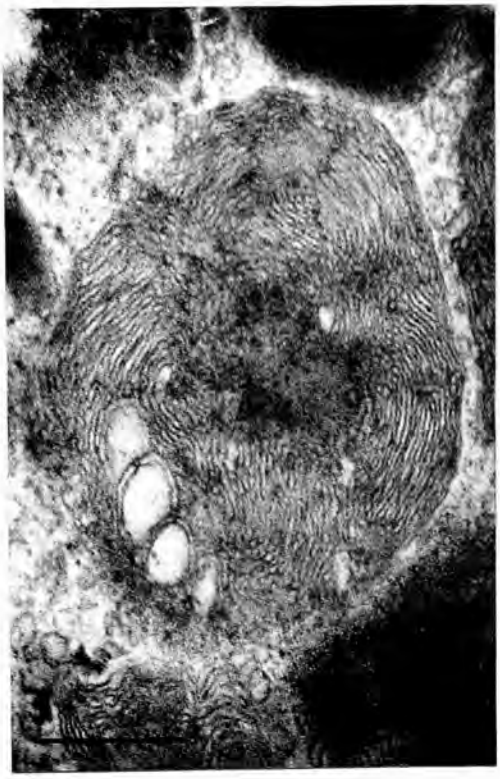

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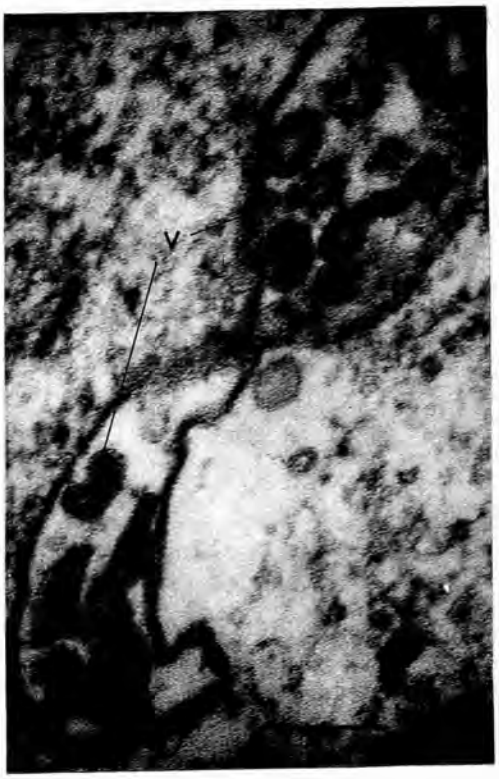

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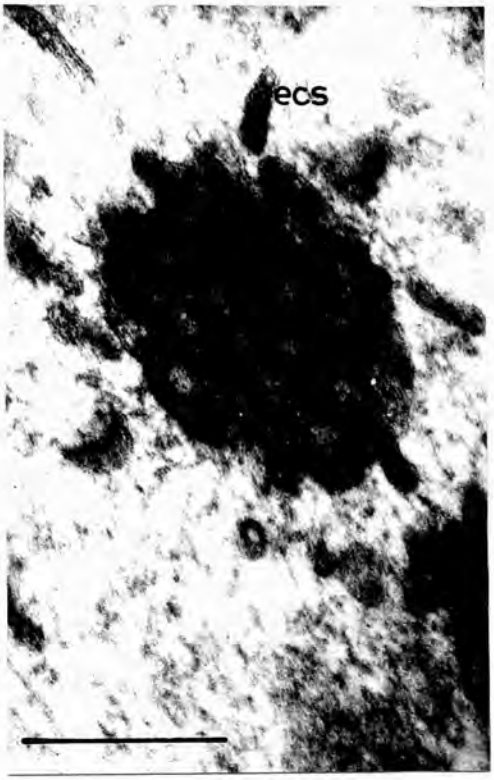
PLATE 4



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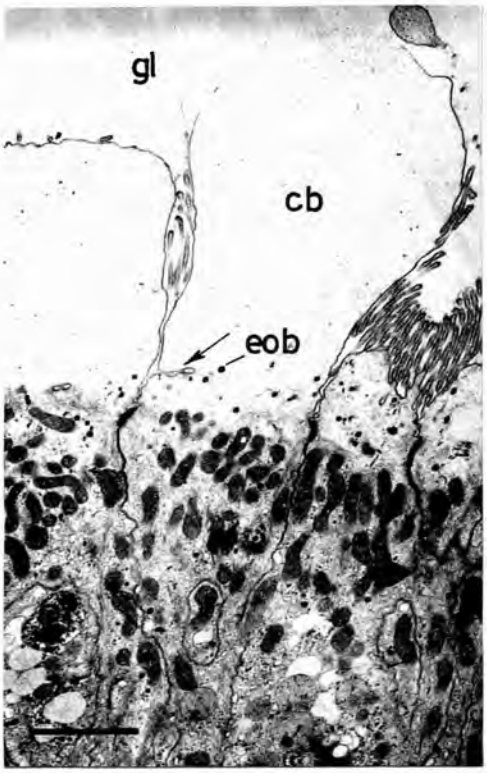

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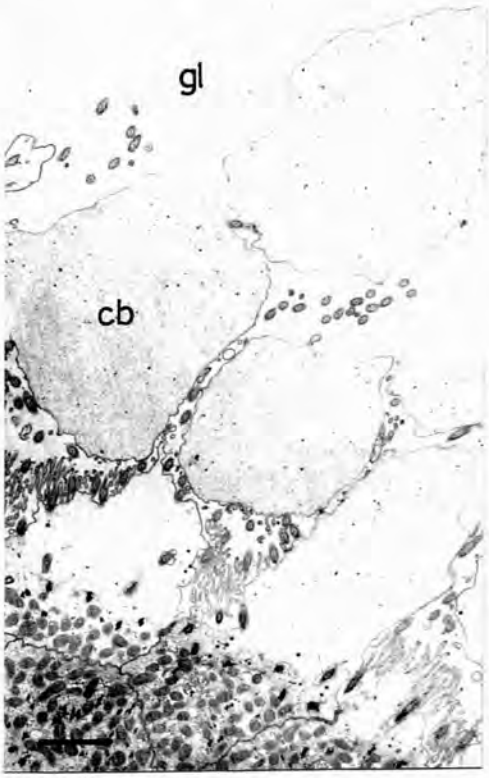

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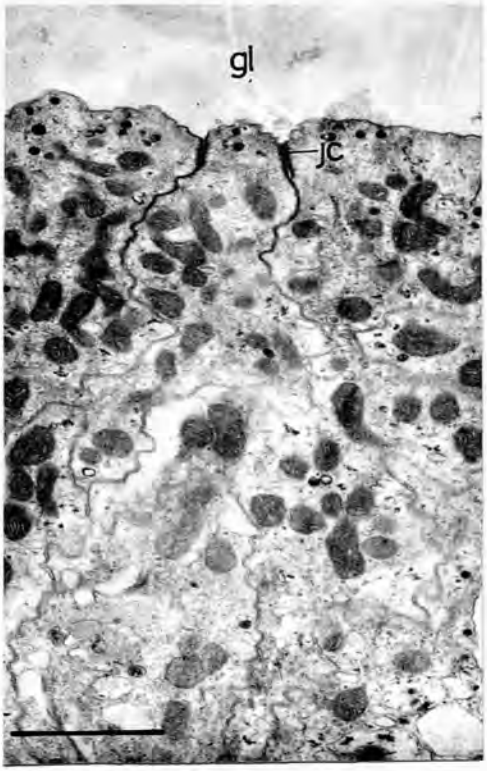

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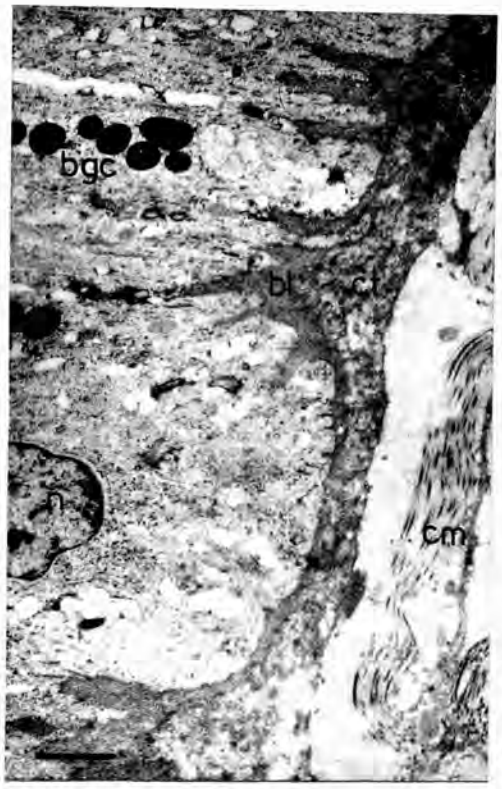
PLATE 5



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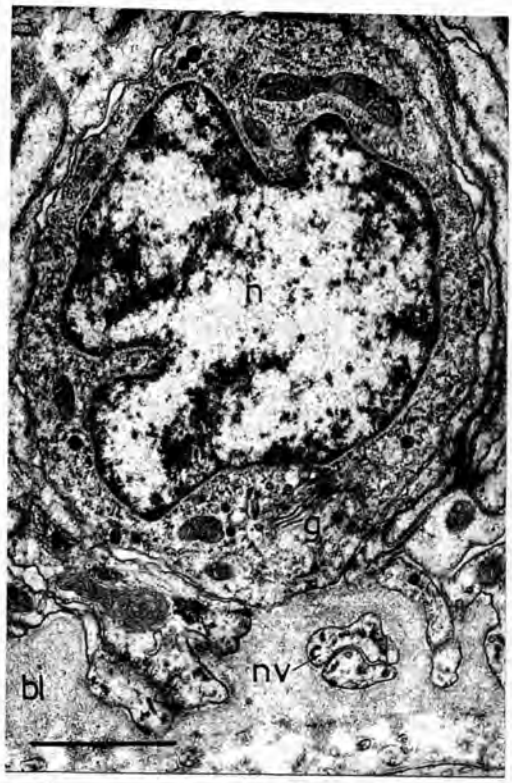

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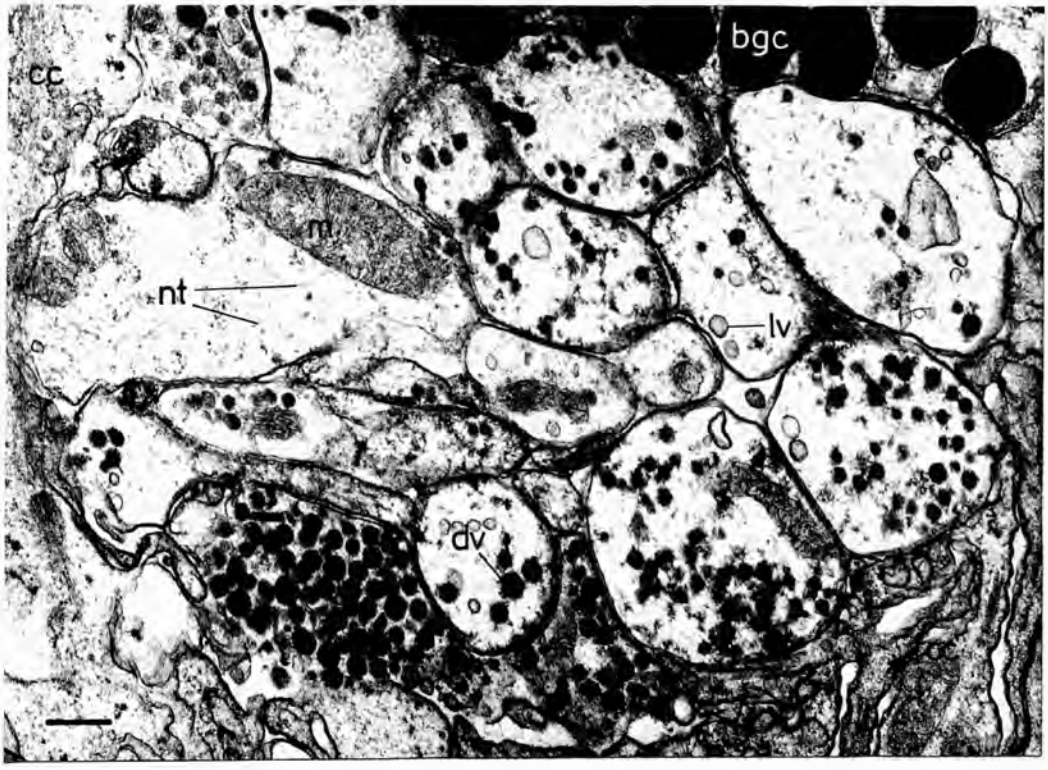

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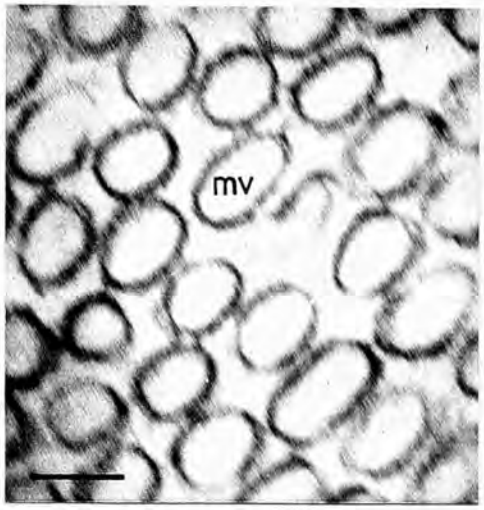
PLATE 6



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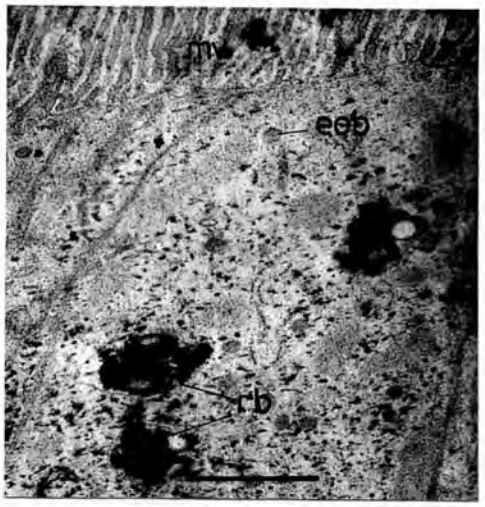

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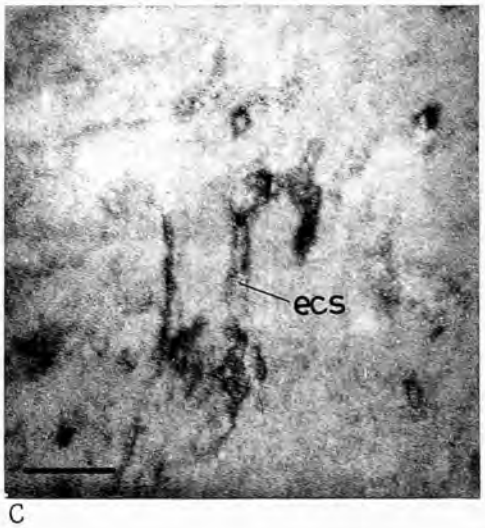

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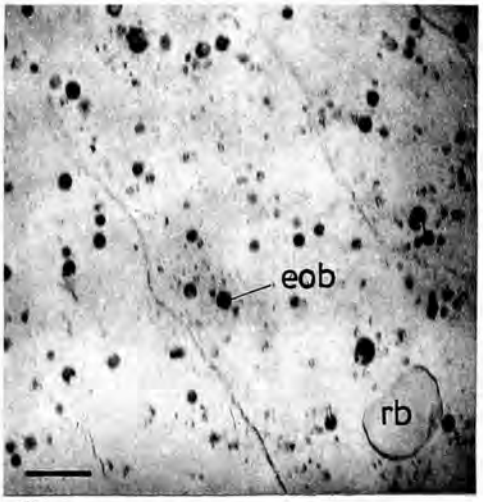

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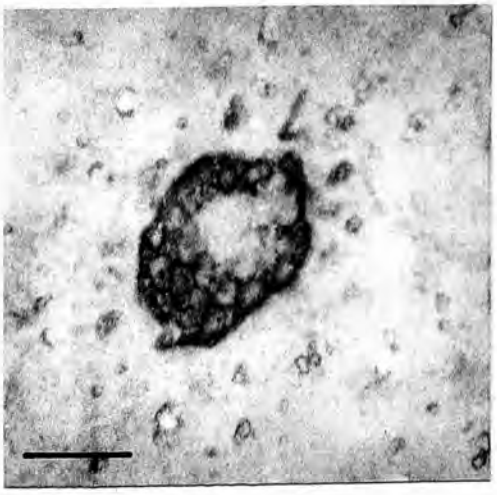

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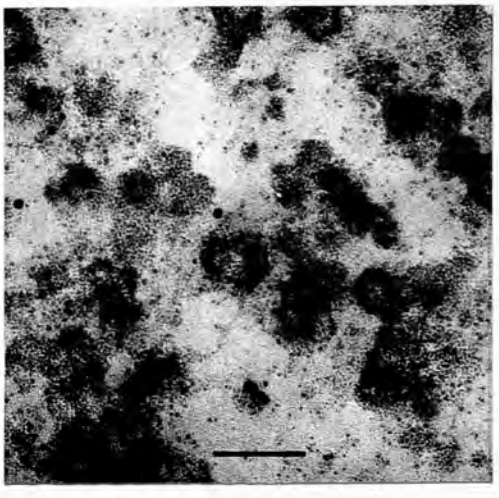

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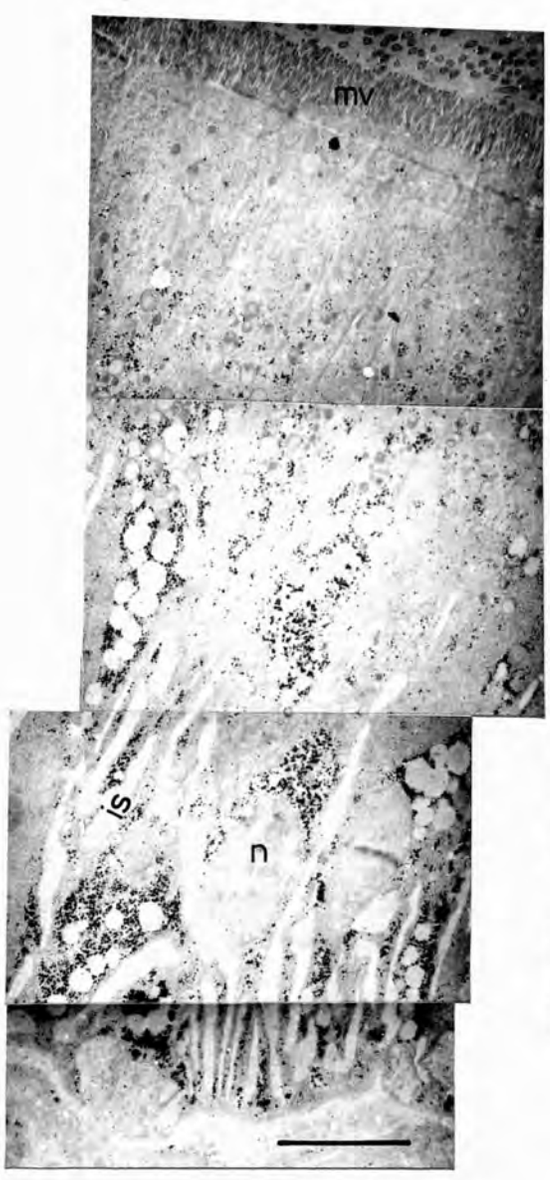
PLATE 7



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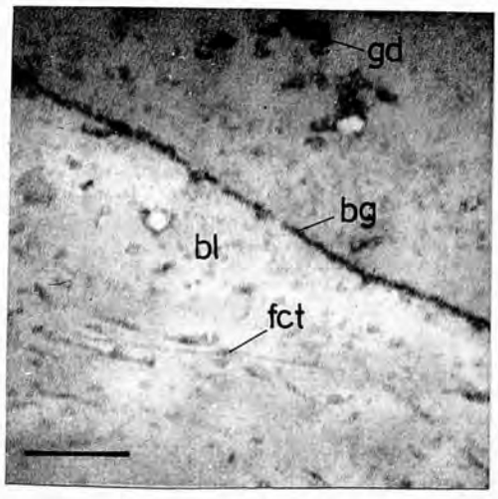

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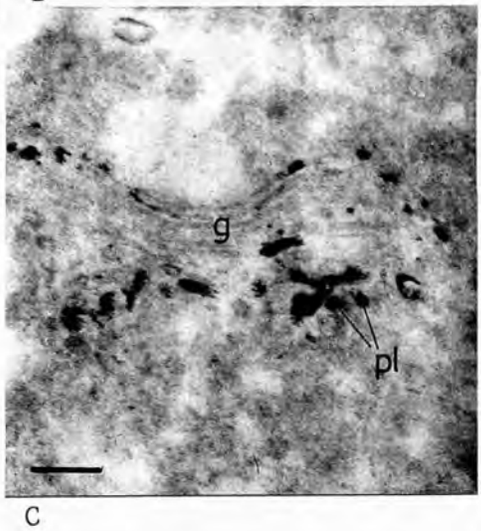

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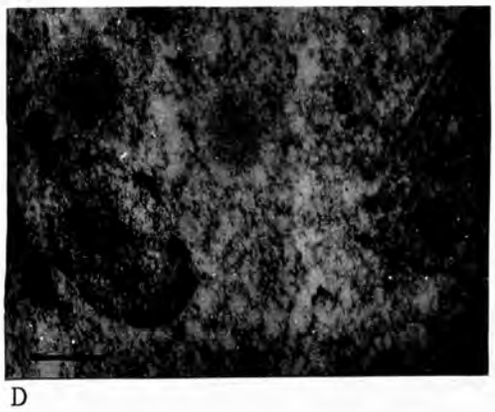

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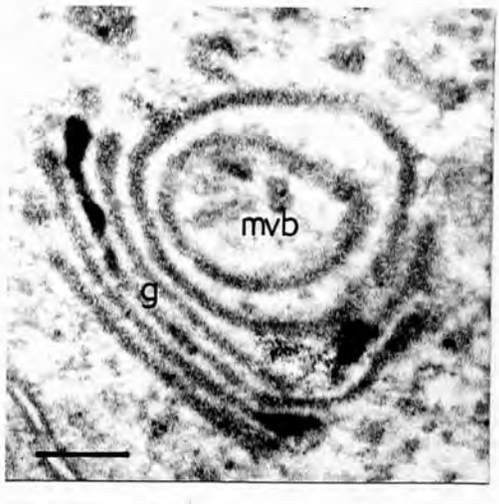


E

THE BUCCAL CAVITY.

This is an anatomical account of the buccal cavity and its associated structures which are situated in the head, a short neck confers mobility and allows the snout to extend beyond the shell margin for grazing. The ventral mouth is subterminal on the snout and is surrounded by a large fleshy outer lip that encircles a pair of inner lips, composed of left and right halves that meet medially (fig 6). A horseshoe-shaped jaw is attached to the outer surface of the antero-dorsal wall of the cavity by short slips of muscle, it serves to maintain the correct spatial relationship of the lips and the shape of the mouth.

The most conspicuous structure lying in the buccal cavity is the buccal mass, consisting of five pairs of odontophore cartilages, the radula and a complicated series of muscles (Graham,1964) (plate 8A). The radula running over the dorsal surface of the buccal mass, consists of a wide subradular membrane in which are embedded numerous transverse rows of teeth, the distal end of each tooth is turned dorsally and develops into a cusp(s) (plates 8A & B, 9A). Each transverse row contains twelve teeth, two pairs of laterals, one pair of pluricuspids, three pairs of marginals and no rachidian tooth and so has the radular formula:

3+D(1+1)+2+0+2+D(1+1)+3

(Jones, Hawkins, Watson, Jack and Kyriakides, 1984), this differs from that quoted by Fretter and Graham (1962) who give the formula as;

3+D+2+R+2+D+3.

Lateral teeth are approximately 50μm wide and 300-500μm long and bear a single cusp about 150μm high. The largest teeth are the pluricuspids (80μm wide by 450-500μm long) which are tricuspid; the central cusp is dominant (100μm tall), the inner cusp is small (45μm) and the outer one slightly larger (80μm). Evidence of fusion indicates the possible derivation of the pluricuspid tooth from a

bicuspid tooth and a single cusped tooth represented by the outer cusp of the three. Between the inner and central cusp is a small hole of unknown significance (plate 9A). The marginal teeth are the least conspicuous, their reduced cusps and breadth give them a spatulate appearance; the outer teeth are broader (80µm) than the inner ones (40µm) and the middle teeth tend to lie on the inner ones.

At the tip of the buccal mass is the "licker", ventral to it is the sublingual pouch and dorsal to it is the most complex region of the buccal cavity wall. Arising in the latter position are two dorsal folds that subsequently extend posteriorly into the oesophagus delineating the dorsal food channel between them (fig 6). Each fold originates from a thickened pad of tissue formed from the roof and sides of the buccal cavity on either side of the mid-line.

The two pairs of salivary ducts running along the anterior oesophagus and buccal cavity open onto these pads (plate 8B). They are difficult to trace to their apertures because they become enveloped by the cephalic musculature (dorsal buccal protractor and the dilator muscles of the radular diverticulum and dorsal food channel) (plate 9B). Once located, the apertures which appear inconspicuous amongst the folded ciliated wall of the buccal cavity, are seen to open onto the pad.

The dorsal posterior limit of the buccal cavity is considered by Fretter and Graham (1962) to coincide with the appearance of the two dorsal folds and its ventral limit is said to be where the radula passes posteriorly into the radular diverticulum, a broad shallow space anterior to the radular sac proper. Separating the diverticulum from the opening of the anterior oesophagus is a transverse sheet of tissue that forms the roof of the diverticulum and the floor of the anterior oesophagus (plate 8A). Arising a little posterior to this sheet are the two oesophageal ventral folds that run back into the oesophagus and fuse to form the bifid ventral fold (fig 6).

The post-buccal length of the radula lies in the blind-ending radular sac, which itself is enclosed within the radular artery, a branch of the anterior aorta. Emerging posteriorly through the neck into the visceral mass, the radula passes ventral to all other organ systems, it swings to the left and then curves to the right. After bending back upon itself, it retraces its path and ends just posterior to the buccal mass.

ABBREVIATIONS FOR PLATES 8-9.

asd..Aperture of salivary ducts.

df...Dorsal fold.

dfc..Dorsal food channel.

h....Hole in tooth.

lsd..Lateral salivary duct.

lt...Lateral tooth.

mrd..Mouth of radular diverticulum.

msd..Medial salivary duct.

mt...Marginal tooth.

pt...Pluricuspid tooth.

r....Radula.

rao..Roof of anterior oesophagus.

s....Saliva.

sg...Salivary gland.

sm...Subradular membrane.

tst..Transverse sheet of tissue.

LEGENDS TO PLATES 8-9.

THE BUCCAL CAVITY.

PLATE 8.

A. SEM of the buccal mass. x96.

B. SEM: left x82, inset shown magnified on the right.

right x330, apertures of right salivary ducts.

Scale bars: A=210μm; B(left)=240μm; (right)=60μm.

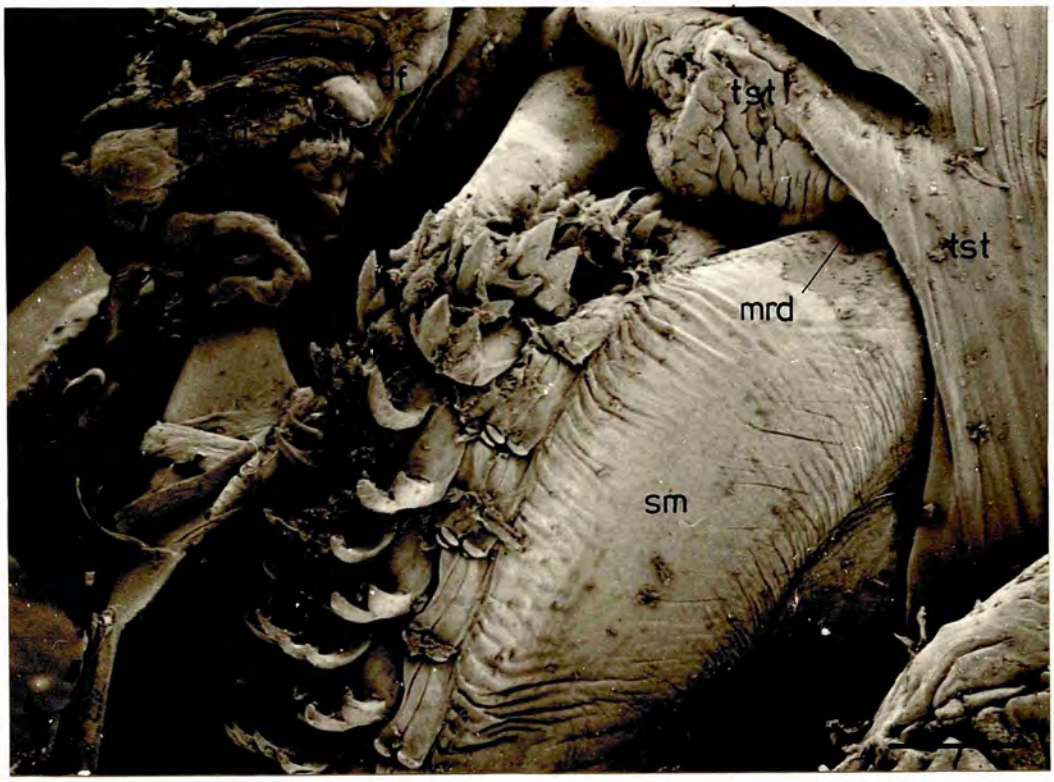
PLATE 9.

A. SEM of the radula. x163.

B. SEM, dorsal view of head after removal of cephalic integument. x53.

Scale bars: A=75μm; B=380μm.

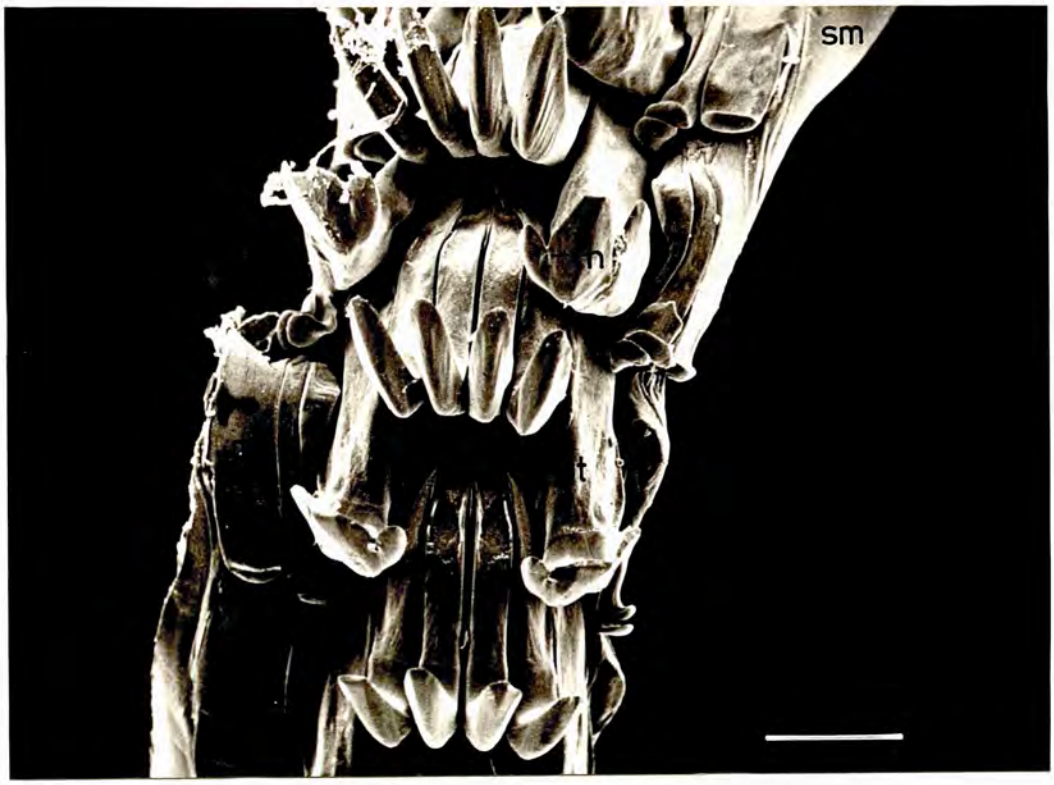
PLATE 8



A

B

PLATE 9



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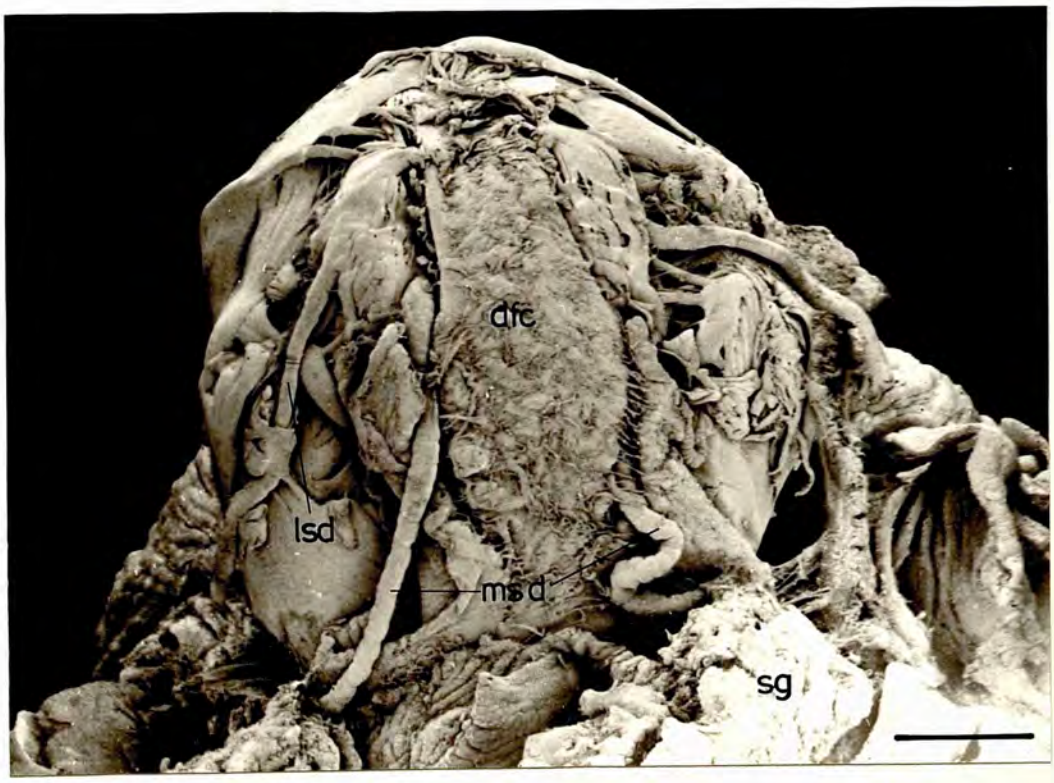


B

THE SALIVARY GLANDS.

The salivary glands of healthy specimens are bright orange or yellow (Graham, 1932; Pugh, 1963), but once the limpets have spent a few weeks in the aquarium, they adopt a creamy colour.The bulky masses of convoluted tubules lie either side of the oesophagus at the anterior of the visceral mass. Two pairs of salivary glands with an identical histology open into the buccal cavity by four ducts that run forwards along the anterior oesophagus (fig 2; plate 9B). One pair from the dorsal glands take a medial path, whilst the lateral pair emerge from the ventral glands and open anterior to the medial pair into the buccal cavity where the oesophageal dorsal folds arise (fig 6; plate 8B).

1-THE DUCTS.

Each duct can be traced back to the gland where it subdivides into narrower branches (<100μm in diameter) that penetrate in to it (plate 10A). The main ducts have a diameter of at least 200-300μm. Each duct may develop swellings (100-250μm) along its length (plate 10A), though they are commonest in the regions nearest the gland. They correspond to the "lobules of glandular tissue" reported by Graham (1964). The lobules are enveloped by connective tissue and are essentially localised outpushings of the duct epithelium, their lumina are confluent with that of the main duct.

It was not possible to slit the ducts open to observe ciliary currents using graphite suspensions. Instead the proximal end of a duct was placed in a droplet of the suspension and occasionally a few particles could be seen moving slowly along the duct. The ciliary activity may appear to be insignificant, but the narrowness of the lumen and high viscosity of the saliva indicate that the cilia must be working against large drag forces.

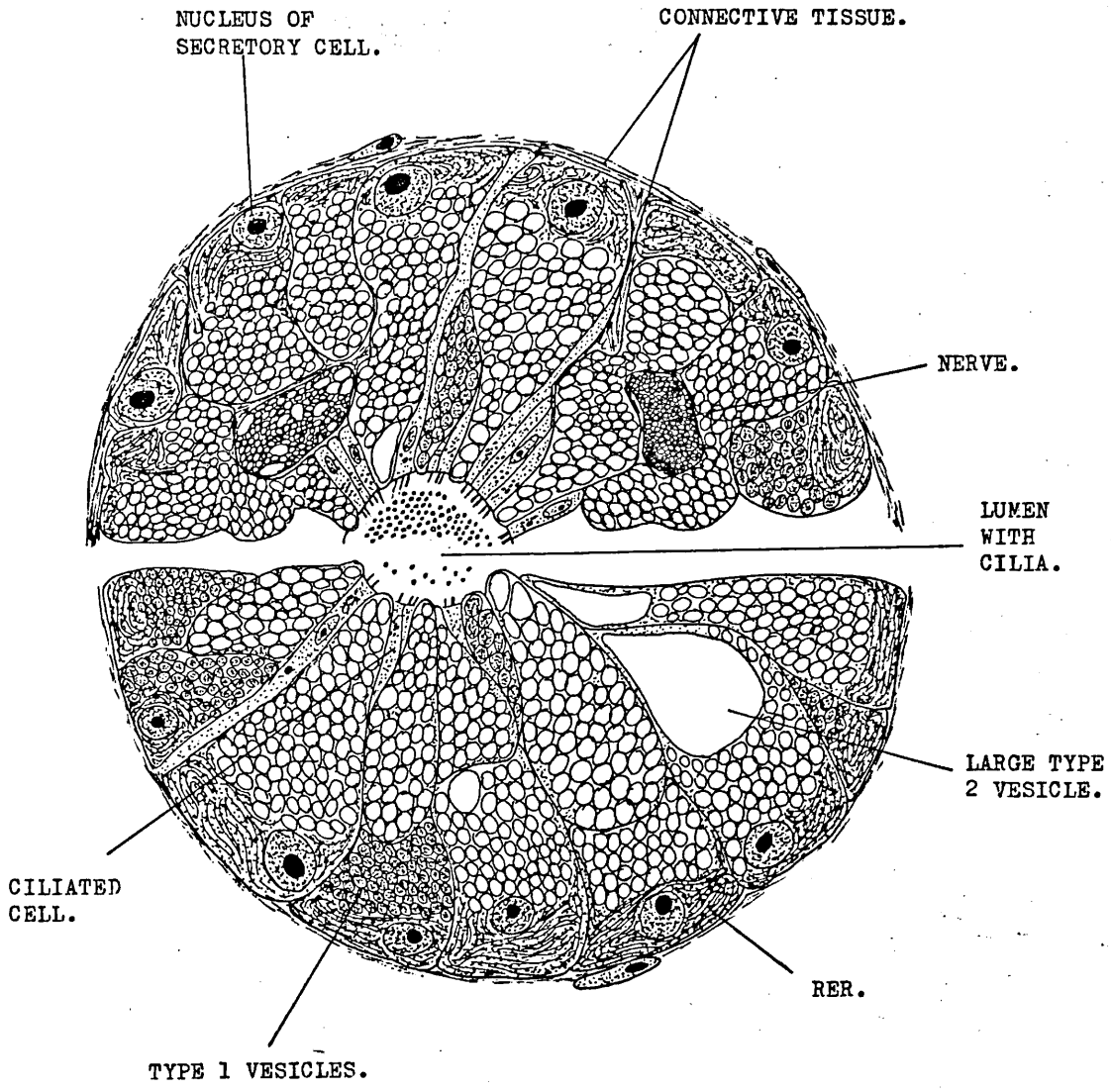
A). HISTOLOGY AND ULTRASTRUCTURE. Examination of teased lengths of ducts reveals little information because the delicate tissue quickly

disintegrates into a mass of globules. Short lengths of duct treated with Liang's leucofuchsin method reveal 2 or 3 nerves coursing through the epithelium, they cannot be traced into the glandular lobules and their fine branches are difficult to trace within the mass of tubules. The epithelium of the ducts is formed of the same two cell types as the tubule epithelium and so have similar fixation properties. The epithelium is poorly fixed with Bouin, but well preserved with glutaraldehyde. Figure 4 shows a diagrammatic representation of the epithelium as it occurs in the ducts and tubules, in the latter the secretory cells outnumber the ciliated cells. The secretory cells are often so distended that they obscure the ciliated cells (plates 10B & 12A). Both cell types rest on the basal lamina (150nm thick) and slips of the sub-epithelial connective tissue (3-5μm thick) penetrate the epithelium to bind and support it.

The numerous long cilia have their distal ends arranged parallel to the long axis of the duct and are thus seen in transverse section; whereas the proximal ends are cut obliquely as they emerge from ciliated cells (plates 10B & 11B). Ultrastructurally the ciliated cells resemble the type described in section 1 except for the following differences. A brush border is absent and the flat apical membrane lacks a glycocalyx (plates 10B, 11B & 12A), the endocytotic canal system is absent and there is no evidence of pinocytosis. The restricted basal regions contain few organelles, most are located in the apical portions. When the cells are traced to the basal lamina the basal membrane rests against it; hemidesmosomes, a cytoskeleton and extracellular spaces are absent. Blebs of cytoplasm are not produced. The secretory cells are described in part 2.

Large nerves (10-20μm diameter) were encountered in thin resin sections (plate 10B), these possibly correspond to the nerves demonstrated with Liang's technique. They contain hundreds of small (300-400nm) axons or fewer, larger (800-1200nm) ones that resemble

FIGURE 4. A DIAGRAMMATIC REPRESENTATION OF SALIVARY DUCT (TOP) AND TUBULE EPITHELIA SEEN IN T.S.



those described in section 1. There is intimate contact between the neurilemma and the plasma membranes of adjacent cells, but synaptic junctions have not been located; secretion may be under neural control.

2-THE TUBULES.

A). HISTOLOGY AND ULTRASTRUCTURE. Each gland is composed of numerous tubules ramifying in all directions to form a convoluted mass of secretory tissue. Individual tubules are closely packed together and bounded by a thin (1μm) connective tissue layer similar to that binding the ducts. The pH of the homogenised gland is 6.1. In sections the tubules are circular with a diameter of 60-80μm, the narrow (5-10μm) lumen is central (fig 4; plate 11A).

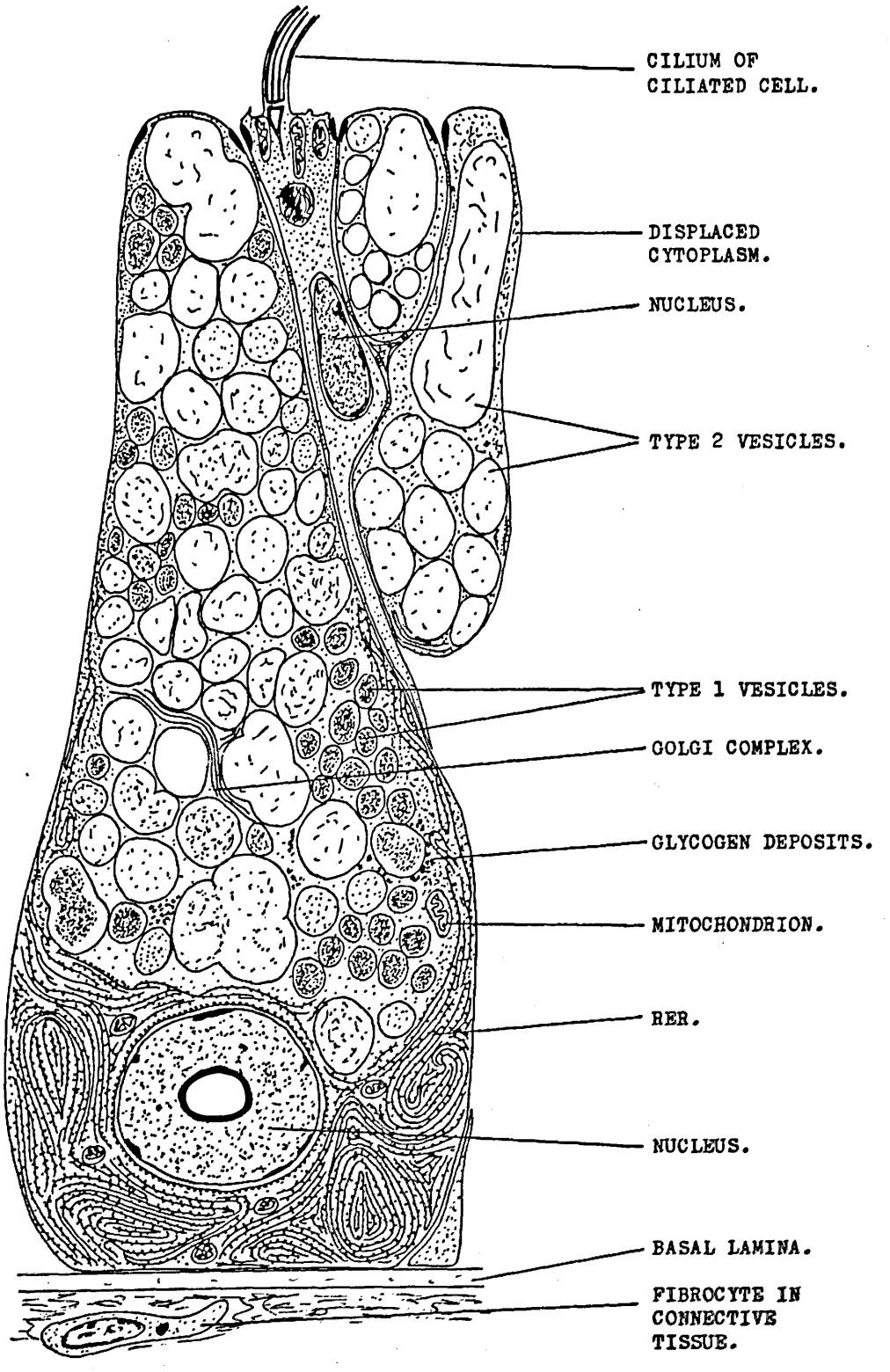
Examination of stained sections of both duct and tubule epithelia gives the impression that there are two types of secretory cell. One contains vesicles that are reactive to most stains used and the other type contains droplets that are only weakly stained (plates 10B, 11A & B, 12A), these correlate to cell types 1 and 2 respectively reported by Pugh (1963). Thick resin sections stained with toluidine blue show the secretory cells full of vesicles (1-3μm), some of them unstained, others coloured blue-green and very few dark blue. The abundance of the blue-green vesicles varies from cell to cell and they usually occur in a more basal position with respect to the colourless ones; the basophilic cytoplasm stains purple. In Mallory-stained wax sections many cells stain bright blue, others are blue-black and only a few orange, the blue-black cells contain dark blue vesicles 1-1.5μm in diameter. Following Heidenhain's haematoxylin, the majority of cells stain a steel-blue colour and contain blue-black vesicles 1-1.5μm in diameter, some cells appear a straw-brown colour. Unstained droplets 3-4μm in diameter are visible in most cells. These staining reactions indicate the presence of proteinaceous and mucoid components in the vesicles.

Ultrastructurally, the two cell types proposed by Pugh differ only in their vesicle content. The following description applies to both cell types from ducts or tubules (fig 5). Various distended shapes occur, only occasionally could the entire cell be traced from the basal lamina to the lumen. The cells are widest at their base and taper towards their apex, so they are pear or flask-shaped. Microvilli and cilia are absent from the slightly domed apical surface of the cells which are joined to their neighbours by typical junctional complexes. Few organelles are visible and the cytoplasm is displaced to the peripheries of the cell by numerous secretory vesicles (plate 12A).

The basophilic cytoplasm appears moderately electron-opaque and granular due to the abundant RER and free ribosomes. RER occurs as extensive curvilinear whorls and the cisternal space (20nm) is full of a moderately electron-opaque, finely granular-filamentous material (plates 12B & C). Small spherical mitochondria are located mainly in the basal cytoplasm; profiles of the golgi complex, consisting of a few indistinct cisternae, are usually found between secretory vesicles and not in the peripheral cytoplasm. A large (4-5μm) spherical, basal nucleus with a conspicuous nucleolus (2μm) is characteristic of the cells; little heterochromatin is present in the nucleoplasm and the outer membrane of the nuclear envelope is studded with ribosomes(plate 12B).

Cell type 1 typically contains numerous membrane-bound, finely granular vesicles (1-1.5μm) that characteristically display a peripheral, electron-lucent halo surrounding the granular matrix (plate 12C). This halo is wider (up to 150nm) in the paler vesicles, but only 20-40nm wide in the most opaque ones.The difference in opacity is due to the amount of material sequestered in each vesicle. In lower densities the secretory material adopts a flocculant appearance rather than the granular form in the dense vesicles.

FIGURE 5. A DIAGRAMMATIC REPRESENTATION OF SALIVARY GLAND SECRETORY AND CILIATED CELL TYPES.



The lucent membrane-bound vesicles common in the type 2 cells vary in size; the smallest may be 800nm and the largest can occlude most of the cell, however, 2μm is a common size (plates 10B, 11A&B, 12A). Larger vesicles are formed by the fusion of smaller ones. Sometimes vesicles similar to those in cell type 1 occur amongst these lucent vesicles. At higher magnifications (plate 10B) low densities of a faint flocculant material occur in the lucent vesicles. It seems likely, therefore, that the two putative cell types are different phases of a single type of cell as suggested by Pugh (1963). This conclusion was reached because of the similar ultrastructure and the similarity of the secretory product that occurs in varying densities giving rise to vesicles of different electron-opacity. The phases are not related to the tidal cycle since both are always present in any one tubule at any time.

Exocytosis of the secretion occurs when the bounding-membrane of the vesicle fuses with the apical plasma membrane and the secretory material diffuses into the lumen. The lucent type 2 vesicles are released intact (plate 11B).

B). HISTOCHEMISTRY.

Summarised results of the confirmatory tests are presented in table 7.

i).CARBOHYDRATES. PAS-treated sections display a clear positive reaction for 1,2-glycol groups, secretory cells within a particular tubule stain various shades of red and pink. This reaction is due to the presence of numerous red vesicles (1.0μm diameter =type 1 vesicles) in the cells. The reactions with alcian blue and aldehyde fuchsin demonstrate the presence of carboxylated and sulphated acid mucopolysaccharides. With all three techniques, certain cells within a single tubule react more strongly than others. An attempt was made to differentiate the PAS-positive and alcianophilic components of the secretion using the AB pH2.5-PAS method. A majority of the tubules

TABLE 7.

SUMMARISED HISTOCHEMICAL RESULTS OF THE SALIVARY SECRETORY CELL.

PROCEDURE	RESULT	CONCLUSION
SHIFF	-	NO ALDEHYDE GROUPS
P.A.S	+	1,2-GLYCOL GROUPS
ACETYL/P.A.S	+R	" " PARTIALLY BLOCKED
DIASTASE/ P.A.S	+	NO GLYCOGEN DEMONSTRATED
AB pH 2.5	+	COOH & SO_4-ACID MUCOPOLY-SACCHARIDES (A.M.P.)
M/AB pH 2.5	-	" " BLOCKED
M/S/AB pH 2.5	+R	COOH A.M.P. UNBLOCKED
AB pH 1.0	+	SO_4 A.M.P.
M/AB pH 1.0	-	" " REMOVED
M/S/AB pH 1.0	+W	NOT ALL SO_4 A.M.P. REMOVED BY M, SO THESE REACT WITH AB
AF	+	SO_4 A.M.P.
M/AF	-	" " REMOVED
M/S/AF	+W	NOT ALL SO_4 A.M.P. REMOVED BY M, SO THESE REACT WITH AF
AB pH 2.5/ P.A.S	+/+	COOH & SO_4 A.M.P. & 1,2-GLYCOL GROUPS
PA-TSC-SP	+	1,2-GLYCOL GROUPS
BROMOPHENOL BLUE	+W	SLIGHT PROTEIN CONTENT
DE-NH_2/BROMO PHENOL BLUE	+W	GROUPS OTHER THAN NH_2 PRESENT
DNFB	-	TYROSINE, SH & NH_2 ABSENT
DIAZOTIZ'TN-COUPLING	+	TYROSINE PRESENT
IODITIZATION/ DIAZ-COUPLING	+R	PARTIAL BLOCK
SUDAN BLACK B	-	NEUTRAL LIPIDS AND PHOSPHOLIPIDS ABSENT
NAPHTHOL AS-BI PHOSPHATE	+	ACID PHOSPHATASES PRESENT
GOMORI MEDIUM pH 4.9	-	ACID PHOSPHATASE INACTIVE
HUGON-BORGERS MEDIUM pH 9.0	-	ALKALINE PHOSPHATASE ABSENT

NOTATION:

AS FOR TABLES 5 & 6.

stained this way contain cells with mauve or purple vesicles and only a few stain red or blue. These results indicate that the two components are produced in the same cell and not by separate cell types. Furthermore, because some cells stain for only one component at any time this indicates that the two components are probably synthesised at different times.

PA-TSC-SP staining reveals the presence of glycogen deposits in the secretory cell cytoplasm, especially around the lucent vesicles; diastase digestion removes the deposits. The type 1 vesicles stain weakly (plate 12D), but the golgi complex does not.

ii).PROTEINS. Sections stained with bromophenol blue appear pale blue and devoid of any strongly reactive vesicles, areas of displaced cytoplasm stain more strongly. Only the diazotization-coupling technique yields a strong positive result for tyrosine, the reactive vesicles are stained and distributed similarly to the PAS and alcianophilic-reactive vesicles.

iii). ENZYMES. The naphthol AS-BI phosphate method gives a specific localization of acid phosphatase activity in the mid region of those secretory cells that had an affinity for the methyl green counterstain used. The precise location of the enzyme could not be deduced from the frozen sections because the Gomori incubation method for the cytochemical demonstration of the enzyme failed to give specific results. The positive result is probably due to the golgi complex.

ABBREVIATIONS FOR PLATES 10-12.

bs....Blood space.

c.....Cilia.

cc....Ciliated columnar cell.

dl....Lumen of duct.

l.....Lumen of tubule.

m.....Mitochondrion.

nv....Nerve.

rer...Rough ER.

S1,2..Salivary cells containing type 1 & 2 vesicles.

sd....Salivary duct.

sdl...Lobule of salivary duct.

v1,2..Vesicle types 1 & 2.

LEGENDS TO PLATES 10-12.

THE SALIVARY GLANDS.

PLATE 10.

A. SEM of salivary gland and its duct system. x82.

B. Section of duct epithelium showing the ciliated and two secretory cell types. 5.25K.

Scale bars: A=240μm; B=4μm.

PLATE 11.

A. Transverse section of a salivary tubule. 2K.

B. Tubule lumen, arrows indicate release of secretion. 10K.

Scale bars: A=10μm; B=2μm.

PLATE 12.

A. Two cells containing type 2 vesicles. 11.5K.

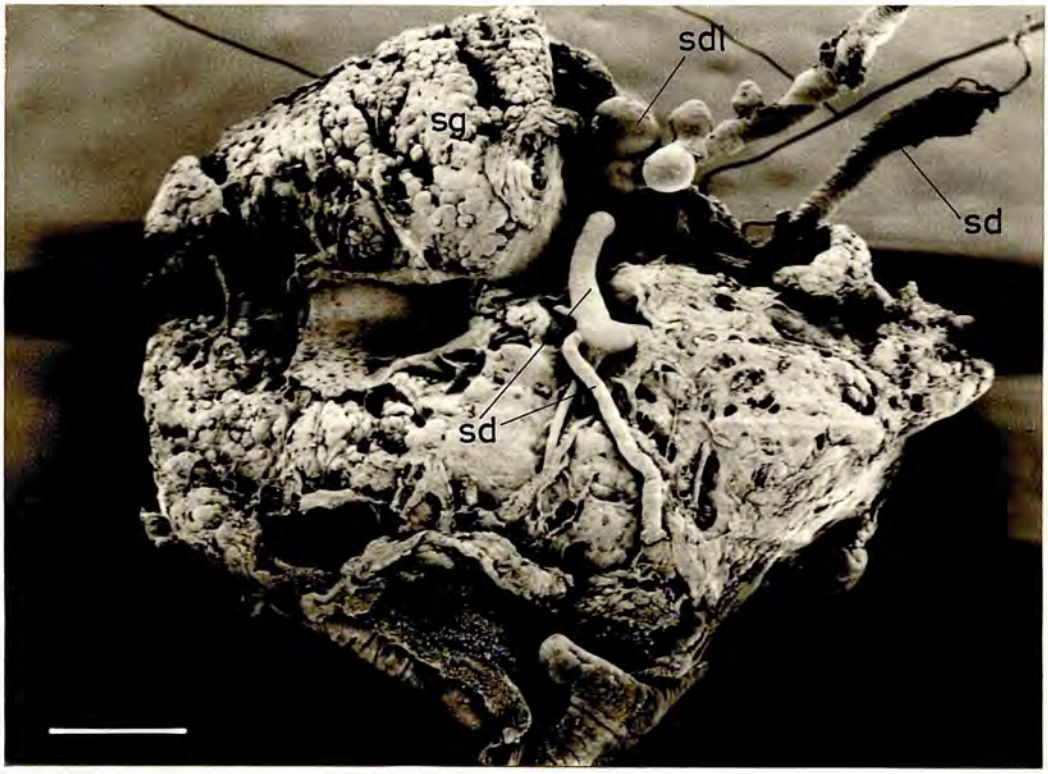
B. Basal regions of secretory cells. 5.5K.

C. A type 1 vesicle, arrow indicates lucent halo. 88K.

D. Type 1 vesicles stained by the PA-TSC-SP method. 39K.

Scale bars: A,B=3μm; C,D=500nm.

PLATE 10



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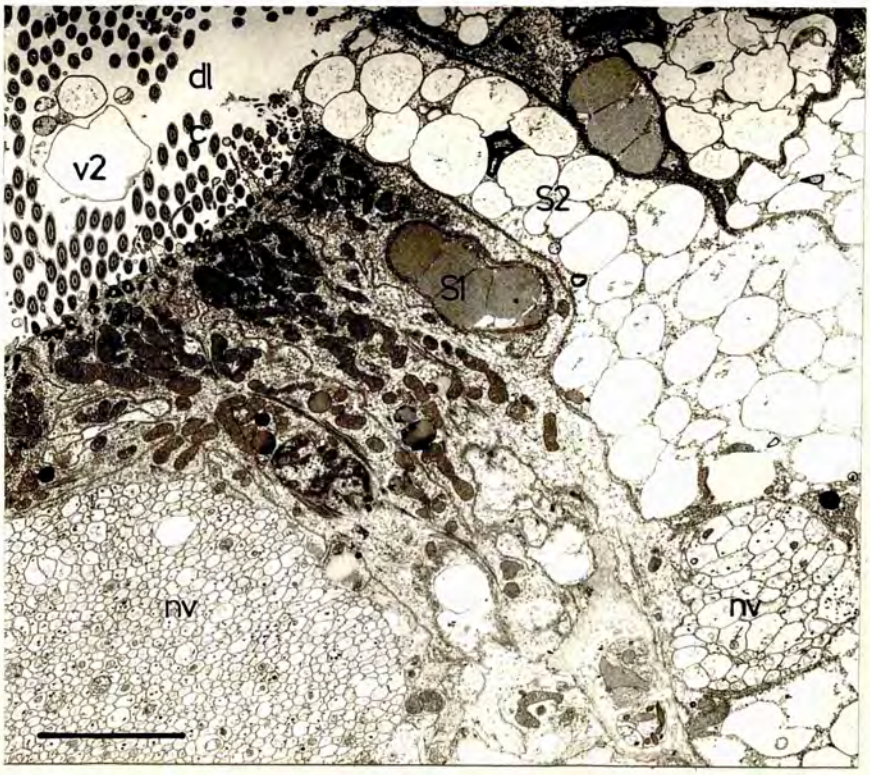

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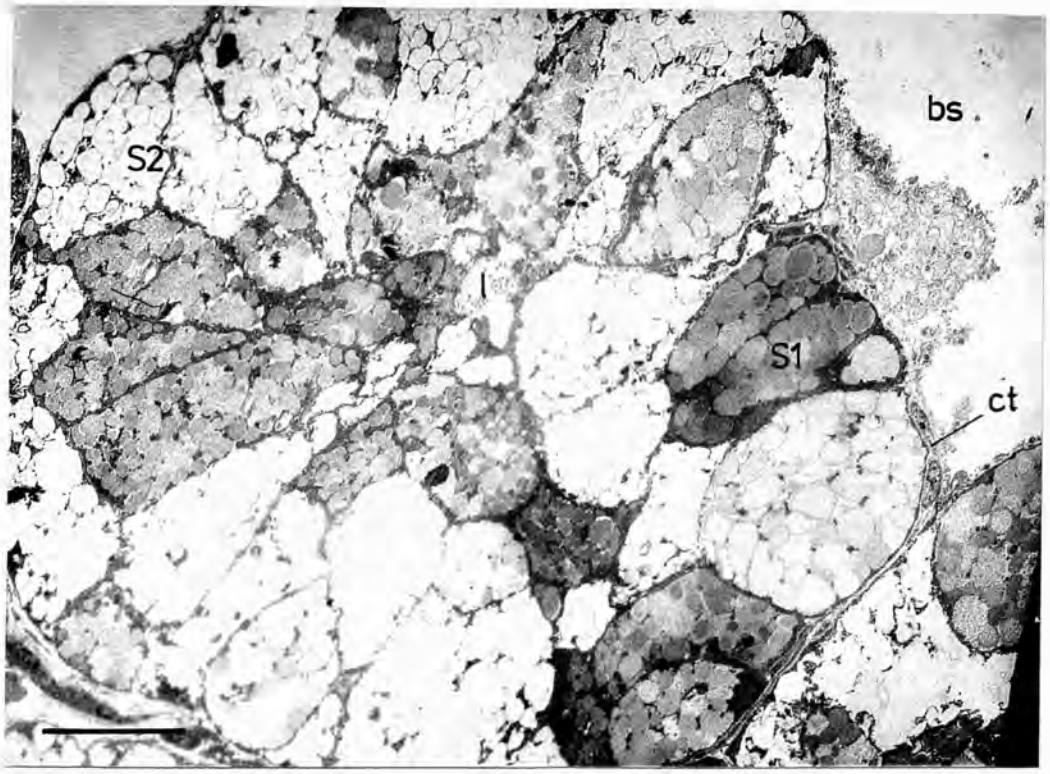
PLATE 11



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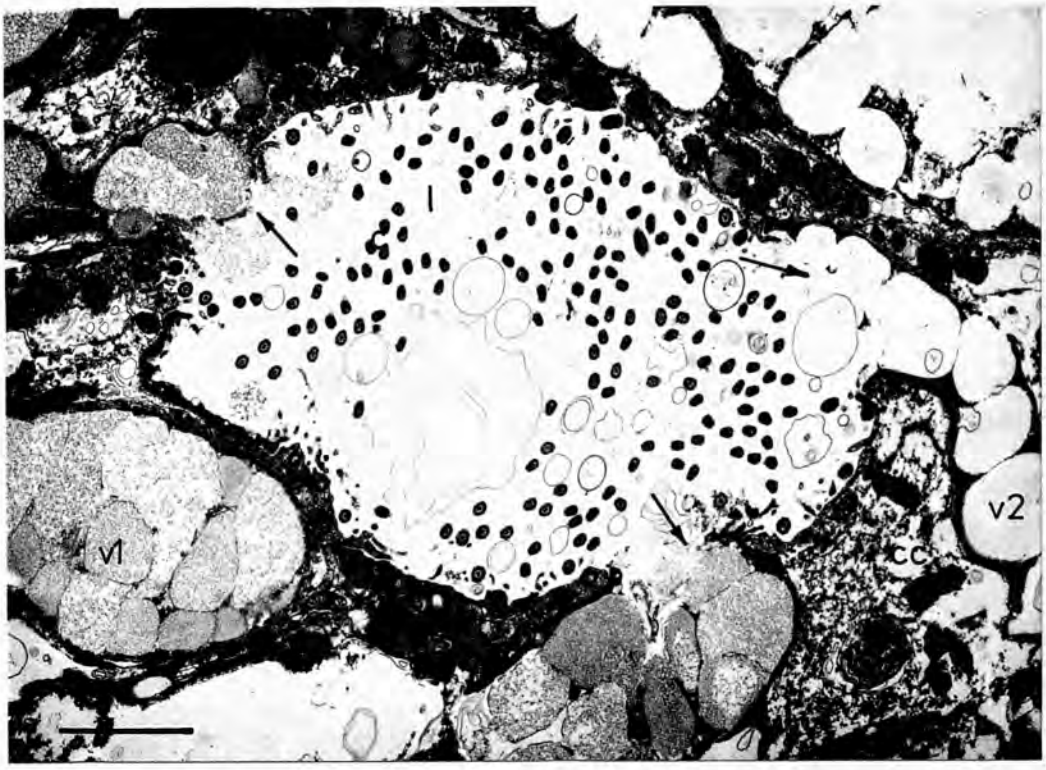

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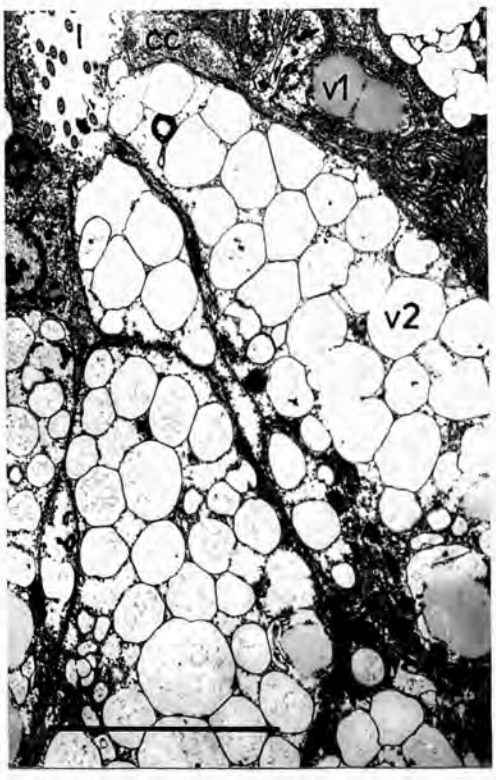
PLATE 12



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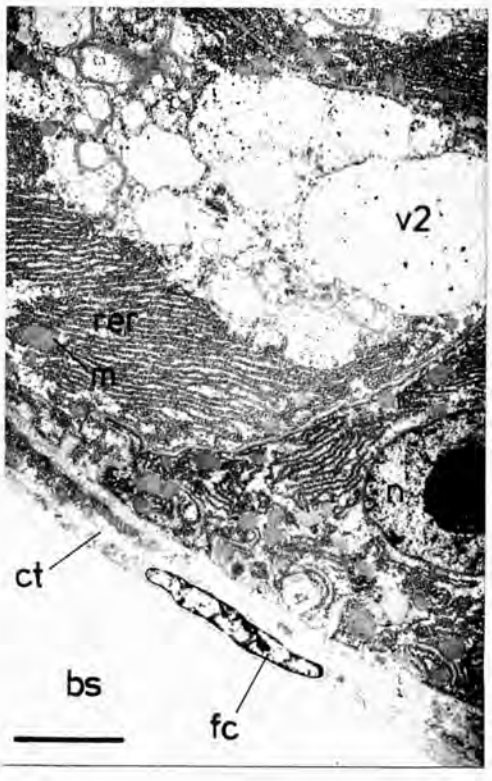

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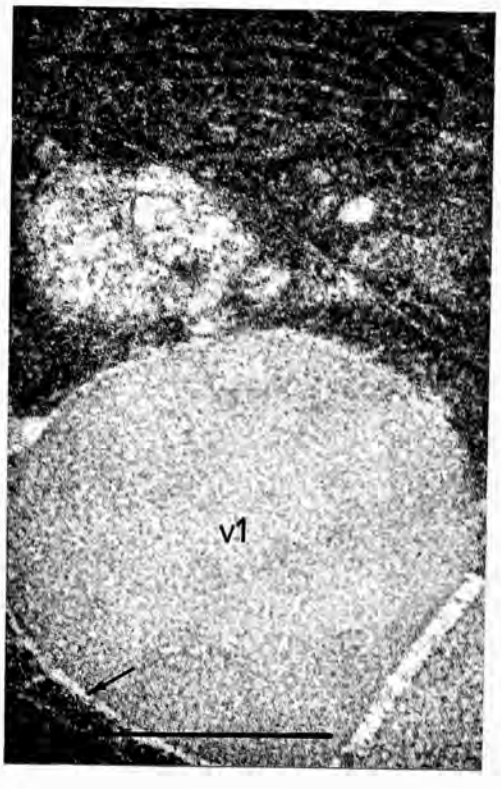

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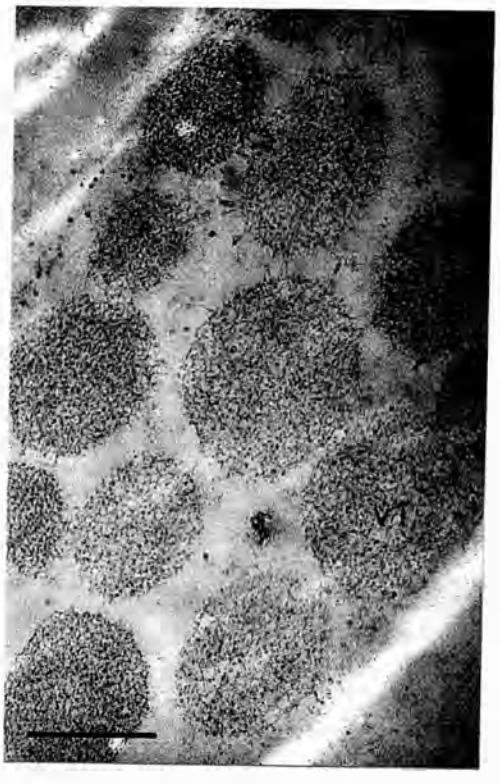


D

THE OESOPHAGUS.

1-ANATOMY.

That part of the gut in a prosobranch gastropod which is involved in torsion is the oesophagus; it connects the buccal cavity to the stomach and is divisible into three morphologically distinct sections, the anterior, mid- and posterior regions (Graham, 1939; Fretter and Graham, 1962) (fig 6). The oesophagus is a conspicuous part of the gut upon dissection because of its large size and pink colour. As it passes posteriorly out of the head through the short neck, it curves in an horizontal plane through the visceral mass to join the stomach on the animal's right. Only the gonad, right kidney, radular sac and ventral loops of intestinal sections B and C lie ventral to the oesophagus. The internal folds are visible by transparency and the contents have a pH of 6.0.

The anterior oesophagus lies behind the posterior limit of the buccal cavity limited ventrally by the mouth of the radular diverticulum and dorsally by the openings of the salivary ducts. Arising from the dorsal wall just behind these openings are the two ciliated, dorsal folds that run the length of the oesophagus delineating the dorsal food channel between them. From the ventral wall just behind the opening of the radular diverticulum, arise two ciliated folds that run diagonally across the wall to fuse in the mid-line forming the bifid ventral fold. This follows the dorsal ones and its free bifid lip forms a "lid" to the dorsal food channel, thus sealing it off from the lateral portions of the oesophageal lumen (fig 6).

The dorsal folds may attain a height of 500-600μm and the ventral up to 1mm (plates 13A & B). The width of the food channel is 250-350μm and that of the bifid lip of the ventral fold is 300-400μm. Two cell types line the food channel; typical ciliated columnar cells and subepithelial mucous gland cells (plates 14A & 15).

LEGEND TO FIGURE 6.

THIS STEREOGRAM DISPLAYS THE ALIMENTARY TRACT AS SEEN FROM THE POSITION INDICATED IN FIGURE 2. THE LEFT SIDE OF THE CEPHALIC INTEGUMENT, BUCCAL EPITHELIUM AND ANTERIOR OESOPHAGEAL EPITHELIUM HAVE BEEN REMOVED TO REVEAL THE INTERNAL FOLDS AND BUCCAL MASS. THE MID-OESOPHAGUS HAS BEEN SECTIONED TO DISPLAY THE EFFECT OF TORSION ON THE INTERNAL STRUCTURES. TO FACILITATE THIS VIEW THE LEFT HALF OF THE VISCERAL MASS HAS BEEN REMOVED AND THE DIGESTIVE GLAND OMITTED ENTIRELY.

NOTATION:

aoe	ANTERIOR OESOPHAGUS.
bc	BUCCAL CAVITY.
be	BUCCAL EPITHELIUM.
bm	BUCCAL MASS.
ci	CEPHALIC INTEGUMENT.
ct	CEPHALIC TENTACLE.
dfc	DORSAL FOOD CHANNEL.
f	FOOT.
il	INNER LIP.
int A,B,C,D	INTESTINAL SECTIONS.
j	JAW.
lf	LATERAL FOLD.
lp	LATERAL POUCH.
m	MANTLE.
mo	MOUTH.
moe	MID-OESOPHAGUS.
mrd	MOUTH OF RADULAR DIVERTICULUM.
n	NECK.
ol	OUTER LIP.
poe	POSTERIOR OESOPHAGUS.
r	RADULA.
rdf	RIGHT DORSAL FOLD.
re	RECTUM.
rs	RADULAR SAC.
rsa	RIGHT SALIVARY APERTURE.
s	SNOUT.
sg	SALIVARY GLAND.
slp	SUBLINGUAL POUCH.
srm	SUBRADULAR MEMBRANE.
st	STOMACH.
tst	TRANSVERSE SHEET OF TISSUE.
vf	VENTRAL FOLD.

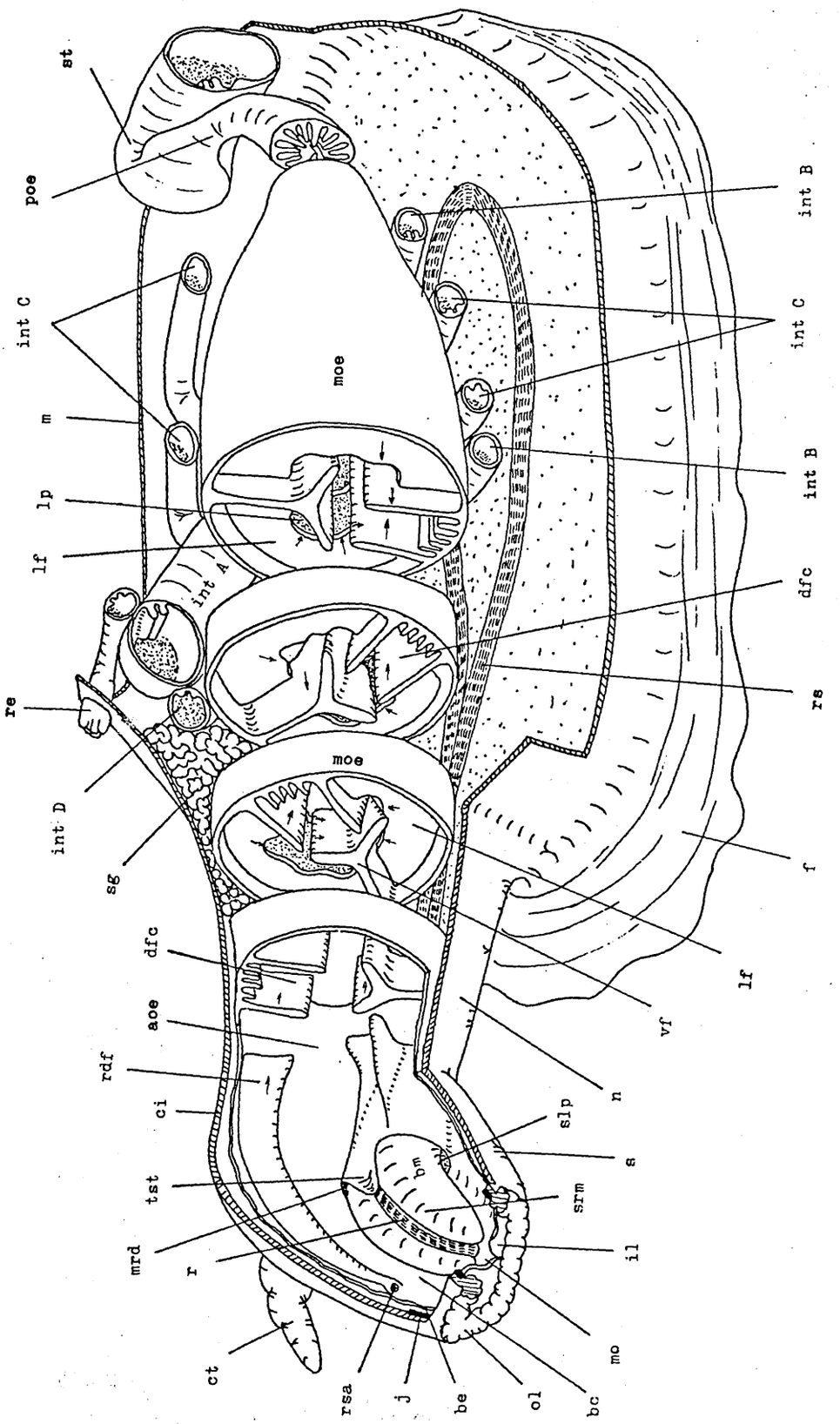

st
poe
int C
m
lp
lf
int A
re
int D
sg
dfc
aoe
rdf
ci
tst
mrd
r
ct
rsa
j
be
ol
bc
mo
il
srm
slp
s
n
vf
lf
f
rs
dfc
int B
int C
int B
moe
moe

FIGURE 6.

The anterior limit of the mid-region is that point where, from the lateral walls, the first of a series of transverse, lamellate lateral folds arises (plates 13A & B). They create a number of separate lateral pouches that are lined by a ciliated, glandular epithelium composed of two cell types: typical ciliated cells and gland cells secreting an amylase (plate 14B). The lateral pouches constitute the oesophageal gland. The lateral folds join the longitudinal dorsal and ventral folds so that each pouch constitutes a separate compartment communicating with paired lateral oesophageal channels (plate 13A), one either side of the food channel, by a wide, centrally directed mouth. The more posterior pouches are smaller than the anterior ones (plate 13B).

Following the mid-section and connecting it to the stomach is the narrower, simpler posterior section lined by numerous tall (150-250μm), wide (70-100μm), ciliated longitudinal folds (plate 16A). Histologically, they are composed of a similar epithelium to that lining the food channel. The dorsal and ventral folds diminish in size so that they can not be distinguished from the other folds, as this occurs the food channel disappears so that the lumen is reduced to narrow channels between the large folds (plate 16A).

The structural complexity of the oesophagus is further complicated by torsion (fig 6). The dorsal folds which originate from the morphologically dorsal wall of the buccal cavity curve around the left side of the mid-oesophagus to lie in the topographical mid-ventral line. Similarly, the ventral fold comes to lie in the mid-dorsal line after curving over the right side. Furthermore, the lateral pouches are also twisted, so that the series originating on the morphological left side, comes to lie on the topographical right side of the oesophagus and vice versa. The overall effect is a counter-clockwise twisting of the oesophagus through 180° when seen from the posterior aspect.

The oesophagus is divisible into three morphologically distinct regions, but histologically and functionally only two distinct regions occur. The food channel and posterior oesophagus, which are histologically similar, accumulate and transport food to the stomach. The lateral glandular pouches are functionally and histologically distinct from the food channel and produce the only extracellular enzyme in the gut.

2-CILIARY CURRENTS.

When an oesophagus is opened a few minutes after force feeding an animal with graphite suspensions, a graphite-laden mucus string is found in the food channel. Particles are not found outside the channel. Pipetting suspensions onto the luminal surface of an opened oesophagus reveals the currents accurately described by Graham (1932) (fig 6). Powerful currents on the surfaces lining the food channel are directed towards the stomach, whilst weaker ones on the lateral folds carry particles out of the pouches onto the free edges of these folds. From there, particles are taken onto the outside face of the morphologically right dorsal fold by a counter-clockwise current (viewed from posterior aspect) on the free lip of the lateral folds of the morphological right side. In the case of the lateral folds of the morphological left side, particles are carried along the free edge by a counter-clockwise current onto the outside surface of the ventral fold. The direction of these currents and the effect of torsion are all counter-clockwise.

Particles and mucus strings on the outer surfaces of the dorsal and ventral folds are carried anteriorly until they are gradually gathered into the food channel by the transversely beating cilia on the crests of the folds (plate 17A). On the longitudinal folds of the posterior oesophagus cilia beat powerfully towards the stomach. Graphite particles in this region are all bound into a bulky mucus string. The narrow diameter and reduction of the lumen by the tall folds creates a

"bottle-neck" effect so that the mucus accumulates into a large mass. Rhythmic contractions of the posterior oesophagus occur spontaneously, persist for a few seconds and then cease. This squeezing will release soluble nutrients from the mass and help its entry into the stomach.

3-THE EPITHELIUM OF THE FOOD CHANNEL.

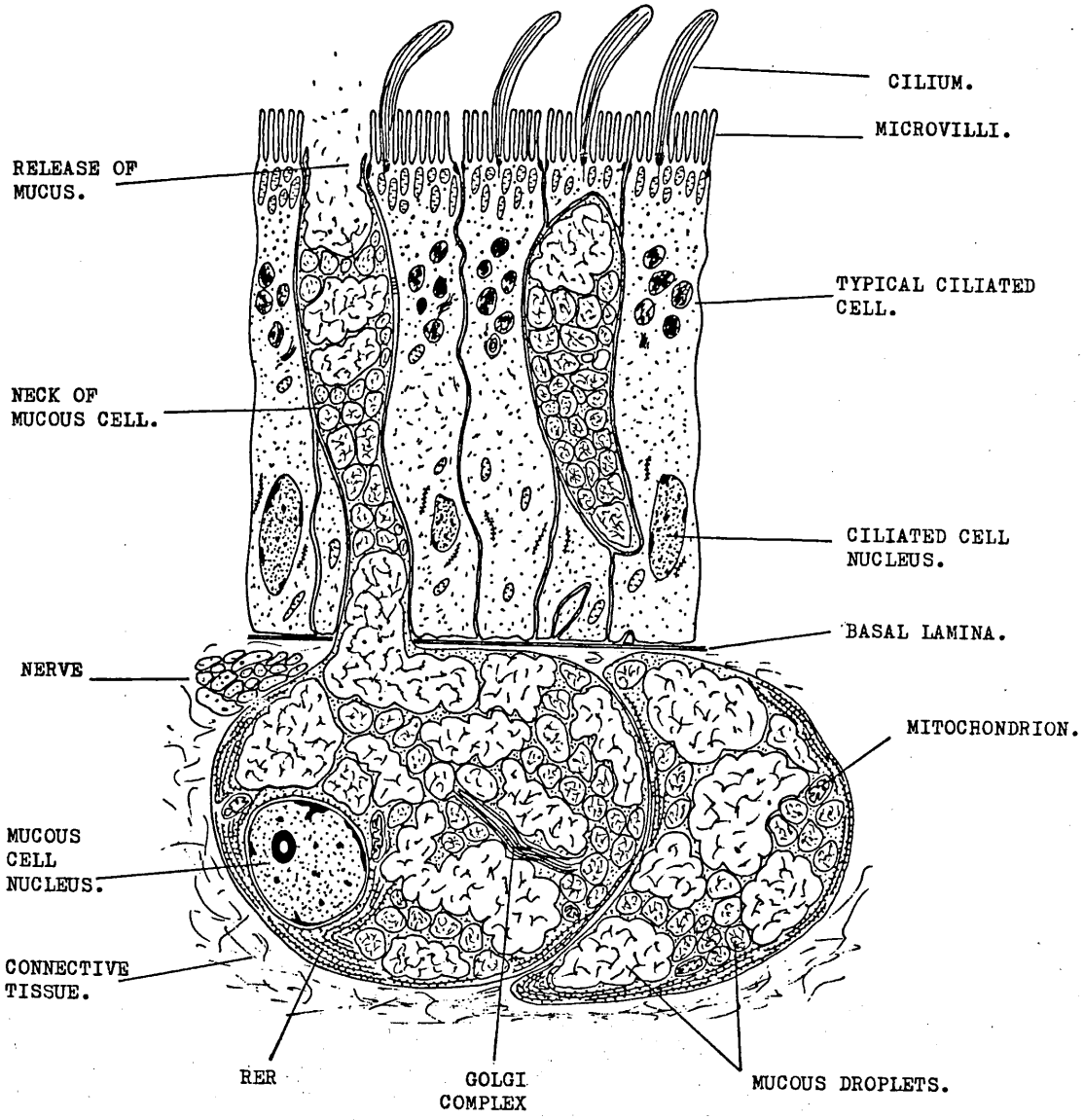
This lines the inner surfaces and crests of the dorsal, ventral and posterior longitudinal folds of the oesophagus (fig 7; plates 13A & 16A). Examination of living tissue reveals a thick covering of active cilia coated with the sticky, clear mucus from the subepithelial mucous cells, the necks of which penetrate the ciliated epithelium and open into the food channel (plates 15 & 16B).

A). THE CILIATED CELLS. The ciliated columnar cells vary in height, the tallest being on the crests of the longitudinal folds (table 4). They are similar to those described in section 1 except in the following respects: the small, rare residual bodies sometimes contain PTA-reactive areas (plate 6B), the apical cytoplasm stains positively with the naphthol AS-BI phosphate method, secretory blebs are rarely encountered, there is no evidence of a cytoskeleton and intercellular channels are absent.

B).THE MUCOUS GLAND CELLS. The distribution of these cells was checked using serial wax sections stained with alcian blue. They are first encountered in the subepithelial connective tissue of the anterior oesophagus where the dorsal and ventral folds originate by an invagination of the ciliated epithelium. The folds increase in height as they pass posteriorly, the mucous cells proliferate, so they are more abundant in the posterior section (plates 13A & 16A).

The large (10-30μm), globular cell body of a typical mucous cell contains most of the cytoplasm and cell organelles (plate 15). The nucleus (4μm diameter) frequently has a nucleolus (1.5μm diameter) with a great deal of heterochromatin against the inner membrane of the nuclear envelope, the outer one is studded with ribosomes. Mucous

FIGURE 7. A DIAGRAMMATIC REPRESENTATION OF THE OESOPHAGEAL DORSAL FOOD CHANNEL EPITHELIUM.



droplets accumulate in the central portions of the cell body and in the neck, thus restricting the cytoplasm to the periphery of the cell (plate 15). Here lie the large whorls of RER cisternae interspersed with mitochondria, although some cisternae may intrude into the central regions of the cell. Profiles of the golgi complex occur as a concave stack of six to seven cisternae surrounding large numbers of mucous droplets (plates 17B, C & D).

The smallest vesicles (300-500nm) can fuse with others to form large, membrane-bound vesicles of diverse shapes and sizes; single vesicles are commonly 1-2μm in diameter. All vesicles, irrespective of size, have a characteristically pale, speckled appearance when stained with uranyl acetate and lead citrate (plates 14A & 15). At higher magnifications it is clear that this appearance is due to a moderately electron-opaque coagulum of a thread-like lattice with minute globules of secretion (plate 17D). This appearance persists irrespective of position in the cytoplasm, so there is no ultrastructural evidence to suggest that the vesicles mature as they pass along the neck.

Exocytosis of the mucus occurs by fusion of the vesicular membrane with the cell membrane followed by the diffusion of the secretion into the food channel; the speckled appearance of the mucus is lost. This merocrine secretion may be under neural control because the subepithelial nerves have a close association with the mucous cells (plate 15); however, no connection with them was found.

In thick resin sections, the mucus stains consistently positive for carbohydrates, but not for proteins or lipids. Staining with the PAS method, alcian blue at pH 2.5 and 1.0 and aldehyde fuchsin demonstrates the presence of 1,2-glycol groups and acid mucopolysaccharides (AMPS) (table 8). Differentiation of carboxylated and sulphated AMPS was attempted with the alcian blue pH2.5/aldehyde fuchsin method. Alcianophilia was exhibited by the largest vesicles whilst the

TABLE 8.

SUMMARISED HISTOCHEMICAL RESULTS OF THE MUCOUS GLAND CELL.

PROCEDURE		RESULT	CONCLUSION
SCHIFF		-	NO ALDEHYDE GROUPS
P.A.S		+	1,2-GLYCOL GROUPS
ACETYL/P.A.S		-	" " BLOCKED
DIASTASE/ P.A.S		+	NO GLYCOGEN DEMONSTRATED
AB pH 2.5		+	COOH & SO_4 ACID MUCOPOLY-SACCHARIDES (A.M.P.)
M/AB pH 2.5		+R	PARTIAL BLOCK OF " "
M/S/AB pH 2.5		+	COOH UNBLOCKED
HY/AB pH 2.5		+	HYALURONIC ACID & CHONDROITIN SULPHATE ABSENT
AB pH 1.0		+	SO_4 A.M.P. PRESENT
M/AB pH 1.0		-	" " REMOVED
M/S/AB pH 1.0		+R	NOT ALL SO_4 A.M.P. REMOVED BY M,SO THESE REACT WITH AB
HY/AB pH 1.0		+	HYALURONIC ACID & CHONDROITIN SULPHATE ABSENT
AF		+	SO_4 A.M.P. PRESENT
M/AF		-	" " REMOVED
M/S/AF		-	NO SO_4 A.M.P. LEFT TO STAIN
AB pH 2.5/AF		+/+	DIFFERENTIATION OF COOH & SO_4 A.M.P.
M/AB pH 2.5 /AF		-	ALL COOH & SO_4 A.M.P. REMOVED
M/S/AB pH 2.5 /AF		-	COOH A.M.P. UNREACTIVE, ALL SO_4 A.M.P. REMOVED
LOW pH PTA		+	MUCUS
PA-SM		-	1,2-GLYCOL GROUPS UNREACTIVE
PA-TSC-SP		+	1,2-GLYCOL GROUPS REACTIVE
NAPHTHOL AS-BI PHOSPHATE		-	ACID PHOSPHATASE INACTIVE
GOMORI MEDIUM pH4.9		+	ACID PHOSPHATASE ACTIVE
HUGON-BORGERS MEDIUM pH 9.0		-	ALKALINE PHOSPHATASE ABSENT

NOTATION:

AS FOR TABLES 5 & 6.

smallest stained deep purple with the aldehyde fuchsin, which may easily have masked any alcianophilia they exhibited.

The golgi complex and mucous vesicles react specifically to the PA-TSC-SP and the low pH PTA methods (plates 17B & C) and the golgi also reacts to the incubation in the Gomori medium (plate 17D). In both cases the mucus retains its typical speckled appearance, but displays a greater electron-opacity compared to that after normal staining. Frequently the smallest droplets (300-500nm) react very strongly, a similar phenomenom was noticed in the thick resin sections stained for carbohydrates. Perhaps this is a result of a higher density of secretion in the smaller vesicles which thus stain more intensely. A gradient of staining intensity is displayed by the golgi complex with these methods, the trans cisternae appearing more electron-opaque than the cis face indicating a concentration of the carbohydrate moiety at the trans face.

The SM methods stains the mucus, golgi complex, RER, nucleus and cytoplasm non-specifically. Treatment with the sodium borohydride reagent after periodic acid oxidation reduces the staining intensity, indicating that reducing groups other than aldehydes were responsible for the reaction.

4-THE EPITHELIUM OF THE OESOPHAGEAL GLAND.

Examination of the surface of a piece of living lateral fold reveals small clusters of ciliated cells and gland cells where the ciliary activity is absent. The apical cytoplasm of the ciliated cells contains small, refringent, yellow-brown residual bodies. A large cluster (2-5μm) of similar bodies together with smaller single ones occur in the gland cells and also within blebs of cytoplasm shed by the cells; gentle squeezing of the coverslip causes blebs to be released. Isolated lateral folds are capable of muscular contractions.

The typical mosaic of the two cell types can be clearly seen with the SEM (plate 18A). The apical surface of the gland cells, covered

with microvilli, normally bulge out into the lateral pouch (plates 14B & 18D).Evidence from TEM and histological studies indicates that these cells undergo a secretory cycle, in which apical blebs of cytoplasm containing various inclusions are pinched off from the cell by apocrine secretion (plate 18D).

Initially the apical surface swells outwards until the brush border is lost from the central zone of the bleb, yet it remains peripherally. As the bleb swells further, it constricts proximally to form a microvillus-coated neck which becomes pinched off (plates 18B & D). Once the bleb has detached, the central region of the apical plasma membrane is devoid of microvilli. The free smooth-surfaced bleb is then carried out of the pouch and into the food channel by the ciliary currents described earlier (plate 18C).

Each lateral fold is a deep transverse fold of the lateral mid-oesophageal wall, consequently in transverse section it appears as an epithelial layer (30-40μm thick) folded back upon itself sandwiching a subepithelial space in between (plates 13A & 14B). In sections this space appears empty and is in fact a continuation of the visceral blood space, being separated from it by the circumferential connective tissue and muscle layers that envelop the entire oesophagus. The epithelium rests on a thin basal lamina (75nm), below which are a few radial muscle fibres and very small nerves which are continuous with those in the well developed circumferential connective tissue.

Free in the blood space are amoebocytic blood cells of various shapes and sizes (3-10μm) (plate 22A). Their most conspicuous features are the large centrally placed nucleus (3μm), a large vesicle (1.5-2.5μm) containing a granular-filamentous coagulum and numerous looping pseudopodia emanating from the cell body. The nuclei contain a large proportion of heterochromatin associated with the inner membrane of the nuclear envelope and also in the nucleoplasm;

nucleoli are absent. The finely granular cytoplasm lacks free ribosomes, contains few RER cisternae and occasionally small (150-400nm) residual bodies. Mitochondria and small electron-lucent pinocytotic vesicles (80-150nm) containing a diffuse flocculant material, occur throughout the cytoplasm. A small golgi complex consisting of four to five cisternae, reacts for acid phosphatase.

The pseudopodia, enclose between their looping processes, electron-lucent spaces that may contain a filamentous material. Evidence suggests that this material may become included into the cell in the small lucent vesicles and that these fuse with the large vesicle, emptying their contents into it. The cell and vesicular membranes of the amoebocyte could not be seen although those of the epithelial cells were obvious; this may indicate a difference in the plasma membranes of the two cell types. Furthermore, it would suggest that the vesicular and cell membranes are similar due to the derivation of the vesicles by invagination of the cell membrane.

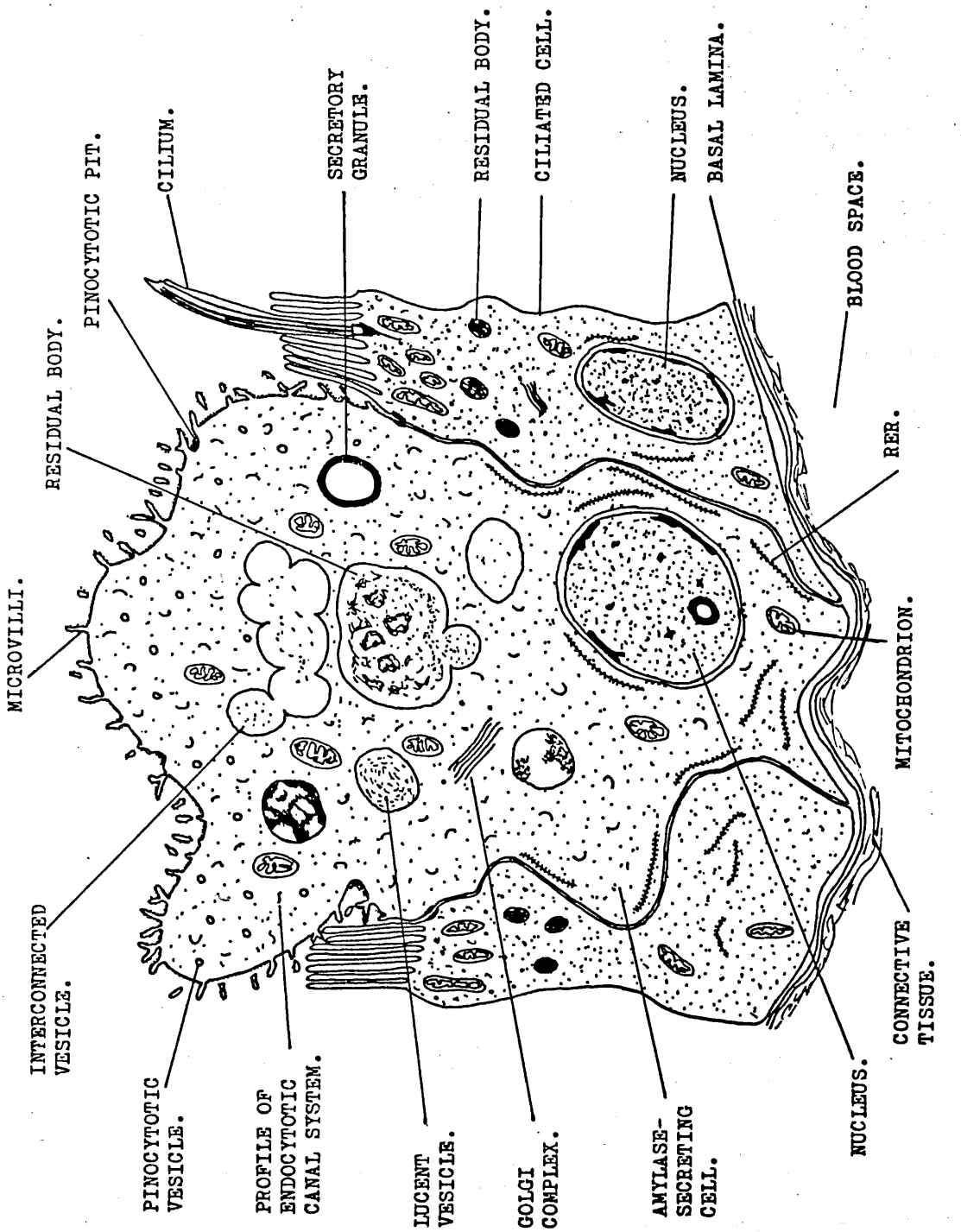
A). THE CILIATED CELLS. Those lining the lateral pouches conform to the typical description given in section 1; they are shorter than those of the food channel.

B). THE AMYLASE-SECRETING CELLS.

These gland cells have a very characteristic ultrastructure (fig 8, plates 14B & 19). They lack cilia but have short microvilli on the convex apical surface, which is sometimes thrown into lobose projections. A diverse population of vesicles, residual bodies and secretory granules occur in the cytoplasm together with profiles of an endocytotic canal system. The cells are irregular in shape and the basal regions are sometimes concealed between adjacent ciliated cells, so that the epithelium appears pseudostratified.

The regularity, size and numbers of the short microvilli (750-1000nm long and 80-250nm in diameter) varies (plates 19, 20A & 21D); they are frequently branched. Tiny vesicles (30nm) occur in the

FIGURE 8. A DIAGRAMMATIC REPRESENTATION OF THE OESOPHAGEAL LATERAL POUCH EPITHELIUM.



homogeneous cytoplasm of the microvilli. A faint attenuate glycocalyx projects from their membrane but central core filaments are absent. The plasma membrane between microvilli demonstrates pinocytotic activity, small amounts of a finely granular-flocculant material being accumulated in depressions of the membrane (plate 20A). The cytoplasmic surface of these depressions is sometimes "coated". In the apical cytoplasm numerous "uncoated" vesicles (130-170nm) containing a material similar to that in the depressions, may occur. Fine fibrils can just be distinguished radiating from the cytoplasmic surface of the vesicular membrane. This ultrastructural appearance closely resembles the endocytotic activity seen in the digestive cell of the digestive gland of Patella (plate 33B). The rarity of such activity in the amylase-secreting cells suggests that it either happens infrequently or that it is of short duration.

The endocytotic canal system occurs throughout the moderately electron-opaque cytoplasm in the form of short, tubular profiles (40-60nm in diameter) varying in shape from straight rods to ring shapes (plates 20B, D & F). Their bounding membranes and granular content stain intensely, so they are conspicuous even at low magnifications (plates 18D & 19). However, paler profiles may occur and be continuous with the strongly stained sections. The profiles show a close association with the apical plasma membrane (plate 20B) and the secretory vesicles into which they discharge their contents (plate 51C).

Tissue incubated in the TARI medium reveals an intensely stained material in the pinocytotic depressions, vesicles and also some of the sections of the canal system (plates 20C & D), suggesting that the canal system is involved in endocytosis. The ferritin experiments endorsed this conclusion. Tissue from the in vitro incubations showed ferritin accumulations against the apical membrane, within the pinocytotic depressions and vesicles, canal system and the lucent

vesicles which they join (plates 20E & F). The in vivo experiments indicate the time course of this activity. After one hour ferritin was present in the pinocytotic vesicles, canal system and lucent vesicles, there is little left in the lumen and the recently formed depressions do not contain any. After six hours ferritin is scarcer in these organelles, but may occur in any residual bodies present.

Apicolateral junctional complexes similar to those described in section 1 join the gland cells to their neighbours. The integrity of the junctions is not affected by the bulging of the apical surface during bleb formation (plate 18D). The basal portions of the cells rest flatly against the basal lamina, hemidesmosomes and a cytoskeleton are not apparent.

Only a brief description of the numerous vesicles will be given in this section, section 10 deals with the derivation and functions of the types described below. There are two populations of large vesicles in the cells and they are related to the two phases of activity (secretory and absorptive) that the cells undergo. The secretory vesicles (1.5-3.0μm) contain an electron-opaque granular matrix that stains intensely, they occur in the apical regions of the cell and also in the released secretory blebs (plates 18D, 19 & 51A). A series of slightly smaller (1-2μm) lucent vesicles together with a large residual body (up to 10μm, usually 6μm) are characteristic of the absorptive phase (plates 21A & B). The vesicles may be lucent or moderately electron-opaque depending on the density of material in their lumen; they commonly join up to form a series of interconnected vesicles (plates 21B & 52A).

Mitochondria occur throughout the cell but are plentiful near the vesicles and residual bodies suggesting a high cellular activity there (plates 19 & 50). Compared to other gland cells of the gut, the amylase-secreting cell has relatively little RER. That present, is in the form of short lengths of cisternae lying in the peripheries of the

cell and around the nucleus (plate 21C). The cisternal lumen contains a faint, thread-like material.

The golgi complex is modest for such an apparently active cell; it consists of five or six cisternae forming concentric whorls, cup-shapes or flat profiles in section (plate 21C). In the latter state a distinct electron-opaque line separates juxtaposed cisternal membranes but this disappears at the distal margins. Transitional ER elements frequently lie close to the cis face and moderately electron-opaque vesicles (40-60nm) are common around the golgi area; these may be primary lysosomes. The spherical, basal nucleus (2-3μm) has a nucleolus (750-1000nm), heterochromatin is clumped against the inner membrane of the nuclear envelope and a few ribosomes stud the outer one.

Summarised results of the histochemical tests are displayed in table 9. Initial staining of wax sections with Mallory and Heidenhain's haematoxylin indicated that the secretory vesicles are proteinaceous. The cells do not react with any of the carbohydrate-specific or lipid stains, except for the low pH PTA technique which stains the apical glycocalyx and profiles of the canal system (plate 21D). The secretory vesicles give positive results with the mercuric bromophenol blue and DNFB methods for proteins. The residual bodies stain with all of the pigment stains tried, indicating the presence of lipofuscins.

Acid phosphatase was demonstrated in the residual bodies and apical cytoplasm by the naphthol AS-BI phosphate method and in the golgi complex by the Gomori incubation; primary lysosomes did not react. An amylase capable of digesting an unfixed starch film was demonstrated in the oesophageal gland by the method of Tremblay and Charest (1968). The digested area of the film compares in shape and size to the serial section cut for comparison (plate 22B).

TABLE 9.

SUMMARISED HISTOCHEMICAL RESULTS OF THE AMYLASE-SECRETING CELL.

PROCEDURE.		RESULT	CONCLUSION
LOW pH PTA		+	GLYCOCALYX & ENDOCYTOTIC CANAL SYSTEM STAINED
BROMOPHENOL BLUE		+	SECRETORY GRANULES (SG) CONTAIN PROTEIN
DE-NH_2/BROMO-PHENOL BLUE		+R	GROUPS OTHER THAN NH_2 ARE REACTING
DNFB		+	TYROSINE, NH_2 & SH PRESENT IN SG
DE-NH_2/DNFB		-	TYROSINE RESPONSIBLE FOR THE POSITIVE REACTION
DIAZOTIZ'TN-COUPLING		-	TYROSINE UNREACTIVE
DMAB-NITRITE		-	TRYPTOPHAN ABSENT FROM SG
DDD		+W	FEW SH GROUPS IN SG
THIOGLYCOLATE/ DDD		+W	-SS- LINKAGES ABSENT
SUDAN BLACK B		-	NEUTRAL AND PHOSPHOLIPIDS ABSENT
SCHMORL		+	MELANIN, LIPOFUCHSIN & SH GROUPS IN RESIDUAL BODY (RB)
LONG ZIEHL-NEELSEN		+	ACID FAST LIPOFUCHSINS PRESENT IN RB
H2O2-NILE BLUE SULPHATE		+	LIPOFUCHSINS PRESENT IN RB
NAPHTHOL AS-BI PHOSPHATE		+	ACID PHOSPHATASE IN RB & APICAL CYTOPLASM
GOMORI MEDIUM pH 4.9		+	ACID PHOSPHATASE IN GOLGI
HUGON-BORGERS MEDIUM pH 9.0		-	ALKALINE PHOSPHATASE ABSENT
STARCH FILM-P.A.S		+	AMYLASE LOCATED IN LATERAL POUCHES

NOTATION:

AS FOR TABLES 5 & 6.

ABBREVIATIONS FOR PLATES 13-22.

A....Anterior.

ab...Amoebocytic blood cell.

asc..Amylase-secreting cell.

b....Bleb.

bl...Basal lamina.

bs...Blood space.

c....Cilia.

cb...Cytoplasmic bleb.

cc...Ciliated columnar cell.

cpd..Coated pinocytotic depression.

cpv..Coated pinocytotic vesicle.

D....Dorsal.

df...Dorsal fold.

dfc..Dorsal food channel.

dg...Digestive gland.

ecs..Endocytotic canal system.

ep...Epithelium.

g....Golgi complex.

ilv..Interconnected lucent vesicles.

L....Left.

lc...Lateral channel.

lf...Lateral fold.

lgt..Longitudinal fold.

lp...Lateral pouch.

lv1,2,3..Types of lucent vesicle.

m....Mitochondrion.

mc...Mucous gland cell.

ms...Muscle.

mu...Mucus.

mv...Microvilli.

n....Nucleus.

nmc..Neck of mucous cell.

nv...Nerve.

og...Oesophageal gland.

p....Pseudopodia.

phv..Phagocytic vesicle.

pl...Primary lysosome.

poe..Posterior oesophagus.

R....Right.

rb...Residual body.

rer..Rough ER.

sgi1,2..Immature secretory granule.

sgm..Mature secretory granule.

spv..Smooth pinocytotic vesicle.

v....Mucous vesicle.

vf...Ventral fold.

LEGENDS TO PLATES 13-17.

THE OESOPHAGUS.

PLATE 13.

A. Light micrograph of a transverse section of mid-oesophagus stained with AB pH2.5-haematoxylin. x100.

B. SEM, mid- and posterior oesophagus opened by a dorsal longitudinal incision . x30.

Scale bars: A=0.3mm; B=1mm.

PLATE 14.

A. The epithelium lining the dorsal food channel. 2.25K.

B. The epithelium lining the oesophageal gland (lateral pouches) 2.2K.

Scale bars=10μm.

PLATE 15.

Montage of the epithelium lining the dorsal food channel. 7K.

Scale bar=3μm.

PLATE 16.

A. Light micrograph of a transverse section of posterior oesophagus stained with AB pH2.5-haematoxylin. x100.

B. SEM of the cut edge of the epithelium lining the dorsal food channel. 3K.

Scale bars: A=0.3mm; B=10μm.

PLATE 17.

A. Mucous strings entering the dorsal food channel. x230.

B-D Mucous gland cell.

B. Mucous vesicles and golgi stained by the PA-TSC-SP method. 50K.

C. As B, but stained with PTA. 50K.

D. Acid phosphatase in the golgi cisternae. 26K.

Scale bars: A=90μm; B,C,D=400nm.

LEGENDS TO PLATES 18-22.

THE OESOPHAGUS.

PLATE 18.

A. SEM of the surface of a lateral fold showing ciliated and inactive amylase-secreting cells. 1.2K.

B. As A, but the amylase-secreting cells are active. 2.7K.

C. SEM of free blebs on the summit ridge of a dorsal fold. 2.4K.

D. Formation of bleb containing secretory granules. 5K.

Scale bars: A,C=10μm; B,D=4μm.

PLATE 19.

An early stage in the secretory phase of an amylase-secreting cell. 11K. Scale bar=4μm.

PLATE 20. Amylase-secreting cell.

A. Pinocytosis. 31.5K.

B. Endocytotic canal system. 47K.

C. Pinocytotic depression and vesicle stained by the TARI method. 72K.

D. Profiles of the canal system stained by the TARI method. 47K.

E. Ferritin (arrowed) in pinocytotic vesicles. 135K.

F. Ferritin (arrowed) in canal system and large vesicle. 80K.

Scale bars: A,B,D=500nm; C,E,F=250nm.

PLATE 21. Amylase-secreting cell.

A. Residual body and vesicle (arrow) emptying contents into it. 11K.

B. Residual body and interconnected vesicles. 6.8K.

C. RER and golgi complex. 21K

D. Apical glycocalyx and canal system stained by PTA method. 59K.

Scale bars: A,B=2μm; C=1μm; D=500nm.

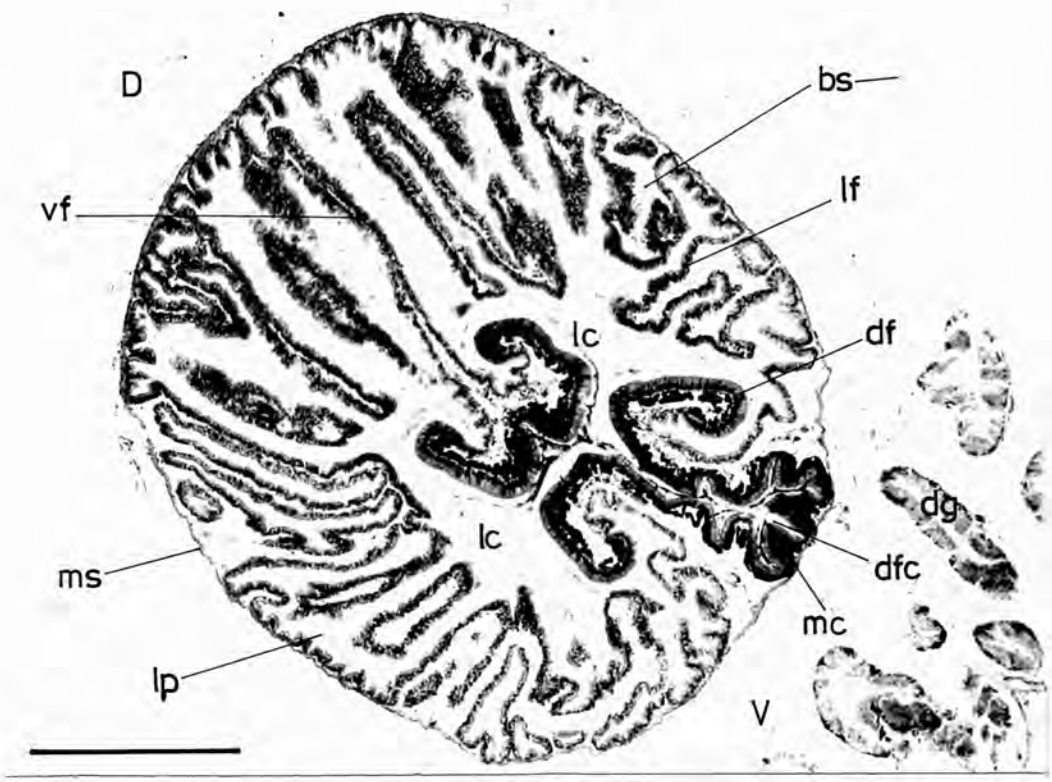
PLATE 22.

A. Blood cell in blood space of oesophageal gland. 14K.

B. Frozen section of oesophagus overlay shows corresponding area of digested starch film. x75.

Scale bars: A=2μm; B=310μm.

PLATE 13



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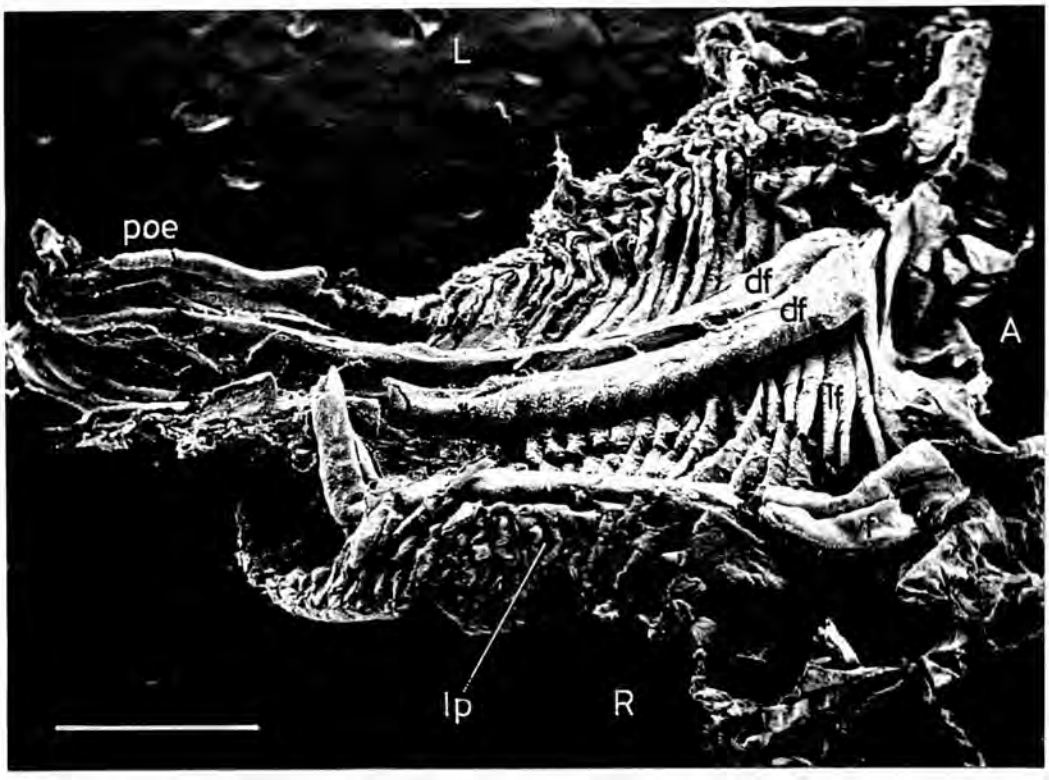

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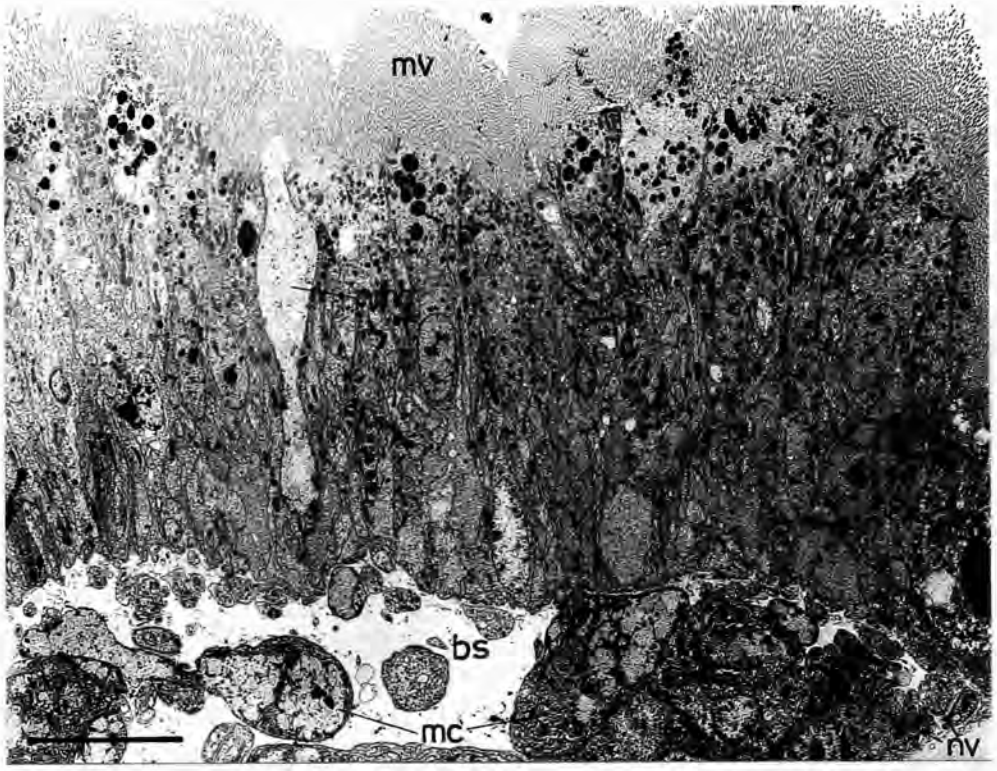
PLATE 14



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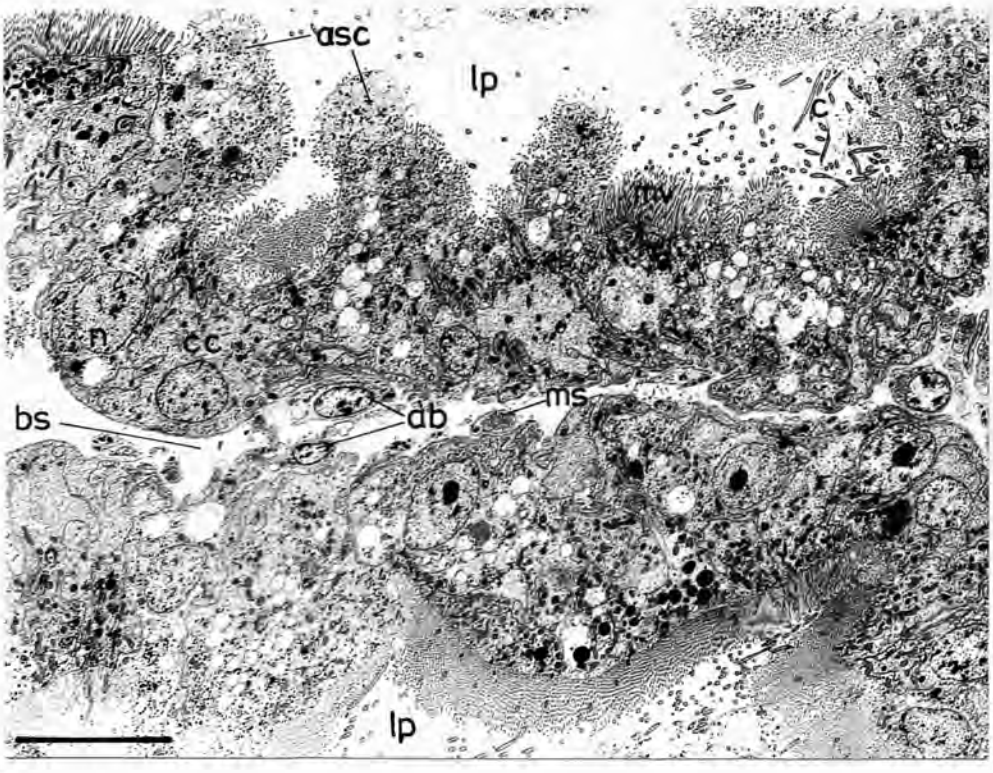

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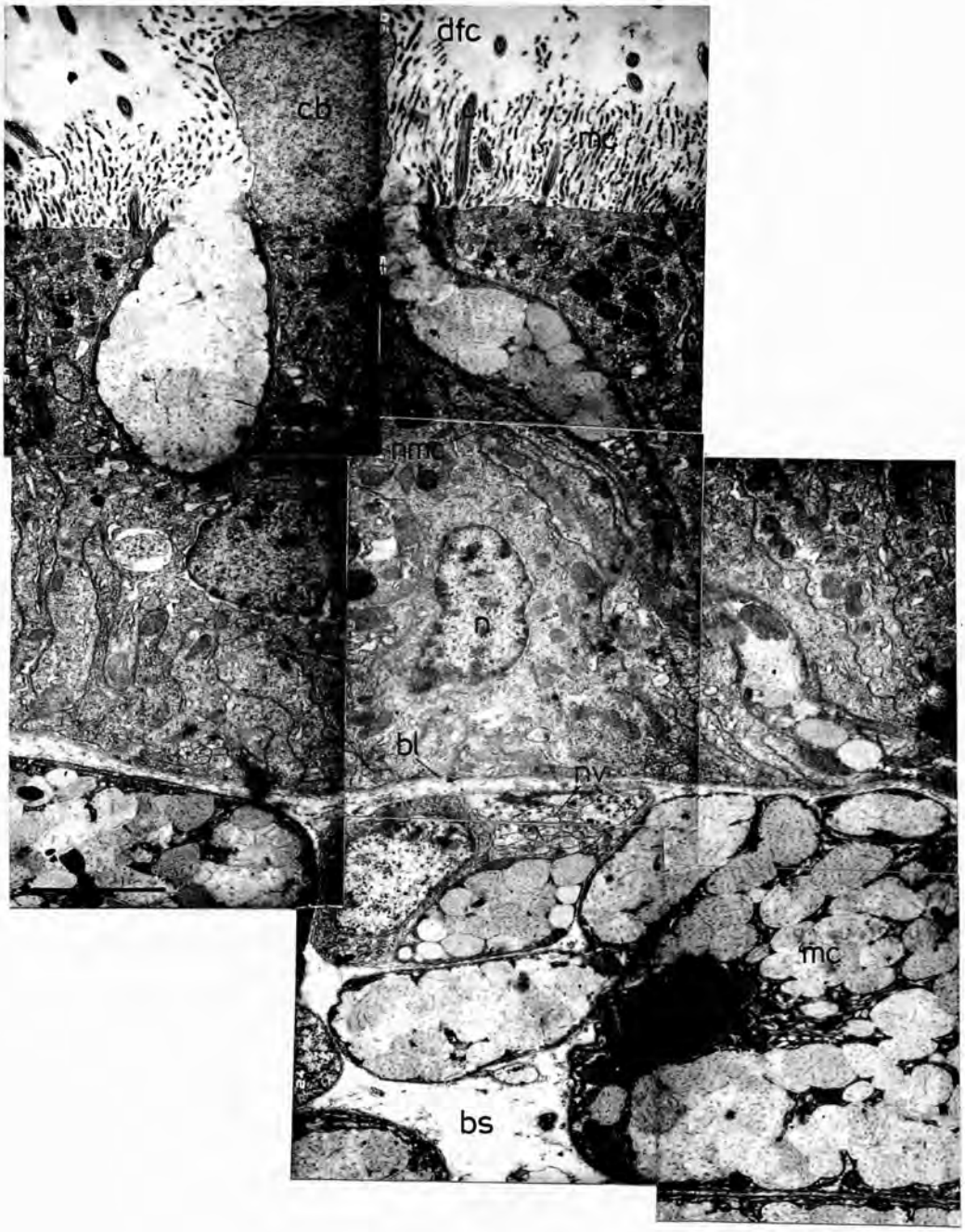
PLATE 15



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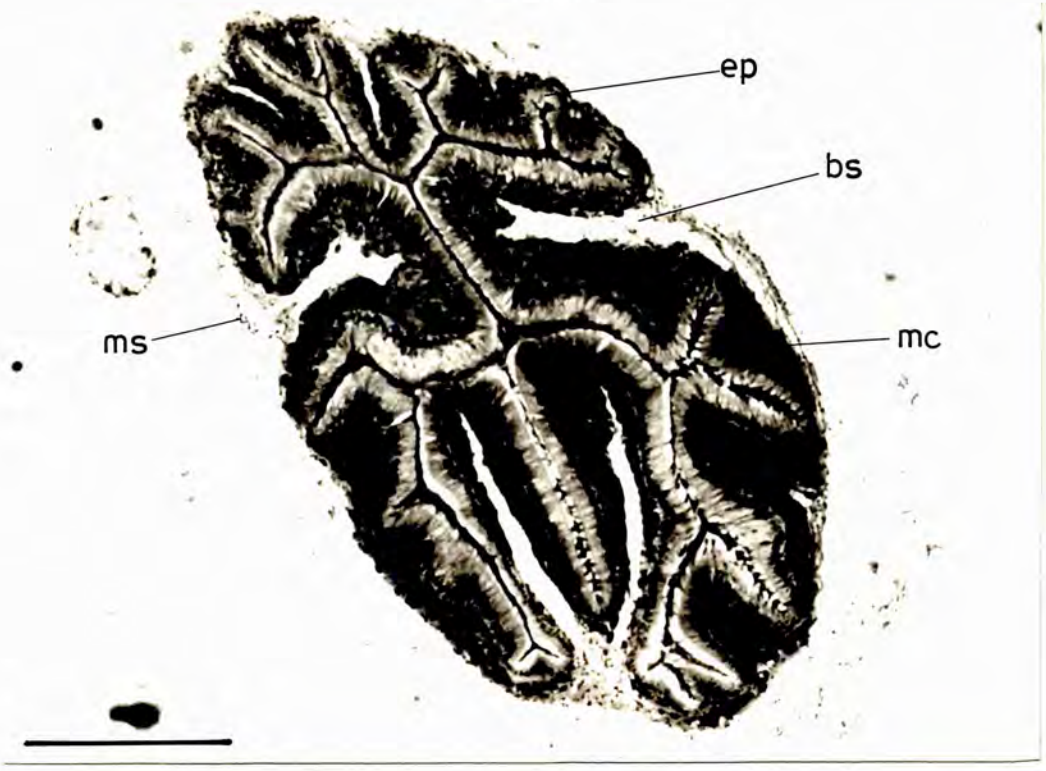

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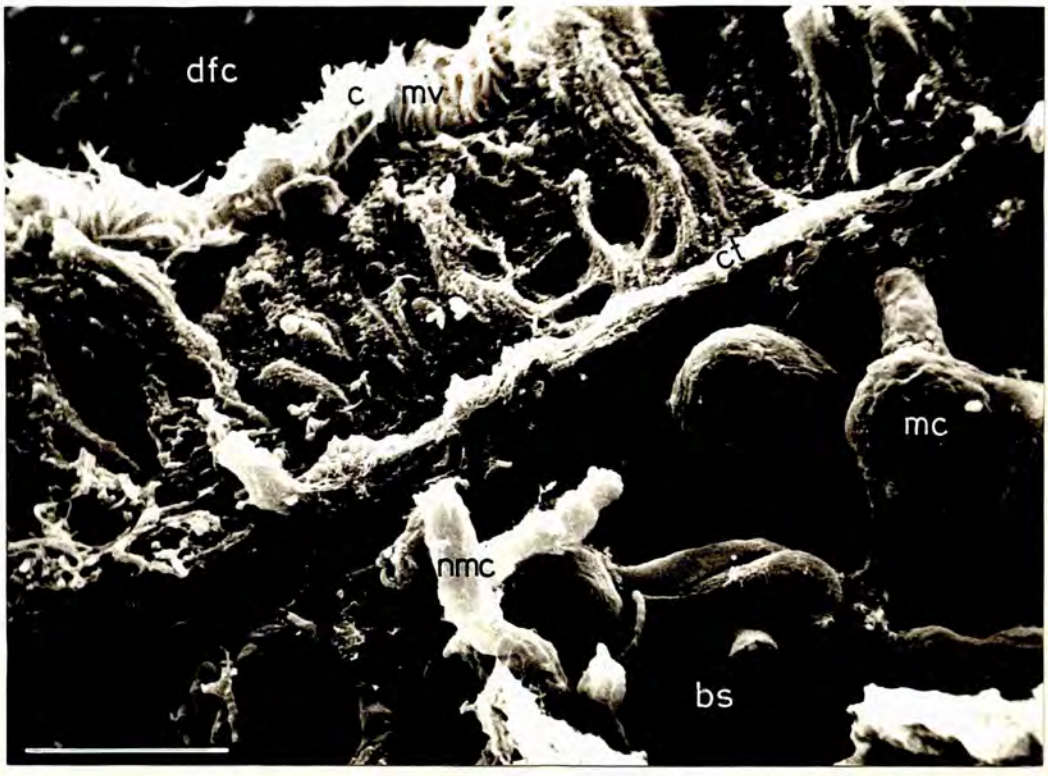

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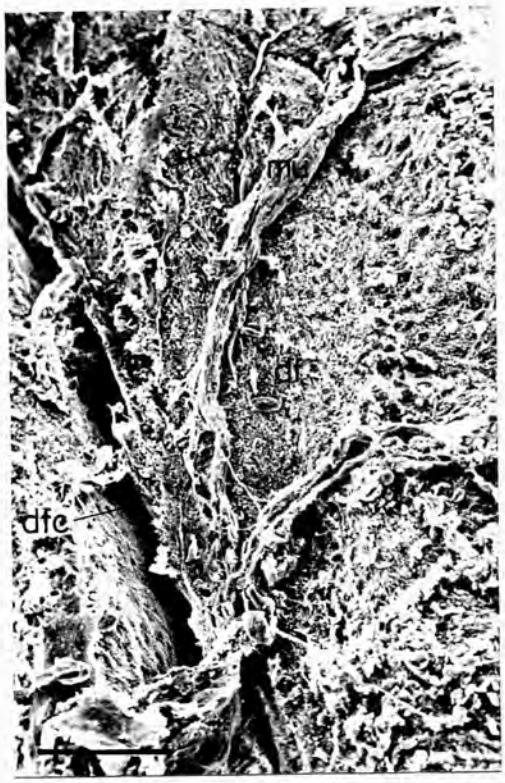
PLATE 17



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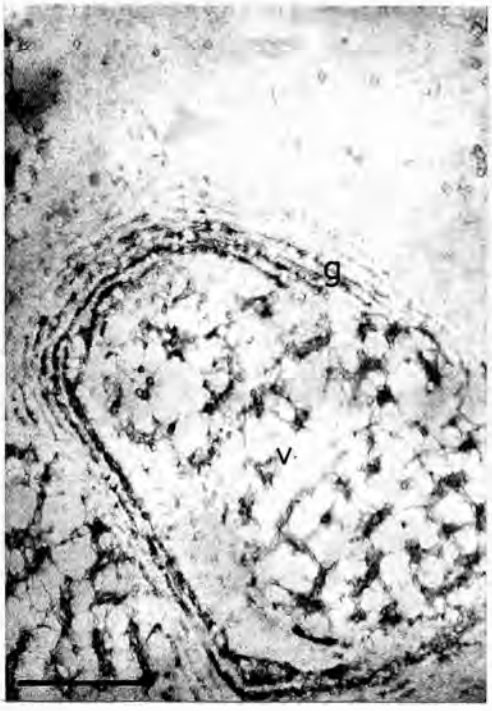

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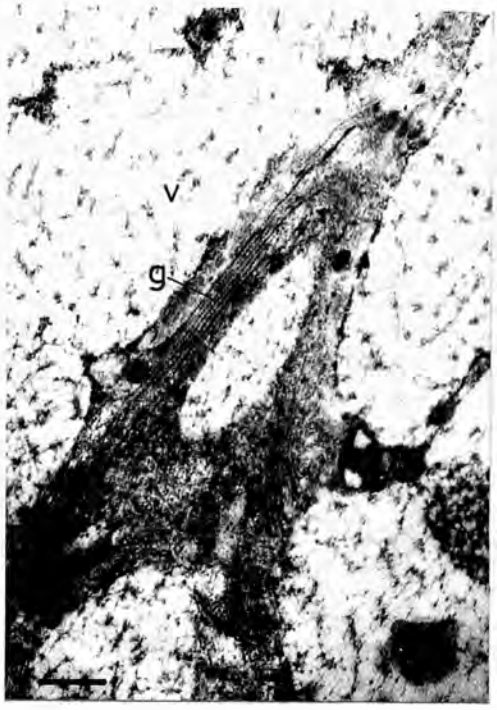
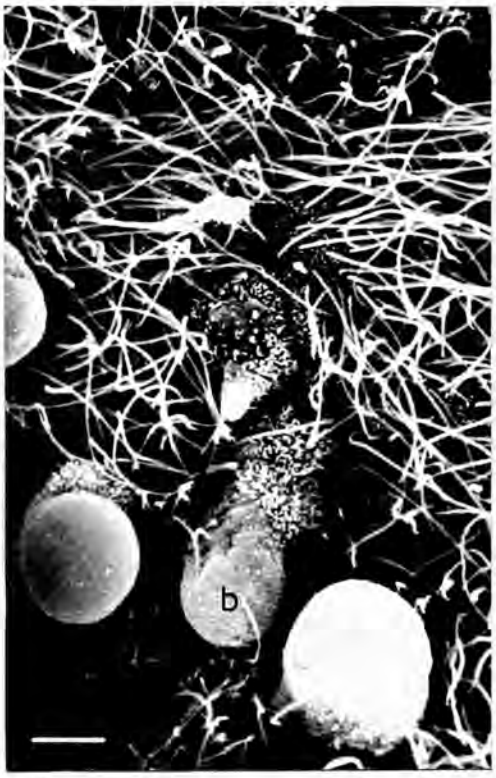


D

PLATE 18

A



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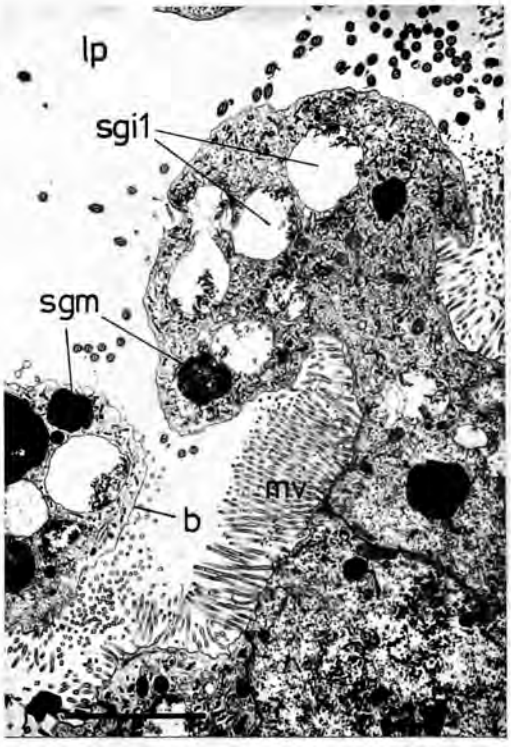

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PLATE 19

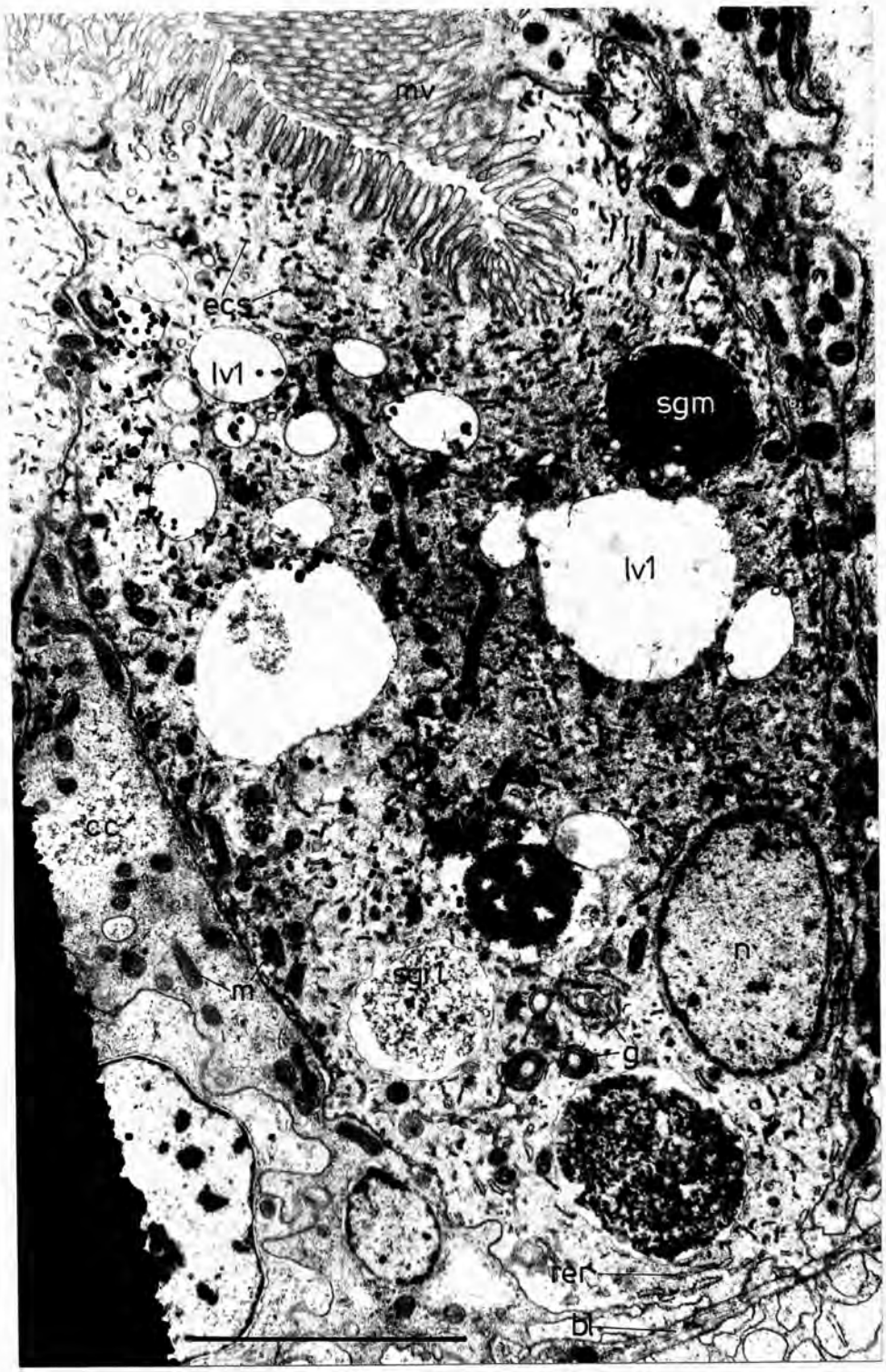
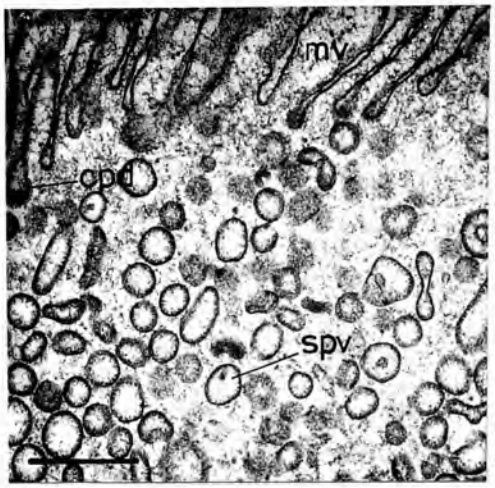


PLATE 20



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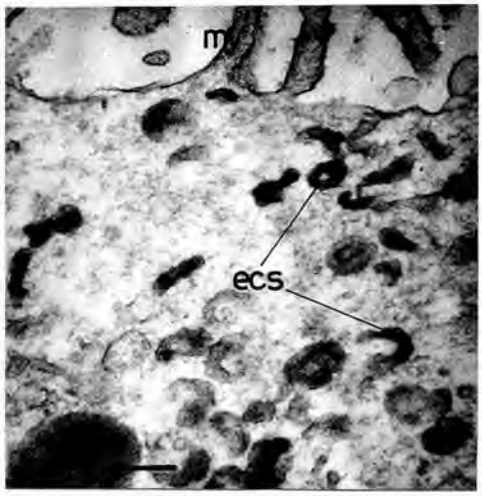

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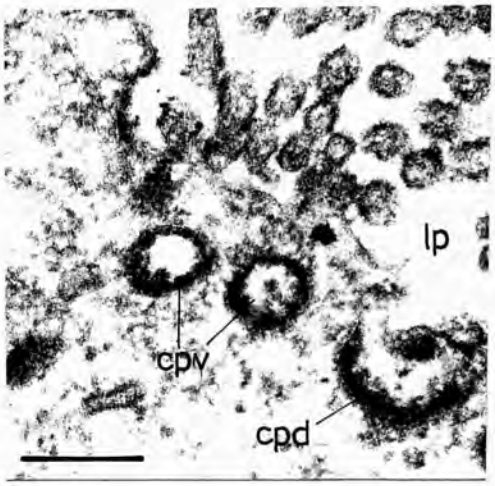

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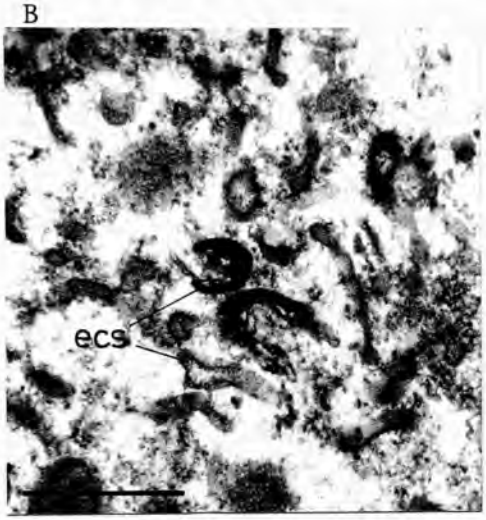

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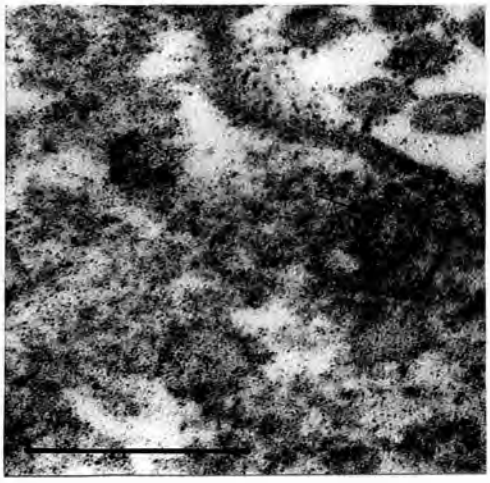

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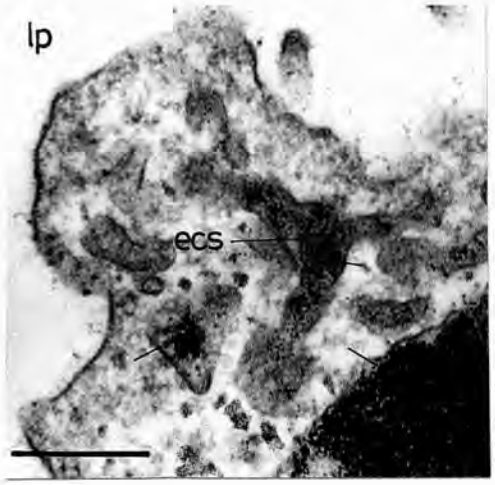
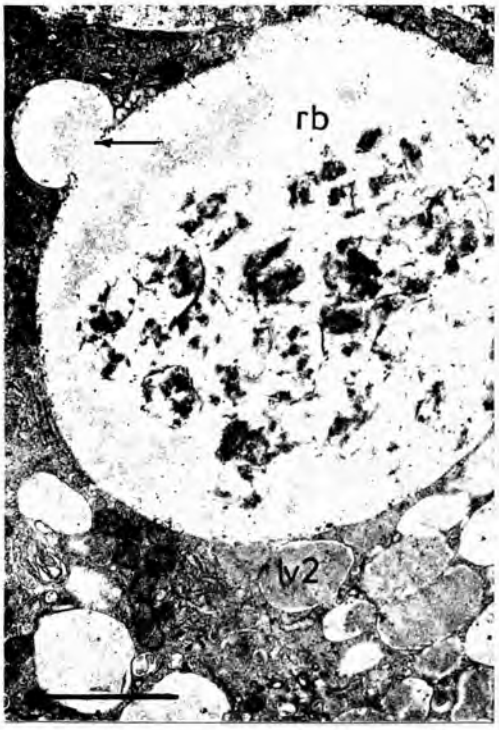


F

PLATE 21



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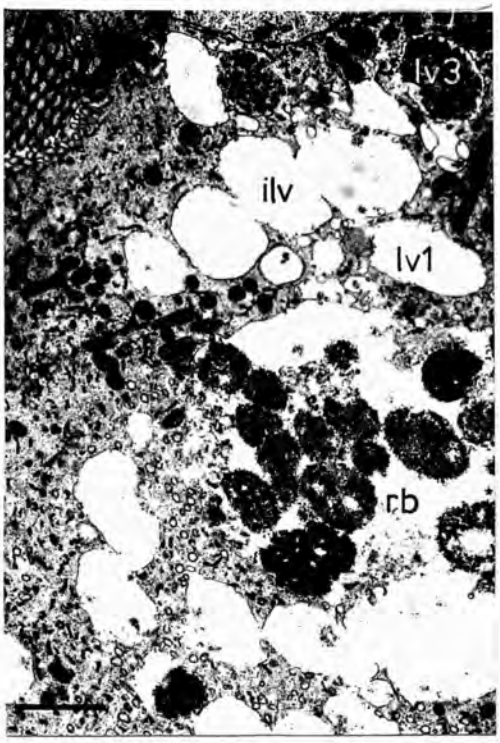

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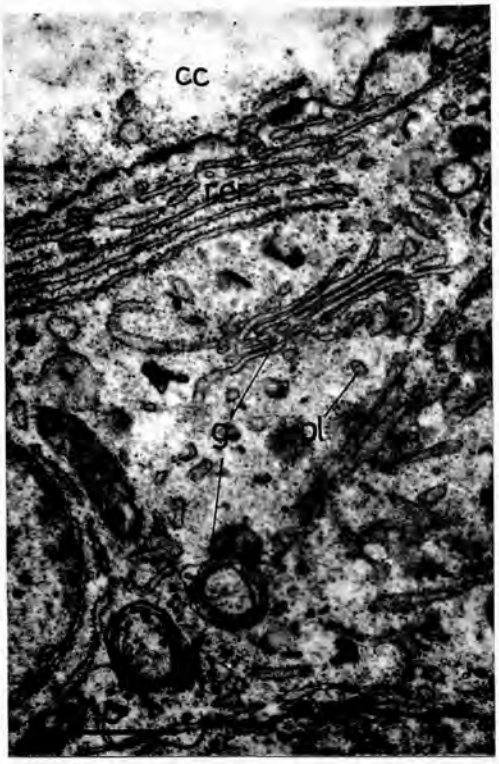

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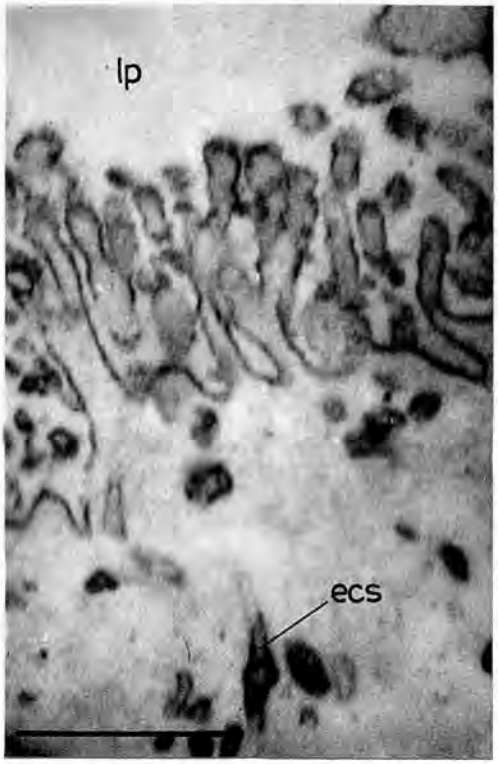

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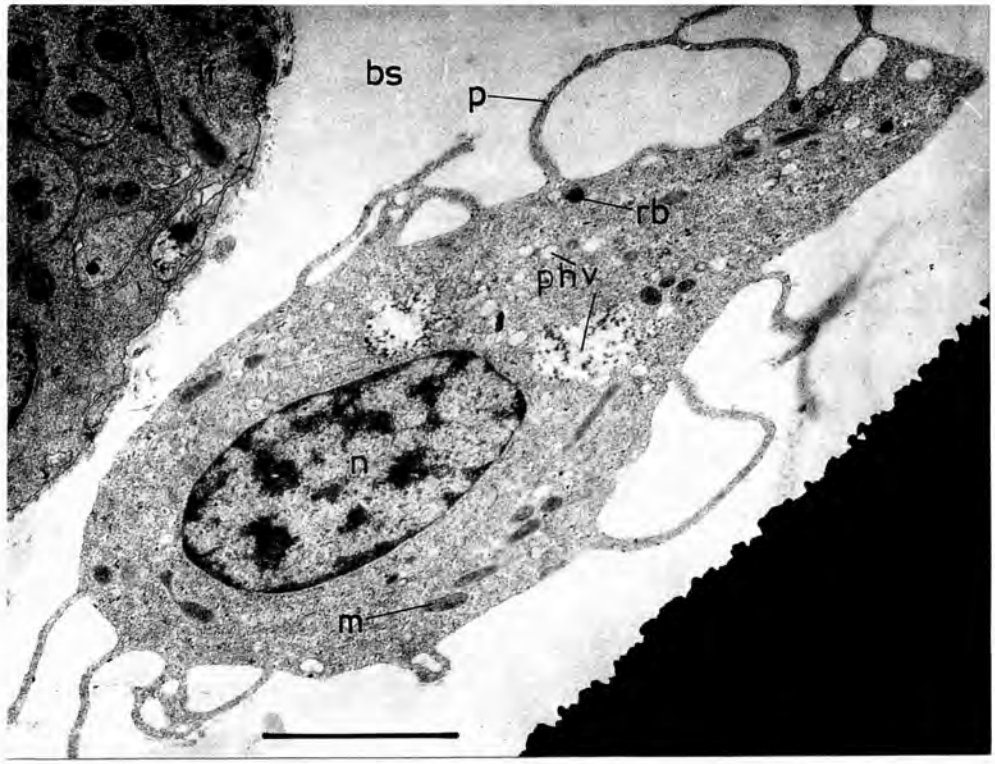
PLATE 22



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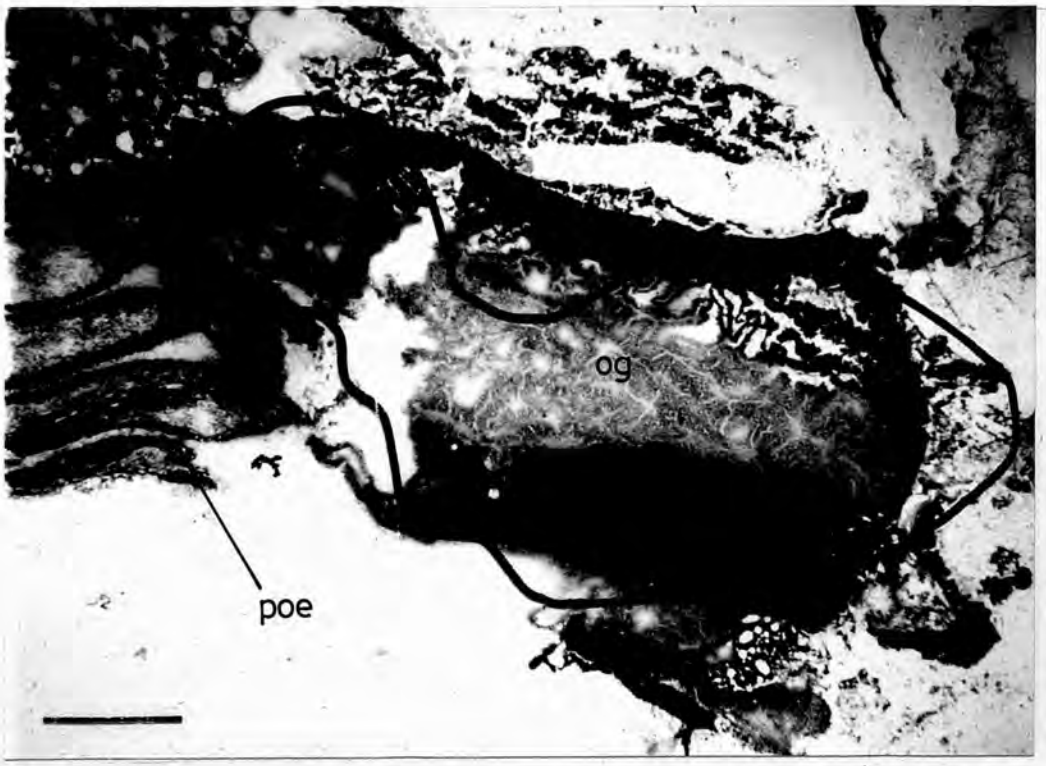


B

THE STOMACH AND STYLE SAC.

The limpet stomach is "..merely a short stretch of alimentary canal placed at a sharp bend where the oesophagus runs into the intestine" (Graham, 1932). It lies peripherally on the right side of the animal, so that its lateral convex surface and the style sac lie against the right kidney and mantle (figs 2 & 6). Viewed externally, both are heavily pigmented sections of gut that curve sharply through 180° in an horizontal plane. The dark brown pigmentation obscures any internal detail. The posterior oesophagus curves from left to right and opens into the stomach anteriorly, whilst the style sac leaves posteriorly and curves immediately to the left forming a tight bend. A crystalline style is absent.

1-SEM.

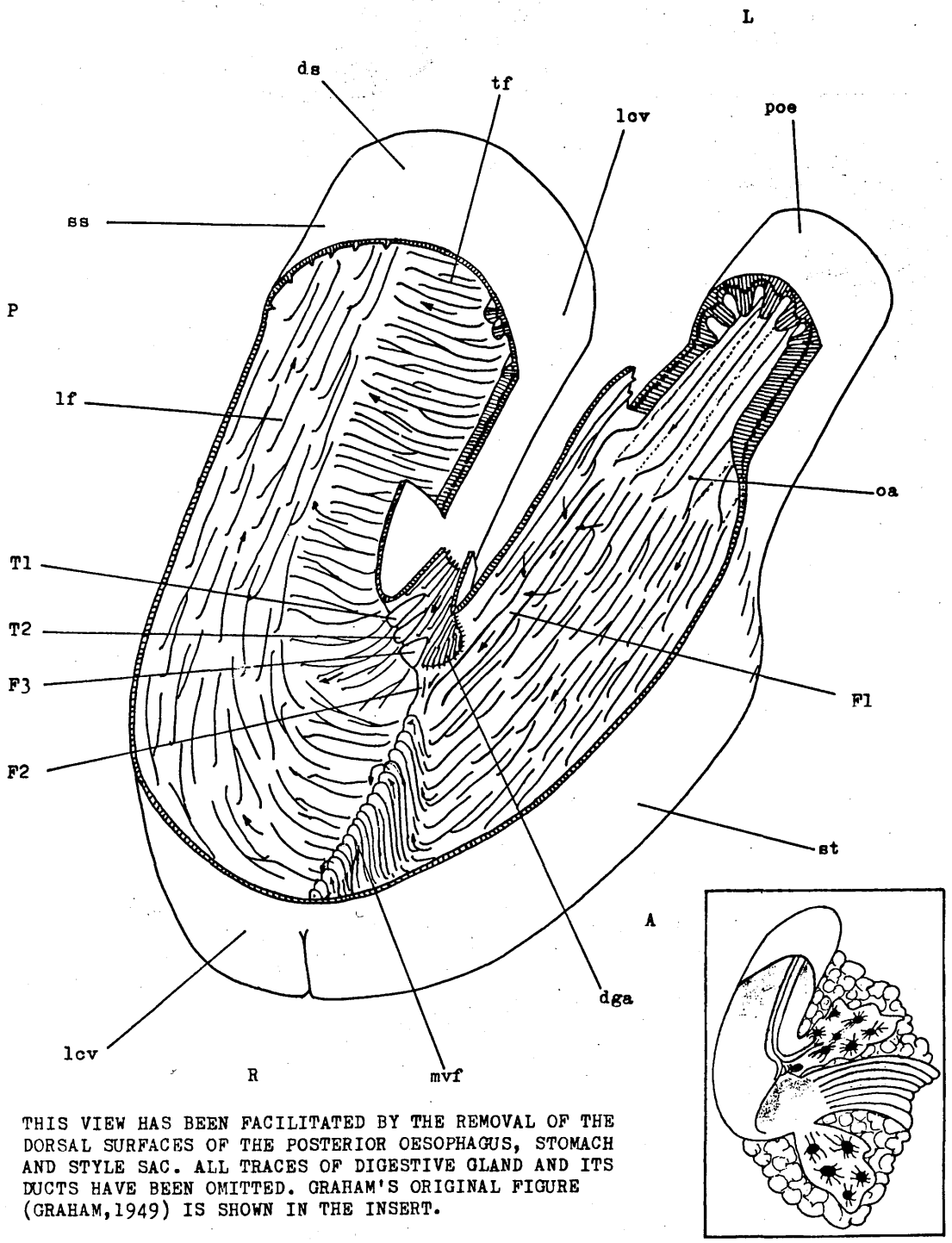
The structural simplicity of the stomach and style sac compared to a typical prosobranch is demonstrated when they are opened longitudinally (figs 9, 10 & 11). At its opening from the oesophagus, the tall oesophageal folds merge smoothly with the smaller and more numerous longitudinal folds of the stomach (plate 23). The glandular epithelium of the oesophageal food channel is replaced by a ciliated columnar epithelium without gland cells.

The longitudinal stomach folds are 40-50μm tall in wax sections and cover the entire luminal surface. A taller fold (100μm), F1 (plate 23A) arising on the concave lateral wall near the oesophageal aperture, runs to the bend in the stomach, where it fuses with the lip of the muscular fold (fig 9). This fold arises ventral to the single dorsal opening of the duct of the digestive gland (plates 23B, 24A & B) and extends across the ventral wall to the other side, projecting deep into the lumen along its path and so functions as a valve. The longitudinal folds of the ventral wall pass over the valve and continue into the style sac. The lip of the valve bears taller epithelial cells (100μm) and so appears swollen. Another much smaller

ABBREVIATIONS TO FIGURES 9, 10 & 11.

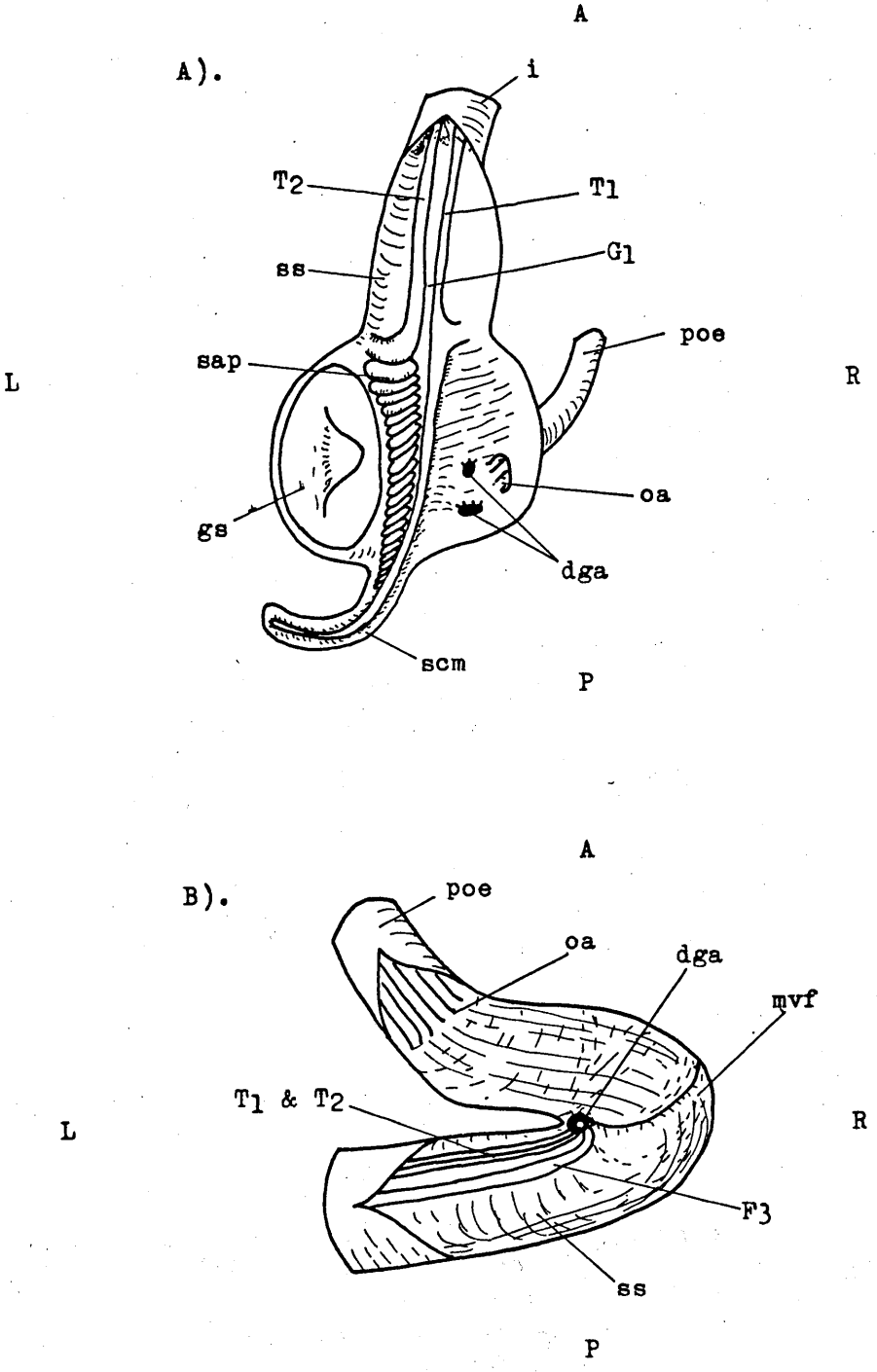
A	ANTERIOR.
dga	APERTURE OF DIGESTIVE GLAND DUCT.
ds	DORSAL SURFACE OF STYLE SAC.
F1,2,3	LARGE FOLDS.
Gl	INTESTINAL GROOVE.
gs	GASTRIC SHIELD.
i	INTESTINE.
iss	INTESTINAL END OF STYLE SAC.
L	LEFT SIDE.
lcv	LATERAL CONCAVE WALL.
lcx	LATERAL CONVEX WALL.
lf	LONGITUDINAL FOLD.
mvf	MUSCULAR VALVE FOLD.
oa	OESOPHAGEAL APERTURE.
P	POSTERIOR.
poe	POSTERIOR OESOPHAGUS.
R	RIGHT SIDE.
sap	POSTERIOR SORTING AREA.
scm	SPIRAL CAECUM.
sct	SECRETORY TRACT.
ss	STYLE SAC.
st	STOMACH.
T1	MAJOR TYPHLOSOLE.
T2	MINOR TYPHLOSOLE.
tf	TRANSVERSE FOLDS.
→	CILIARY CURRENTS.

FIGURE 9. A DIAGRAMMATIC REPRESENTATION OF THE STOMACH AND STYLE SAC.



THIS VIEW HAS BEEN FACILITATED BY THE REMOVAL OF THE DORSAL SURFACES OF THE POSTERIOR OESOPHAGUS, STOMACH AND STYLE SAC. ALL TRACES OF DIGESTIVE GLAND AND ITS DUCTS HAVE BEEN OMITTED. GRAHAM'S ORIGINAL FIGURE (GRAHAM,1949) IS SHOWN IN THE INSERT.

FIGURE 10. DIAGRAM OF THE STOMACH OF (A) A GENERALIZED PROSOBRANCH OPENED MID-DORSALLY (BASED ON FRETTER & GRAHAM, (1962)) AND (B) PATELLA.



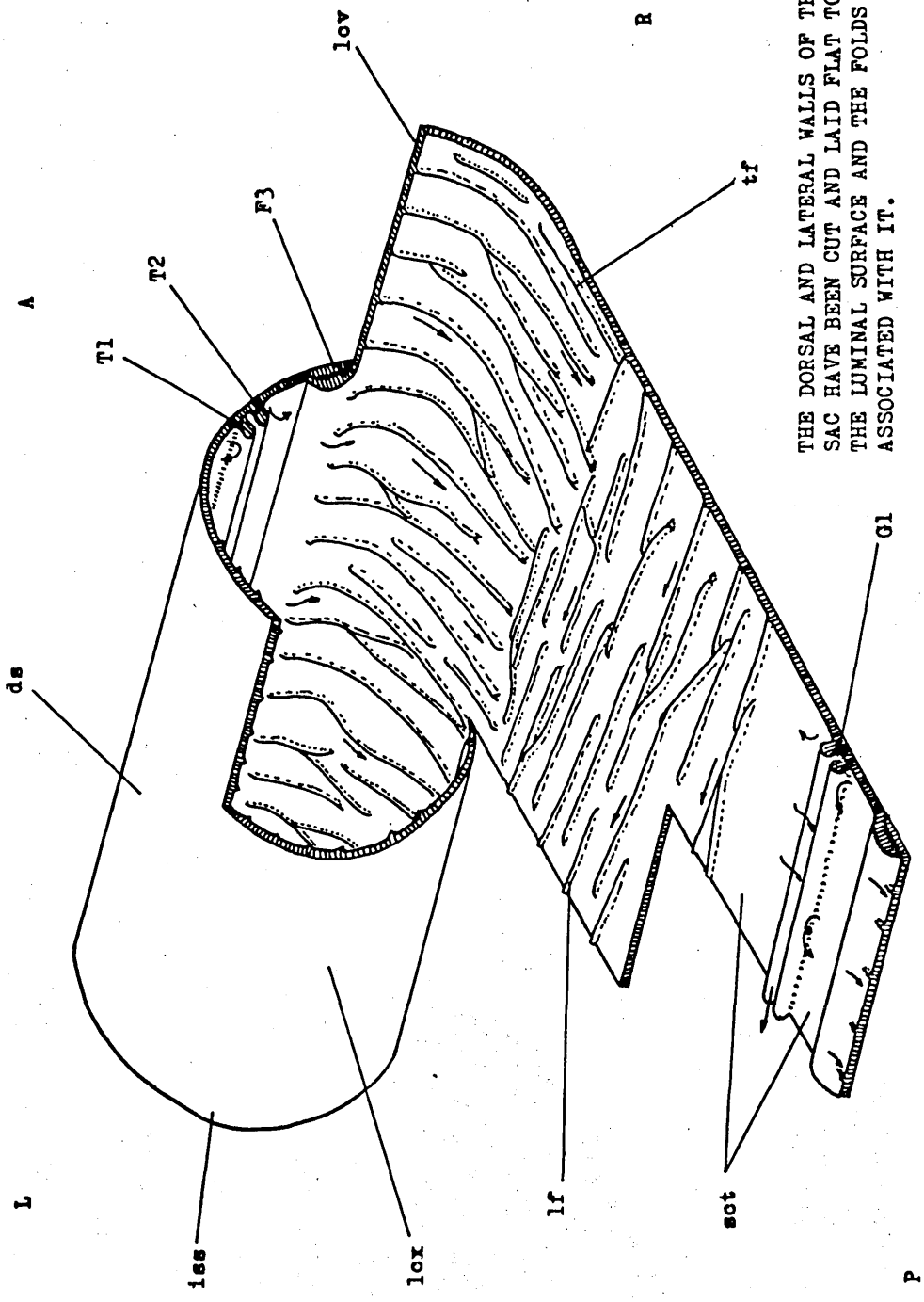


FIGURE 11. A DIAGRAMMATIC REPRESENTATION OF A SECTION OF STYLE SAC. THE DORSAL AND LATERAL WALLS OF THE STYLE SAC HAVE BEEN CUT AND LAID FLAT TO EXPOSE THE LUMINAL SURFACE AND THE FOLDS ASSOCIATED WITH IT.

fold, F2, arises ventral to the aperture of the digestive gland duct and runs to the valve and merges with it (fig 9, plate 24A).

The valve fold has well developed subepithelial muscles composed of an inner layer of circular fibres and an outer layer of radial fibres, contraction of the latter reduces the height of the valve. Contraction of the longitudinal stomach fibres reduces the radius of curvature of the convex wall so pulling it towards the concave wall, whilst contraction of the circular fibres would increase the height of the valve and decrease the luminal diameter.

Arising from within the aperture of the duct are three ciliated folds (fig 9, plates 23B, 24A & B). These are the major and minor typhlosoles (T1 and T2 respectively) enclosing the intestinal groove (GI) between them and the third fold, F3, was described as "a slightly thickened area of the wall" (Graham, 1932). Observations on serial wax sections and specimens prepared for SEM revealed that both typhlosoles run out of the duct and curve around the concave lateral wall to run into and along the style sac (figs 9 & 11; plates 23B & 24B).

The major typhlosole is the larger of the two, being 150μm wide by 65μm tall and the minor 100μm by 55μm; T2 remains parallel with T1 and does not run onto the valve as reported by Graham (1932) (plate 23B). Graham (1949) reported a vestige of the posterior sorting area in the space formed by the divergence of the two typhlosoles in the stomach of Patella and other prosobranchs. This sorting area could not be found. It is possible that without the superior resolution of the SEM, an area of the longitudinally folded wall of the digestive gland duct could be mistaken for a vestige of the sorting area. Fold, F3, arises just inside the aperture of the duct and runs parallel with T2 along the wall of the style sac; it is 100μm tall and 350μm wide. Radiating from F3, towards the longitudinal folds continuing from the stomach, is a series of small transverse folds (fig 11, plate 24B). The gastric shield and spiral caecum commonly encountered in other

molluscan stomachs are absent in Patella.

Histologically both typhlosoles can be divided into ciliated and secretory portions (plates 25A & B & 26A); the inner surfaces lining the intestinal groove are heavily ciliated whilst the outer surfaces are composed of unciliated columnar cells which release blebs of cytoplasm by apocrine secretion. The boundary between the two cell types on the crest of each typhlosole is abrupt and conspicuous in the SEM and thick resin sections. Adjacent to each typhlosole is a secretory tract of unciliated cells continuous with those of the outer typhlosole surface; the one between T2 and F3 being clearly defined, the one next to T1 being less distinct (fig 11). This secretory epithelium is again abruptly replaced by the ciliated epithelium of the fold F3, but more gradually by that of the folded convex lateral wall.

During these observations it was clear that the ciliated areas are covered with a thick layer of a white secretion that forms a continuous sheet, except over the typhlosoles and secretory tracts (plate 26B). The ciliated epithelium of the duct of the digestive gland is covered by a similar layer continuous with that in the style sac. The layer is composed of cytoplasmic blebs which have coalesced and been worked into the layer by ciliary activity. Initially in the duct the cilia perforate the layer of secretion, but as it is carried into the style sac, it comes to overlie the cilia (plate 26B). The persistence of the perforations in specimens is presumably an indication of an increased viscosity caused by a change in pH from 5.3 in the digestive gland to 6.4 in the style sac.

2-HISTOLOGY, ULTRASTRUCTURE AND HISTOCHEMISTRY.

Histologically, the ciliated epithelial cells lining the stomach and style sac are similar and conform to the description given in section 1. The type of epithelium described by Fretter and Graham (1962) as characteristic of the prosobranch style sac, occurs in the

stomach, style sac and intestinal sections A and B of Patella and so it is not possible to differentiate style sac from the other two sections on this basis. The folds described previously, except the valve, are produced by an increse in cell height which varies from 6-100μm (table 4). In serial sections, the fold, F1, was observed to produce blebs of cytoplasm similar to those of the unciliated cells.

The epithelium of both typhlosoles shows minor differences from the typical epithelium discussed in section 1. In thin resin sections each cell has at least six cilia (10μm long), an abundance only paralleled by the rectal cells. The microvilli of the unciliated secretory cells are longer (2μm) than those of the ciliated typhlosole cell (1μm). The endocytotic canal system, electron-opaque bodies and multivesicular bodies are more prolific in the unciliated cells.

Central oval nuclei (7μm by 2μm) occur in the taller cells, but lie basally in the shorter cells. The basal regions of both ciliated and unciliated cell types are developed into extensive infoldings that contain mitochondria; the intercellular spaces are also well developed, more so in the unciliated epithelium where they extend into the apical third of the epithelium (plate 27A). Small nerves occur above the thick (400nm) basal lamina between the basal infoldings and larger nerves occur in the subepithelial connective tissue. One of the most striking differences between the two cell types is the secrteory potential of the unciliated cells, ciliated cells rarely shed blebs.

The cells of the typhlosoles are histochemically similar to the typical ciliated cells except that the brush border lacks alcianophilia, the basal lamina is PAS-reactive and alcianophilic and glycogen deposits are inconspicuous.

3-CILIARY CURRENTS.

When the stomach is opened by a longitudinal incision and graphite suspensions pipetted onto the luminal surface, two distinct series of ciliary currents can be traced into the style sac. By far the greater

proportion of particles are carried by the currents of the longitudinal folds of the concave wall and passed onto the fold, F1, by oblique currents in its vicinity. Loose particles become compacted into a stomach string (Carriker, 1946) as they pass to the valve via F1. The string and any free particles are carried onto and over the free lip of the valve and onto fold, F3, in the style sac (fig 9). Cilia on the transverse folds radiating out from F3 carry them onto the longitudinal folds of the convex wall of the style sac (fig 11). Those particles not caught up in these currents are transported around the convex lateral and dorsal walls, avoiding the valve.

When small volumes of the graphite suspensions are injected into the oesophagus and the stomach is opened 10-15 minutes later, the resulting graphite-laden mucous string can be traced along the route normally taken in an opened stomach. After gently opening the stomach and stle sac, the mucous string is associated with F1, the valve, F3 and the longitudinal folds of the style sac. The strings are always continuous and there is never any evidence to suggest that they pass into the digestive gland duct. The stomach does not squeeze these strings.

If graphite particles are pipetted into or near to the duct aperture in an opened stomach, they are removed by three ejection routes. The most effective of these is via the intestinal groove, particles are swept into and along it by a very strong anally directed current that persists wherever the typhlosoles border the groove. Other particles are caught up in the currents associated with the valve and are thus taken onto the longitudinal folds by F3 and the transverse folds. Thirdly, the few particles that escape these currents are removed across the convex lateral and dorsal walls into the style sac.

During these observations, on three separate occasions when stomachs were opened, a yellow string of faecal material passed out of

the aperture of the duct onto F3 and thence to the longitudinal folds of the style sac; this was the "liver string" (Carriker, 1946). One of these specimens was prepared for SEM, but the string became detached from the luminal surface and had to be examined separately (plate 27B). The distal end in the style sac had a diameter of 350μm whereas the proximal end was 100μm in diameter. This increase is due to additional layers of secretion wrapped around the originally twisted (clockwise as viewed from the stomach) liver string emerging from the duct. Blebs of cytoplasm like those released by the secretory tracts are associatd with the thicker end of the string, some beginning to coalesce with the outer layers of secretion.

The style sac displays five different sets of ciliary currents. Most graphite particles are transported along the intestinal groove. Particles adjacent to the outer surfaces of both typhlosoles are swept away onto either F3 in the case of those particles near to T2 or onto the secretory tract next to T1, if near to that fold. As the outer typhlosole surfaces are unciliated, the currents responsible for this must be vortices produced by the intensely beating cilia of the intestinal groove and centrifugal currents on F3. Particles thrown onto F3 are transported on the transverse folds in a clockwise direction (viewed from the stomach) across the ventral wall to the longitudinal folds. Those particles swept away from T1 are gradually collected by the longitudinal folds and conveyed into the first section of the intestine.

On opening the style sac, the faecal rod is always associated with the transverse folds radiating from F3 and the longitudinal folds onto which they run. The combined effects of the ciliary currents on these folds is to rotate the faecal rod towards the intestine in a clockwise spiraling motion.

The style sac of freshly collected limpets are full of coarse particles, diversely shaped pieces of green and brown algae and

detritus including, in one animal, part of what might once have been a mite (Acari). Graham (1932) reported that the liver string consists of "empty diatom cases, pieces of cellulose from plant-cell walls, a few live diatoms and larger unaltered pieces of plant tissue".

ABBREVIATIONS FOR PLATES 23-27.

b.....Cytoplasmic bleb.

bl.....Basal lamina.

bs.....Blood space.

ce.....Ciliated epithelium.

ct.....Connective tissue.

d.....Duct of digestive gland.

da.....Aperture of digestive gland duct.

dg.....Digestive gland.

dl.....Lumen of duct.

F1,2,3..Large ciliated folds.

g.....Golgi complex.

gl.....Lumen of gut.

is.....Intercellular space.

lf.....Longitudinal folds.

m.....Mitochondrion.

ms.....Muscle.

mv.....Microvilli.

n.....Nucleus.

poe.....Posterior oesophagus.

s.....White secretion.

sct.....Secretory tracts.

st.....Stomach.

sw.....Wall of stomach.

T1,2....Major and minor typhlosoles respectively.

tf.....Transverse folds.

ue.....Unciliated epithelium.

v.....Muscular valve of stomach.

LEGENDS TO PLATES 23-27.

THE STOMACH AND STYLE SAC.

PLATE 23.

A. SEM, Junction of oesophagus and stomach opened by a longitudinal dorsal incision. x67.

B. SEM, Aperture of the digestive gland duct. x150.

Scale bars: A=300μm; B=133μm.

PLATE 24.

A. SEM, dissected aperture of the digestive gland duct. x87.

B. SEM, style sac opened by a longitudinal incision. x60.

Scale bars: A=230μm; B=1mm.

PLATE 25.

A. SEM. Left, typhlosoles, inset shown magnified on the right. x544. Right, unciliated cells not producing blebs. 4.3K

B. SEM, minor typhlosoles with cells producing blebs. x770.

Scale bars: A(left)=100μm, (right)=4μm; B=25μm.

PLATE 26.

A. Light micrograph of the typhlosoles (T.S.). x680.

B. SEM, white secretion formed from blebs (arrows) released by ciliated cells. 3K

Scale bars: A=50μm; B=10μm.

PLATE 27.

A. Montage of the unciliated epithelium forming the secretory tracts adjacent to the typhlosoles. 7K.

B. Liver string, distal end coated with white secretion. x107.

Scale bars: A=2μm; B=190μm.

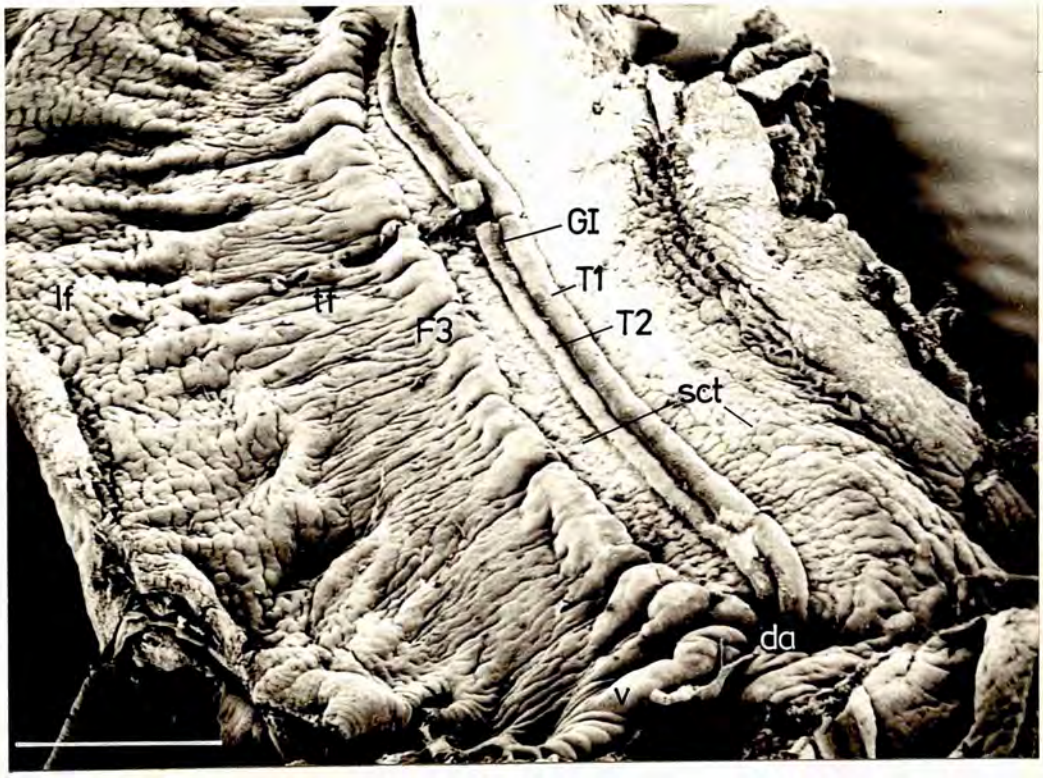
PLATE 23

A

B

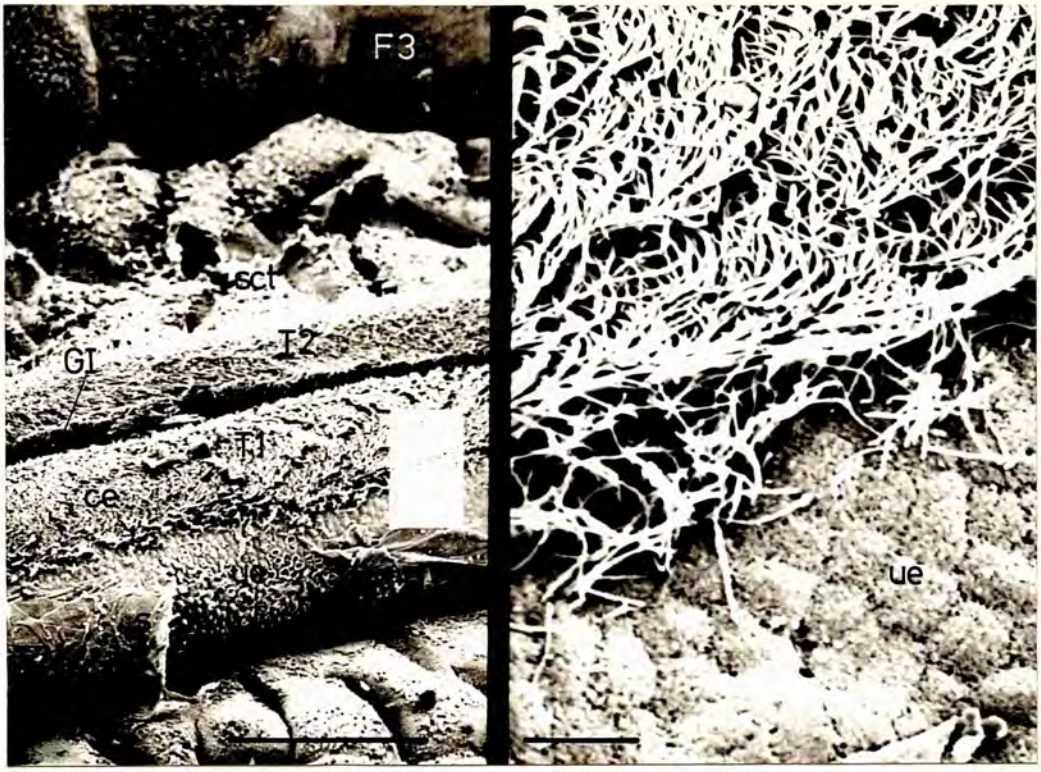
PLATE 24

A



B

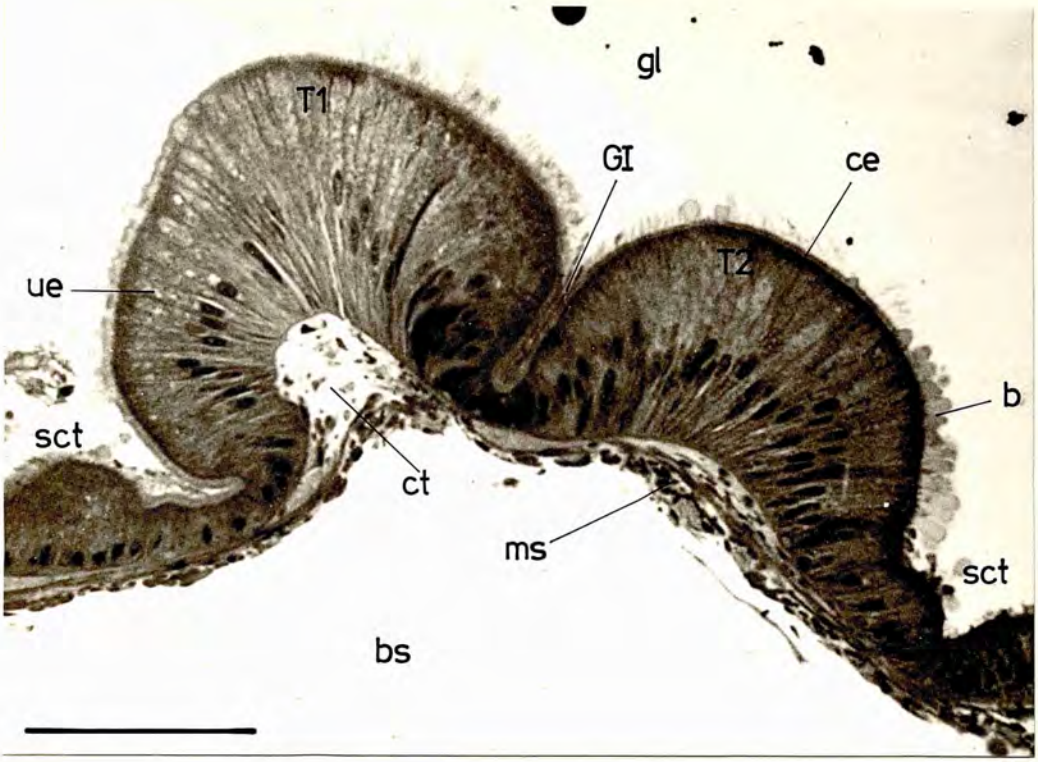
PLATE 25



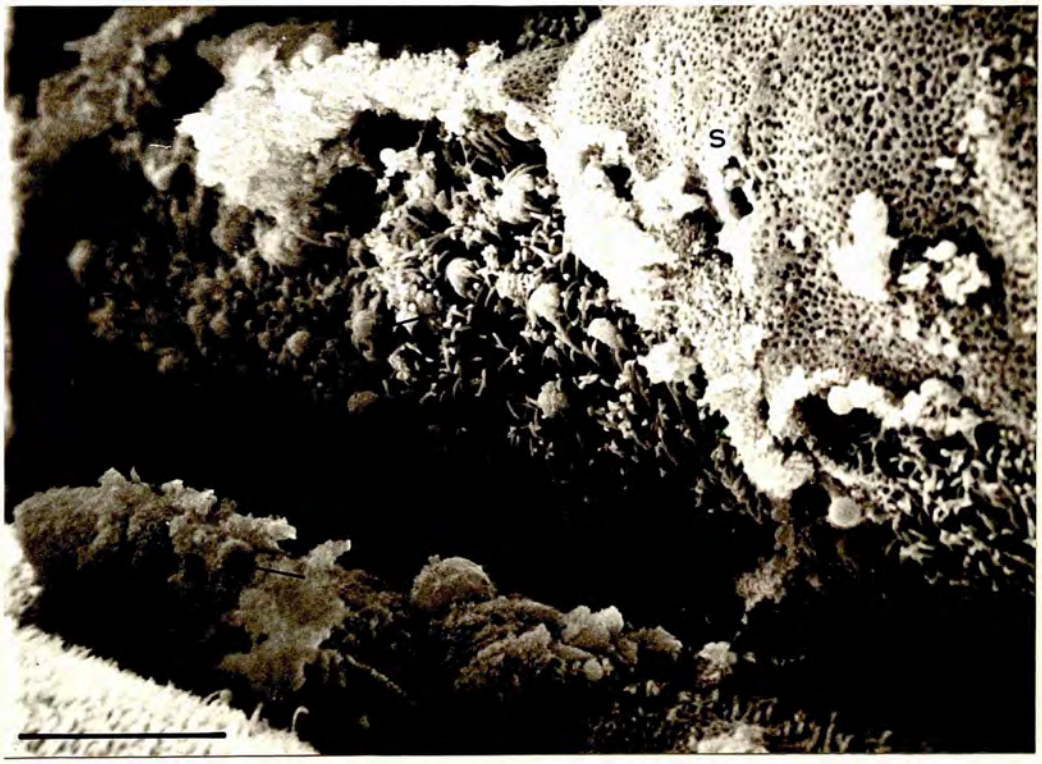
A

B

PLATE 26

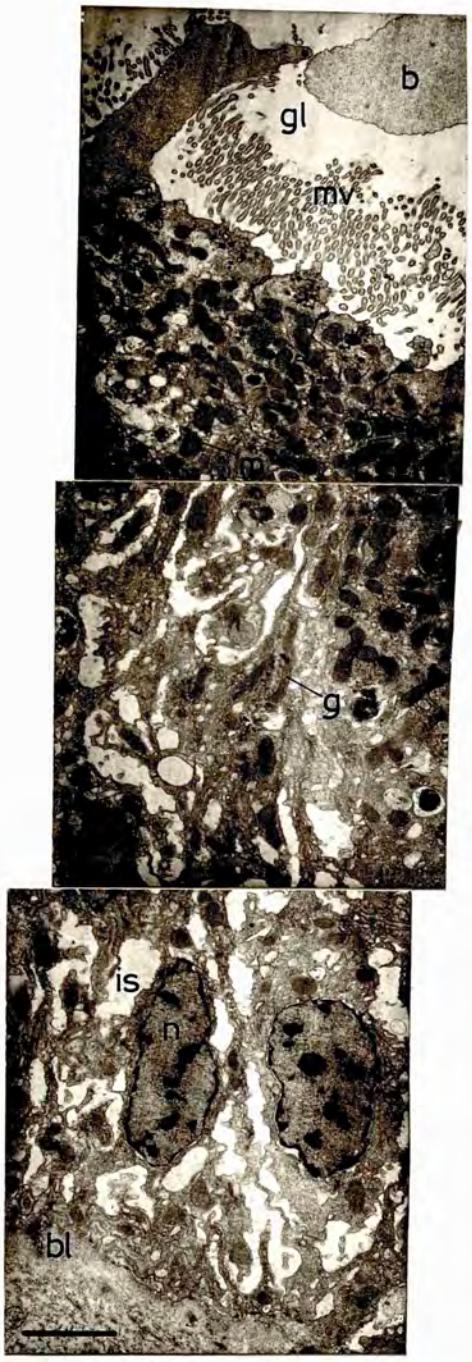


A

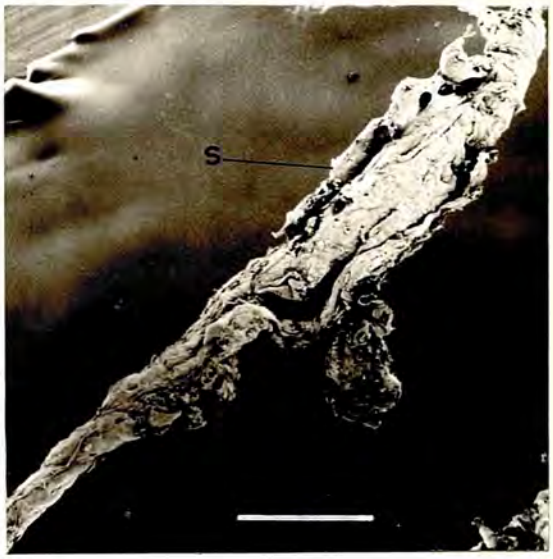


B

PLATE 27



A



B

THE DIGESTIVE GLAND.

The digestive gland is a greenish-brown, voluminous gland occupying the majority of the visceral mass; through and around it wind the many loops of the gut with which it connects by a single duct opening into the stomach. Typically in other prosobranchs, the gland is divided into two unequal lobes each with its own duct opening into the stomach, but in Patella this is not so (Fretter and Graham, 1962). The main duct branches into smaller ones that abruptly join the tubules which compose the bulk of the gland. These blind-ending tubules ramify in all directions so that in sections numerous planes of orientation are encountered.

1-THE DUCTS.

A system of ducts branch from the two main trunks which converge to form the single, wide-mouthed duct that opens into the stomach. In transverse section the ducts are circular or oval in shape and the ciliated epithelium is thrown into numerous longitudinal folds. An increase in cell height and also a slight thickening of the subepithelial connective tissue are responsible for the formation of the folds; most attain a height of 35μm, the range being 20-60μm. All appear ciliated in the SEM, but in sections unciliated folds were found (plate 28A). Both Graham (1932) and Pugh (1963) reported that the ducts are composed of ciliated cells only and Pugh described "..clear globules..being extruded from the epithelium"; these correspond to blebs of cytoplasm.

A). ULTRASTRUCTURE.

The epithelium resembles that of the stomach from which it is derived by evagination (plates 28A & B). Both ciliated and unciliated cells display a typical brush border, but the apical glycocalyx is much more prominent than that of the typical cell described in section 1 (plate 28B). In the apical cytoplasm, the endocytotic canal system is well developed and shows numerous connections with the abundant

electron-opaque bodies. Mitochondria, multivesicular bodies and SER cisternae are also common in this region.

A typical golgi complex lies close to the few residual bodies and above the large numbers of electron-lucent lipid droplets (0.7-2.0μm) (plates 28A & B). In the subnuclear regions, the lateral membranes are loosely attached allowing the development of intercellular spaces between the basal infoldings. Large nerves (>10μm) lie above the thin (70nm) basal lamina and in the connective tissue and well developed circular muscle layer that encircles the duct (plate 28A). The duct is frequently lined by a layer of secretion similar to that in the stomach and style sac, it is composed of blebs of cytoplasm produced by the unciliated cells and worked into a layer by the ciliated cells. The ciliary currents generated on the folds, typhlosoles and the fold, F3, carry the secretion into the stomach.

B). HISTOCHEMISTRY.

The results of the histochemical tests are presented in table 10. The brush border contains a PAS-positive moiety that is resistant to diastase digestion and in thick resin sections it exhibits a weak alcianophilia, indicating the presence of neutral and acid mucopolysaccharides. Residual bodies are PAS-positive but this is partially blocked by diastase digestion. The basal lamina displays a weakly positive PAS result and an alcianophilic one at pH 2.5. Small (<1μm) granules in nerves stained strongly with PAS in thick resin sections, but these cannot be correlated with the nerve ultrastructure.

The bromophenol blue method stains the apical cytoplasm due to the density of organelles there. Frozen sections treated with sudan black B reveal large blue-black areas in the mid- and basal regions of the cells that correspond to the position of the lipid droplets in the thin resin sections. An acid phosphatase resistant to inhibition by sodium fluoride was demonstrated in the brush border by the naphthol

TABLE 10.

SUMMARISED HISTOCHEMICAL RESULTS OF THE DIGESTIVE GLAND DUCT EPITHELIUM.

HISTOCHEMICAL PROCEDURES.			REGION OF CELL/EPITHELIUM. MICROVILLI	APICAL CYTOPLASM	RESIDUAL BODIES	LIPID DROPLETS	BASAL LAMINA	DROPLETS IN NERVES
CARBOHYDRATES.	SCHIFF		–	–	–	–	–	–
	P.A.S.		+	–	*+	–	*+W	*+
	ACETYL/P.A.S.		–	–	–	–	–	–
	DIASTASE/ P.A.S.		+	–	*+W	–	*+W	*+
	AB pH2.5		*+	–	–	–	–	–
	M/AB pH2.5		–	–	–	–	–	–
	M/S AB pH2.5		–	–	–	–	–	–
	AB pH1.0		–	–	–	–	–	–
	PA-TSC-SP		–	–	–	–	–	–
PROTEINS	BROMOPHENOL BLUE		–	+	–	–	–	–
	DE-NH_2 BROMO-PHENOL BLUE		–	+	–	–	–	–
	DNFB		–	–	–	–	–	–
	DIAZOTIZ'TN-COUPLING		–	–	–	–	–	–
	DDD		–	–	–	–	–	–
	SUDAN BLACK B		–	–	–	+	–	–
ENZYMES	NAPHTHOL AS-BI PHOSPHATE		+	–	–	–	–	–
	GOMORI MEDIUM pH4.9		–	–	–	–	–	–
	HUGON-BORGERS MEDIUM pH9.0		–	–	–	–	–	–

NOTATION:

AS FOR TABLES 5 AND 6. *.....Result from thick resin section.

AS-BI phosphate method.

2-THE TUBULES.

The glandular tubules are bathed by the blood in the visceral blood space and each is separated from it and one another by a thin layer of connective tissue. Two distinct cell types compose the tubule epithelium and both are histologically different from the duct cells (plate 28C). The most numerous, the digestive cell (=cell type A; Pugh, 1963), is a columnar unciliated cell and characteristically, diverse membrane-bound vesicles occur in the cytoplasm (fig 13). The second cell type, the basophilic secretory cell (=cell type B; Pugh, 1963), is confined to the crypts of the tubules and is distinguished from the digestive cells by its strong basophilia, pyramidal shape, secretory vesicles and the prominent nucleolus in the large basal nucleus (fig 12).

In pieces of gland teased in seawater, the transparent basophilic cell is easily recognised from the vacuolated digestive cell containing green-brown and refringent vesicles. Homogenised gland gives a pH of 5.3.

A very thin connective tissue layer (1-2μm) surrounds each tubule (plate 28C). The basal lamina resembles that described in section 1, but is 70-100nm thick. A poorly developed muscle layer of circular, longitudinal and oblique fibres encircles each tubule. Amoebocytes do not cluster around the tubules.

The following description presents a static representation of the cells which is necessary to understand the ultrastructure of each cell type. Both cell types display phasic activity which is discussed in section 10.

A). THE BASOPHILIC SECRETORY CELL.

i).ULTRASTRUCTURE.

The broad base of this cell is 20-30μm wide and rests upon the thin, flat basal lamina, the rest of the cell tapers forming a neck,

ABBREVIATIONS TO FIGURES 12 AND 13.

ap	APICAL GRANULE.
bl	BASAL LAMINA.
c	CILIUM.
cd	CONDENSING VACUOLE.
cpv	"COATED" PINOCYTOTIC VESICLE.
ecs	ENDOCYTOTIC CANAL SYSTEM.
gc	GOLGI COMPLEX.
gd	GLYCOGEN DEPOSITS.
hl	HETEROLYSOSOMES.
ld	LIPID DROPLET.
m	MITOCHONDRION.
mv	MICROVILLUS.
n	NUCLEUS.
nu	NUCLEOLUS.
ph.	PHAGOSOME.
rb	RESIDUAL BODY.
rer	RER.
rs	RELEASED SECRETION.
sd	SECRETORY DROPLET.
tv	'TORUS' VESICLE.

FIGURE 12. A DIAGRAMMATIC REPRESENTATION OF A DIGESTIVE GLAND BASOPHILIC SECRETORY CELL.

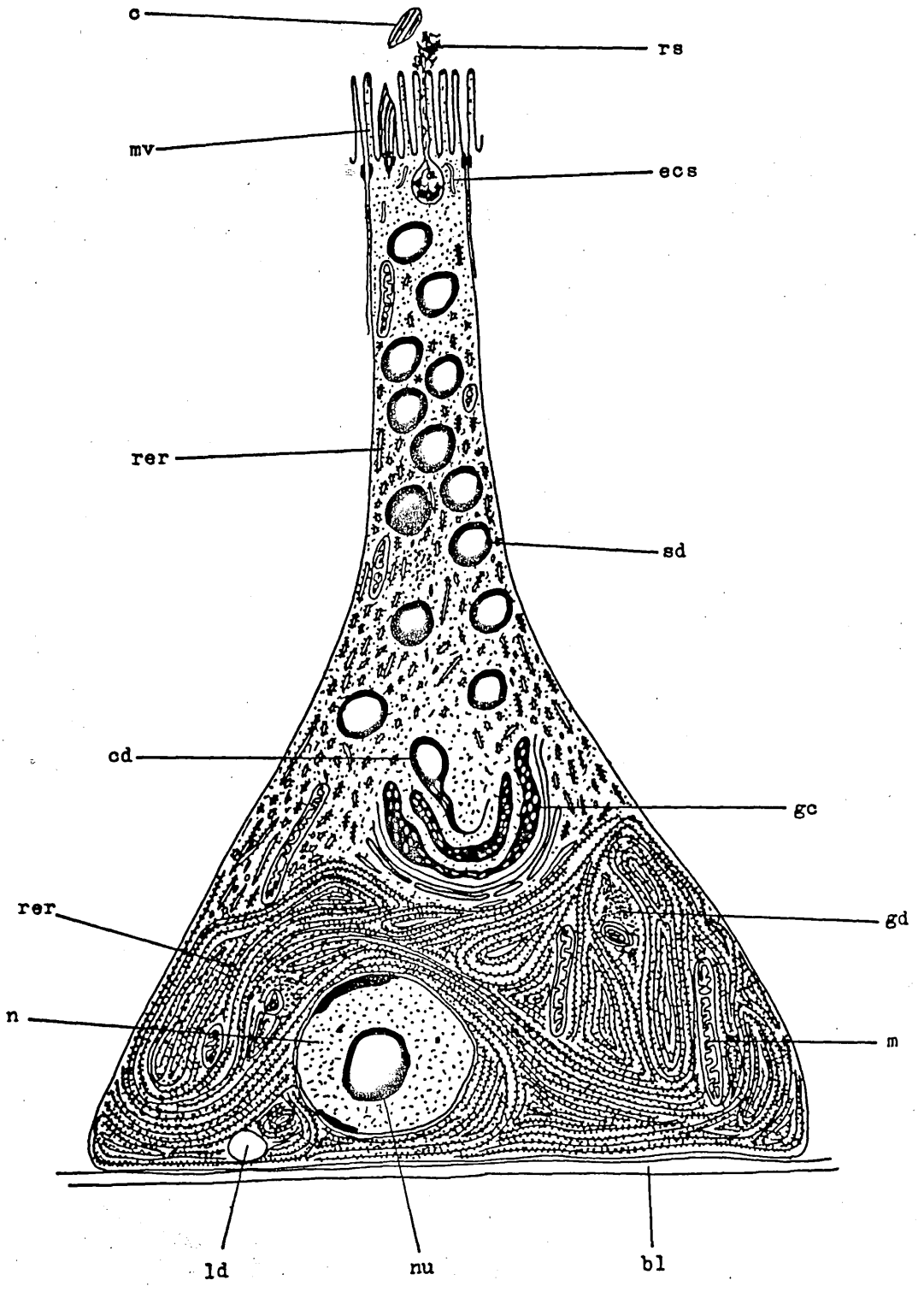
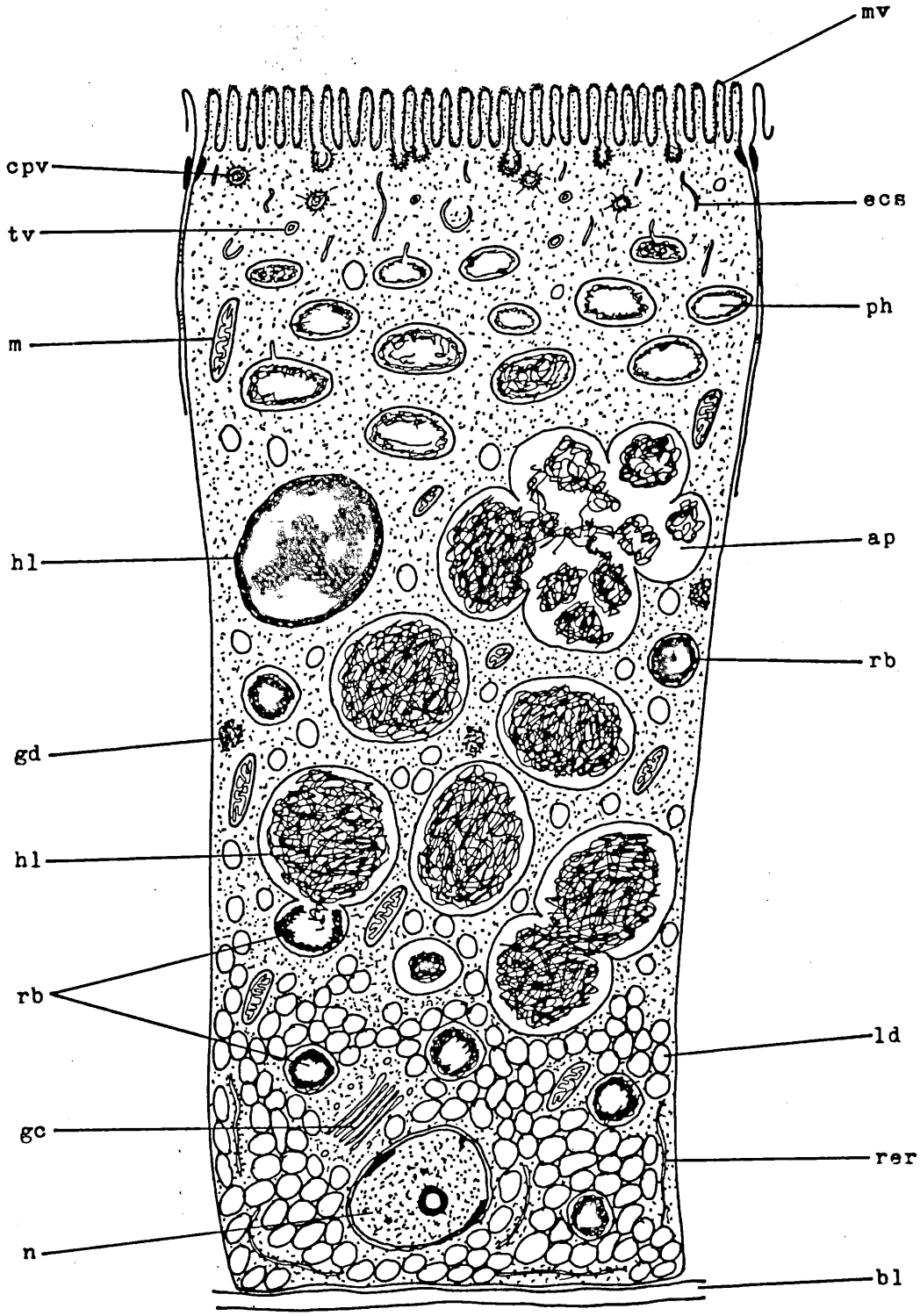


FIGURE 13. A DIAGRAMMATIC REPRESENTATION OF A DIGESTIVE GLAND DIGESTIVE CELL.



the apical surface of which bears microvilli bordering the lumen of the tubule (plates 28C, 29 & 30A). Cell height varies between 30-80μm, depending on the phase of activity. Only with the TEM can the microvilli (1.5-2μm long) and flagellum be seen. At high magnifications a core of microfilaments is visible centrally within each thin microvillus and a slight attenuate glcocalyx projects from the membrane. The microvilli are uniform in diameter (50-60nm) and are sometimes branched at their base, the tips containing an accumulation of finely granular, electron-opaque material (plate 30A). There is no evidence to suggest that this material is pinched off from the tips.

The cells possess at least one flagellum. In sections only the proximal end connected to the basal body is seen together with oblique sections of the flagellum in the lumen (plate 30A). The axoneme has the "9+2" doublet arrangement, but the diameter (250nm) is greater than that of a ciliated columnar cell.

Neighbouring cells are joined to one another by typical junctional complexes (plate 30A). In the apical cytoplasm is an endocytotic canal system manifest as variously shaped membrane-bound profiles (plate 30A). Each is electron-opaque due to a fine granular matrix within the lumen and although the length varies with the plane of section the diameter is a constant 70nm. The canal system is not associated with any organelles and does not take up ferritin.

A highly developed RER system ramifies through the cytoplasm, in the basal regions a curvilinear form occurs, but elsewhere it appears as tubular profiles (plates 29, 30B, 31B & 31C). The cisternae are normally quite distended (50-170nm wide), filled with a very pale granular material and continuous with the nuclear envelope which is also studded with ribosomes. The RER and free ribosomes in the cytoplasm are responsible for the strong basophilia of the cell.

A large cup-shaped golgi complex lies in the supranuclear cytoplasm

and is approximately 6μm across the open trans face directed towards the neck (plates 29 & 30B). Forming the cis face are 2-3 distended (75-225nm wide) cisternae containing a fine material similar to that in the RER (plate 30B). The first of these is discontinuous along its length and is probably derived from flattened TER saccules. Accumulation of these into a linear series followed by their fusion may give rise to the inner cis cisternae, which are more distinct and continuous, yet retain a similar content and appearance.

The remaining trans cisternae are notable because of their intense staining reaction, elaborate structure and distentions that form condensing vacuoles (plate 30B). Their contents are similar to that of the secretory granules, electron-opaque, finely granular and homogeneous. The cisternal space is 30-300nm wide depending on the amount of material. These cisternae are fenestrated (plates 30B & 31A). Round, oval and flattened fenestrae occur (80-100nm), they contain cytoplasm and numerous small, (40nm) electron-opaque vesicles containing a similar material to that in the secretory granules.

A similar trans element together with variously shaped condensing vacuoles occur in the cytoplasm enclosed by the golgi complex. Condensing vacuoles (140-900nm) are commonly seen here and some are still connected to the trans cisternae. The spherical (1.5-2μm), mature secretory granules stain bright red with Mallory and intense blue with toluidine blue, they accumulate in the apical cytoplasm (plate 29). They are bound by a membrane closely applied to the electron-opaque, finely granular matrix (plate 31C). Newly formed granules, those in the cytoplasm enclosed by the golgi, are slightly paler.

Exocytosis of their contents occurs by fusion of single or small numbers of granules with the apical plasma membrane (plate 30A). This was inferred from the different stages of release observed in

sections. Openings of the granules into the tubule lumen has not been observed. In some sections the content of granules close to the apical membrane was diffuse and weakly stained, released secretory material being identified amongst the microvilli. Once released, the secretion stains orange with Mallory and is carried along the tubule by the currents generated by the flagella.

Mitochondria and small amounts of the tubular RER are present in the neck and where the cytoplasm is clear, microfilaments can be seen. The mitochondria are large (up to 4μm long and 500-600nm in diameter) and sometimes hypertrophied (plate 31B), suggesting high activity in these cells.

Observations with the optical microscope reveal pale staining "vacuoles" (1-2μm) in the cytoplasm of some cells and at times a distinct cluster of minute particles (blue after Mallory) occur. These are areas of electron-lucent cytoplasm from which all organelles are excluded, they are not membrane-bound (plates 29 & 31D). The staining reaction of the coarsely granular particles (35-40nm) in these areas varies from weak to intense, ultrastructurally they are reminiscent of β-glycogen particles. Membranous whorls (100-600nm in diameter) are frequently encountered with the glycogen and these may also be stained to varying intensities (plate 31D).

The large (5-6μm), spherical nucleus containing a conspicuous nucleolus (2.5-3.0μm) that stains bright red with Mallory and deep blue after toluidine blue, is situated basally. There is little heterochromatin clumped against the nuclear membrane or free in the nucleoplasm.

ii).HISTOCHEMISTRY.

The summarised results are displayed in table 11.

CARBOHYDRATES. The cytoplasm, nucleolus and secretory granules react very weakly to Schiff and PAS, diastase digestion has no effect and acetylation reduces the reaction only slightly. This may be a

TABLE 11.

SUMMARISED HISTOCHEMICAL RESULTS OF THE DIGESTIVE GLAND SECRETORY CELL.

HISTOCHEMICAL PROCEDURES			SITES EXAMINED: SECRETORY DROPLETS	CYTOPLASM	NUCLEOLUS	LIPID DROPLETS
CARBOHYDRATES	SCHIFF		$+^{W}$	$+^{W}$	$+^{W}$	–
	P.A.S.		$+^{W}$	$+^{W}$	$+^{W}$	–
	ACETYL/P.A.S.		$+^{W}$	$+^{W}$	$+^{W}$	–
	DIASTASE/ P.A.S.		$+^{W}$	$+^{W}$	$+^{W}$	–
	AB pH2.5		–	–	–	–
PROTEINS	BROMOPHENOL BLUE		+	$+^{W}$	+	–
	DE-NH_2/BROMO-PHENOL BLUE		+	$+^{W}$	+	–
	DNFB		$+^{W}$	$+^{W}$	$+^{W}$	–
	DE-NH_2/DNFB		$+^{W}$	–	–	–
	DIAZOTIZ'TN-COUPLING		+	–	–	–
	IODINATION/ DIAZ-COUPLING		–	–	–	–
	DDD		$+^{W}$	–	$+^{W}$	–
	THIOGLYCOLATE /DDD		$+^{W}$	–	$+^{W}$	–
	SUDAN BLACK B		–	–	–	+
ENZYMES	NAPHTHOL AS-BI PHOSPHATE		–	golgi region	–	–
	GOMORI MEDIUM pH4.9		–	–	–	–
	HUGON-BORGERS MEDIUM pH9.0		–	–	–	–

NOTATION:

AS FOR TABLES 5 AND 6.

non-specific reaction towards residual aldehydes from fixation. Acid mucopolysaccharides are not present and the PA-TSC-SP technique leaves the glycogen deposits unstained.

PROTEINS. Very strong reactions to the bromophenol blue and diazotization-coupling methods are given by the secretory granules; but they only react weakly for tyrosine and sulphydryl groups.

LIPIDS. Very few sudanophilic inclusions (1-2μm) were found in the basal regions of the cell, but they could not be correlated to the ultrastructure of the cell.

ENZYMES. Acid phosphatase was demonstrated in the mid-region of the cells by the naphthol AS-BI phosphate method, this may correspond to the golgi complex. Some granules give a very weak reaction.

B-THE DIGESTIVE CELL.

i). ULTRASTRUCTURE.

Digestive cells are columnar, their height varying from 25-80μm depending on the phase of activity. The apical surface borders the lumen of the tubule, bears short microvilli (1-1.5μm long by 95-120nm in diameter) and is either flat, domed or thrown into lobose projections (plates 32A & B). A microfilamentous core is not visible in the microvilli which have electron-opaque, fine granular deposits at their tips and a sparse thread-like glycocalyx (plates 33A & B); they do not contain vesicles. Sometimes they are branched, but diminish as the apical membrane bulges into the lumen during the fragmentation phase.

There is no evidence to suggest that the cells are ciliated. Graham (1932) reported cilia on living digestive cells but suggested that they either retract into the cell or drop off during fixation. Evidence of this has not been found. It is likely that Graham observed the beating flagella of the basophilic cells and at low magnifications attributed them to the digestive cells.

Adjacent cells are joined apically by typical junctional complexes,

the lateral membranes do not interdigitate (plate 32B). In the mid-regions of the cells, intimate associations of the lateral membranes resembling gap-junctions occur and the intercellular space is obliterated by a layer of finely granular, electron-opaque material. There are no hemidesmosomes connecting the cells to the basal lamina.

Digestive cells can be subdivided into apical, mid- and basal thirds each with a relatively typical organelle/inclusion content, that varies ultimately with the phase of activity (fig 13). The most conspicuous organelles are a series of membrane-bound vesicles composing the heterophagic-lysosomal system of the cell (plates 32A & B). It is convenient to separate these diverse vesicles into two classes based arbitrarily upon their size (Owen, 1970), the smaller ones are classified as microvesicles and the larger ones macrovesicles. The terminology adopted by Owen (1972a) is used to differentiate between types in each class.

Microvesicles are small (50-150nm) and include pinocytotic vesicles (=pinosomes) and profiles of the endocytotic canal system (plates 33A, B & C). Extensive endocytosis occurs across the apical plasma membrane; pinocytosis of food and ferritin occurs at the base of the microvilli and via the canal system (plates 33A & 34B).

Occlusion of the tubule lumina due to an increasing epithelial height, concentrates food into a smaller volume and simultaneously brings it into contact with the microvilli (plate 33A). Pinocytotic depressions are "coated" on the cytoplasmic face by closely spaced, bead-like particles (plates 33B & C). As they deepen, the "coat" spreads so as to cover the newly invaginated membrane. Once a vesicle separates from the apical membrane it is surrounded by this "coat", but the region of the membrane directly above, from which it was derived, remains smooth.

A recently formed vesicle is 150nm in diameter and contains a

finely granular, electron-opaque material, the staining intesity and distribution of it depends upon the presence of food in the lumen (plates 33A & B). At this stage a series of fine fibrils (4nm in diameter) radiate from the "coat", they are only noticable in the lucent areas of cytoplasm and disappear as they penetrate denser regions. They may be contractile filaments responsible for pulling the vesicles into the cell. The "coat" disappears as the vesicles move deeper into the cell but the fibrils remain attached.

The endocytotic canal system resembles that already described for the amylase-secreting cells of the oesophageal gland. Profiles range in length from 110-1,300nm and from 40-75nm in diameter, the wider portions occurring in the deeper cytoplasmic ends, especially where the system connects to heterophagosomes (the smallest type of macrovesicle) (plate 34C). On most occasions the canal system is full of a material similar in all respects to the food in the tubule lumen. The system is a permanent feature in all of the phases, but is more prolific during the absorbing and digestive phases.

Some profiles of the system are ring-shaped or form incomplete circles others appear as torus shapes with an external diameter of 120-150nm. A cytoplasmic "coat" and fibrils are not features of the canal system. The various profiles suggest that the canal system is a three dimensional series of repeatedly branched tubules.

Macrovesicles ranging in size from 0.5-10μm in diameter are membrane-bound and contain a heterogeneous matrix. Heterophagosomes, heterolysosomes and residual bodies are included in this group (plates 32A & B).

All microvesicles fuse with the heterophagosomes that occur in the apical third of the cell. Heterophagosomes show a gradation in size; those nearest to the apical membrane being smaller (0.5-1.0μm) than those below them (1.5-3.0μm) (plate 32B). These vesicles correspond to the small pink/pale purple bodies seen in Mallory- stained wax

sections.

Heterophagosomes are normally oval in shape and relatively empty apart from a sparse peripheral halo of material similar to that in the tubule lumen and microvesicles (plate 34A). The opacity and density of this material varies (plate 34C). There is no evidence to suggest that the smaller heterophagosomes fuse to give rise to the larger ones or that the latter fuse to form heterolysosomes.

Heterolysosomes (5-10μm) are frequently situated in the mid-region of the cell and have various contents; depending on the state of intracellular digestion, these appeared electron-opaque and homogeneous or moderately opaque and flocculant (plates 34D & E). Autophagic debris such as membranous whorls and very dense clumps of material do not occur. Frequently, lucent areas (250nm in diameter) without a membrane are present in the matrix which may be separated from the heterolysosome membrane by a lucent halo. The membrane displays small, Ω-shaped evaginations suggesting that pinosomes and/or primary lysosomes have fused with it (plate 34D). In the adjacent cytoplasm, occur small (100-150nm), moderately opaque vesicles which could be pinosomes or primary lysosomes without acid phosphatase activity.

The fusion of heterolysosomes produces a large, irregularly-shaped body with a common lumen, which may fill the apical region of the cell (plate 34F); this corresponds to the "apical granule" described in these cells by Pugh (1963). It contains a moderately opaque matrix that may exist as granules 3-5μm in diameter (plate 56), which appear as a refringent cluster after Mallory staining. This material resembles that in the residual bodies so they may be compound residual bodies.

Residual bodies can be distinguished from the other macrovesicles in Mallory-stained wax sections by their intermediate size (1-3μm) and their purple staining reaction. They are most common in the mid-

and basal regions of the cell (plates 32A & B). Ultrastructurally, various forms are encountered; one type has an electron-opaque peripheral halo (plate 34F), others have a granular matrix clumped in the centre of the lumen (plate 34E), whilst others are filled with a similar matrix (plate 32B).

Residual bodies fuse with heterolysosomes and so contain, together with their typical dense matrix, material from the heterolysosome lumen (plates 34E & F). This is interpreted as the sequestration of indigestible residues from the heterolysosome into the residual body. Ultimately, the residual bodies are ejected from the cells in apical spherules of cytoplasm by apocrine secretion.

Lipid droplets (2.5-3.0μm) are common in the basal half of cells from recently collected limpets (plates 32A, 55 & 56). They are not preserved in wax sections, but in some thick resin sections stained with toluidine blue they are coloured greenish blue. Ultrastructurally, they are homogeneous, lucent and have a pale peripheral halo 80-100nm thick; they show no associations with any of the organelles and are frequently squashed into irregular shapes (plate 34F).

Mitochondria are distributed throughout the cell, but are numerous in the mid-region amongst the heterolysosomes. The basal nucleus is oval (5 by 4μm), with a small nucleolus (1μm) and a little heterochromatin against the inner membrane of the nuclear envelope, the outer one continuous with the RER, is studded with ribosomes. Short, thread-like lengths of RER are present in the basal third of the cell, most of it lying against the basal and lateral membranes or around the nucleus. The cisternal lumen is narrow (40-50nm) and contains sparse amounts of a finely granular, moderately opaque material.

Unexpectedly for a cell actively involved in intracellular digestion, the golgi complex is inconspicuous and rarely seen. It

lies in the basal third of the cell and is modest in size and structure (plate 48C), suggesting that the digestive enzymes may be packaged directly by the RER. It is possible that an active phase was missed. Sparse clusters of β-glycogen occur in the cytoplasm throughout the cell.

ii). HISTOCHEMISTRY.

Summarised results are presented in table 12.

CARBOHYDRATES. 1,2-glycol groups are demonstrated on the brush border by the strong PAS reaction (acetylation blocks the reaction), but acid mucopolysaccharides (AMPS) are absent. The cytoplasm yields a weak PAS result, but the heterolysosomes react strongly, the reaction being blocked by acetylation and unaffected by diastase digestion. They sometimes display an alcianophilia at pH 2.5 indicating the presence of AMPS. "Apical granules" react similarly, but are alcianophilic at both pH 1.0 and 2.5. Only a few glycogen deposits are demonstrated by the PA-TSC-SP method.

PROTEINS. The bromophenol blue method demonstates basic proteins in the heterolysosomes, the reaction is slightly reduced by deamination; they also react for tyrosine and weakly for disulphide groups. "Apical granules" may give a weak reaction to tests for tyrosine.

LIPIDS. The mid- and basal regions show a strong sudanophilic reaction, separate lipid droplets are difficult to distinguish from the large stained areas of the frozen sections.

PIGMENTS. Lipofuscins and melanins were not demonstrated by the tests employed.

ENZYMES. Some heterolysosomes give a positive result for acid phosphatase after staining by the naphthol AS-BI phosphate method. Summarised results of the substrate-film tests for digestive enzymes are shown in table 13. An amylase capable of digesting an unfixed starch film was located in the digestive gland (plate 35A). Both of the photographic film techniques were too insensitive to demonstrate

TABLE 12.

SUMMARISED HISTOCHEMICAL RESULTS OF THE DIGESTIVE GLAND DIGESTIVE CELL.

HISTOCHEMICAL PROCEDURES.		SITES EXAMINED: BRUSH-BORDER	APICAL GRANULE	HETERO-LYSOSOMES	LIPID DROPLETS
CARBOHYDRATES	SCHIFF	–	–	+W	–
	P.A.S.	+	+	+	–
	ACETYL/P.A.S.	–	–	–	–
	DIASTASE/ P.A.S.	+	+	+	–
	AB pH2.5	–	+	+W	–
	M/AB pH2.5	–	–	–	–
	M/S AB pH2.5	–	–	–	–
	AB pH1.0	–	+	–	–
	M/AB pH 1.0	–	–	–	–
	M/S AB pH1.0	–	–	–	–
PROTEINS	BROMOPHENOL BLUE	–	–	+	–
	DE-NH_2/BROMO-PHENOL BLUE.	–	–	+W	–
	DNFB	–	–	–	–
	DIAZOTIZ'TN-COUPLING	–	+W	+	–
	IODINATION/ DIAZ-COUPLING	–	–	–	–
	DDD	–	–	–	–
	SUDAN BLACK B	–	–	–	+
ENZYMES	NAPHTHOL AS-BI PHOSPHATE	–	–	+	–
	GOMORI MEDIUM pH4.9	–	–	–	–
	HUGON-BORGERS MEDIUM pH9.0	–	–	–	–

NOTATION:

AS FOR TABLES 5 & 6.

TABLE 13.

SUMMARISED DIGESTIVE ENZYME HISTOCHEMICAL RESULTS.

TECHNIQUES	TREATMENT		
		EXPERIMENTAL	CONTROLS
STARCH FILM TO DEMONSTRATE AMYLASE. (TREMBLAY & CHAREST, 1968).		+	THERMAL DENATURE OF TISSUE −
			1% BUFFERED DIASTASE +
BLACK AND WHITE FILM TO DEMONSTRATE PROTEASE. (ADAMS AND TUQAN, 1961).		−	THERMAL DENATURE OF TISSUE −
			1% BUFFERED TRYPSIN +
COLOUR SLIDE FILM TO DEMONSTRATE PROTEASE. (FRATELLO, 1968).		−	THERMAL DENATURE OF TISSUE −
			1% BUFFERED TRYPSIN +
STAINED GELATIN FILM TO DEMONSTRATE PROTEASE. (KIERNAN, 1981)		+	THERMAL DENATURE OF TISSUE −
			1% BUFFERED TRYPSIN +
GOMORI'S TWEEN 80 TO DEMONSTRATE LIPASE. (PEARSE, 1980).		+	THERMAL DENATURE OF TISSUE +
			OMIT TWEEN 80 SUBSTRATE +

the protease. Positive results are obtained with the stained gelatin-film method when frozen sections are incubated for at least 30 minutes (plate 35B). Experimental and control sections incubated in Gomori's tween 80 medium to demonstate lipase activity, give positive reactions (speckling with a black precipitate). Repeated experiments with freshly made reagents gave similar results, which were considered inconclusive and so biochemical methods were tried (table 14).

The experimental and control incubations of Agrawal's method gave similar inconclusive results. It was concluded that either the sodium hydroxide or bromothymol blue was causing hydrolysis of the lipid in the milk to form fatty acids, lowering the pH of the medium to cause the colour change. The experiments were repeated using an indicator with a lower pH range, so that the sodium hydroxide could be omitted; similar false positive results were obtained by all procedures. The method used by George, based on a different mechanism was tried. This gave negative results in all procedures, therefore suggesting that a lipase capable of digesting olive oil is not present in the digestive gland.

FERRITIN EXPERIMENTS. Tissue treated by *in vitro* incubation, revealed very little ferritin in either the duct or tubule lumina, suggesting that during the 30 minute incubation period only a small amount of ferritin was transferred into the gland. Ferritin accumulated against the apical membrane of the digestive cells and on very rare occassions in "coated" pinosomes (plate 34B) and the canal system. This low pinocytotic activity reflects the low ferritin concentration in the lumen. This may be due to abnormal conditions; in an intact animal ferritin suspended in the stomach fliud could be forced into the gland by muscular contractions, whilst the stomach valve sealed off the style sac. In isolated pieces of tissue the cilia of the duct would prevent entry of ferritin into the gland.

Following the *in vivo* feeding experiments, ferritin was totally

TABLE 14.

BIOCHEMICAL LIPASE DEMONSTRATIONS.

AGRAWAL'S METHOD USING BROMOTHYMOL BLUE AS INDICATOR.

	TREATMENTS			
			CONTROLS	
COLOUR OF MIXTURE	EXPERIMENTAL (extract, milk, indicator, NaOH).	1 (boiled extract, milk, NaOH, indicator).	2 (milk, NaOH, indicator).	3 (milk, indicator).
INITIAL	CLOUDY PALE SKY BLUE	CLOUDY DEEP SKY BLUE	BRIGHT SKY BLUE	CLOUDY GREEN-BLUE
FINAL	CLOUDY PALE YELLOW-GREEN	AS EXPT.	YELLOW-GREEN	CLOUDY YELLOW-GREEN
	FLOATING ON THE SURFACE OF ALL THESE MIXTURES WAS A YELLOW LAYER THAT DISPERSED ON SHAKING.			

MODIFIED AGRAWAL'S METHOD USING BROMOCRESOL PURPLE AS INDICATOR.

	TREATMENTS		
		CONTROLS	
COLOUR OF MIXTURE	EXPERIMENTAL (extract, milk, indicator).	1 (boiled extract milk, indicator).	2 (milk, indicator).
INITIAL	CLOUDY PALE PURPLE	AS EXPT.	CLOUDY DEEP PURPLE
FINAL	PALE PURPLE-YELLOW FLOATING ON THE SURFACE OF ALL THESE MIXTURES WAS A YELLOW LAYER THAT DISPERSED ON SHAKING.		

GEORGE'S METHOD.

	TREATMENTS			
			CONTROLS	
COLOUR OF OIL DROPLETS	EXPERIMENTAL (extract, oil).	1 (boiled extract, oil).	2 (distilled water, oil)	3 (oil)
INITIAL	PINK			
FINAL	PINK			

absent from the gland of all experimental animals. The ferritin "meal" may have been regurgitated or passed straight into the style sac.

ABBREVIATIONS FOR PLATES 28-35.

bb...Basal body.

bc...Basophilic cell.

bl...Basal lamina.

bs...Blood space.

cm...Circular muscle.

cp...Coated pinosome.

cpv..Coated pinocytotic depression.

ct...Connective tissue.

cv...Condensing vacuole.

dc...Digestive cell.

dl...Lumen of duct.

ecs..Endocytotic canal system.

eob..Electron-opaque body.

f....Flagellum.

fe...Fenestration in golgi cisterna.

fi...Fibril.

g....Golgi complex.

gd...Glycogen deposits.

gf...Gelatin film.

gy...Glycocalyx.

hl...Heterolysosome.

hp...Heterophagosome.

l....Lipid.

m....Mitochondrion.

mb...Membranous body.

mv...Microvilli.

p....Pinosome.

rb...Residual body.

rer..Rough ER.

s....White secretion.

sf...Starch film.

sg...Secretory granule.

sp...Smooth pinosome.

ter..Transitional ER.

tl...Lumen of tubule.

tp..."Torus" profile of ecs.

v....Unspecified vesicle.

LEGENDS TO PLATES 28-32.

THE DIGESTIVE GLAND.

PLATE 28.

A. The unciliated duct epithelium. 2K.

B. Montage of ciliated duct cells. 7K.

C. Light micrograph of a tubule stained with toluidine blue. x335.

Scale bars: A=10μm; B=2μm; C=100μm.

PLATE 29.

Section of tubule epithelium showing both basophilic and digestive cell types. 3.3K. Scale bar=10μm.

PLATE 30. Basophilic cell.

A. Necks of cells with vesicles (arrows) releasing secretion. 16K.

B. Golgi complex. 16K.

Scale bars=2μm.

PLATE 31. Basophilic cell.

A. Fenestrated golgi cisternae. 55K.

B. Hypertrophied mitochondrion. 70K.

C. Secretory granule. 37K.

D. Lucent areas of cytoplasm containing glycogen and a membranous body 37K.

Scale bars: A,C,D=500nm; B=200nm.

PLATE 32.

A. Montage of the tubule epithelium during the absorbing phase. 2K.

B. Apical portion of a digestive cell during the early digesting phase. 5K.

Scale bars: A=10μm; B=4μm.

LEGENDS TO PLATES 33-35.

THE DIGESTIVE GLAND.

PLATE 33 Digestive cell.

A. Apical region during the absorbing phase. 54K.

B. Formation of coated pinosomes. 91K.

C. Endocytotic canal system. 59K.

Scale bars: A=500nm; B,C=250nm.

PLATE 34 Digestive cell.

A. Heterophagosomes and canal system in apices of cell. 28K.

B. Pinocytosis of ferritin (arrows). 108K.

C. Canal system connected to a heterophagosome. 28K.

D. Heterolysosomes during the absorbing phase, note the evaginations of the bounding membrane (arrows). 16K.

E. Residual bodies connected to heterolysosomes during the digesting phase . 8.5K.

F. Several connected heterolysosomes. 4.3K

Scale bars: A=500nm; B=100nm; C=250nm; D,E=2μm; F=3μm.

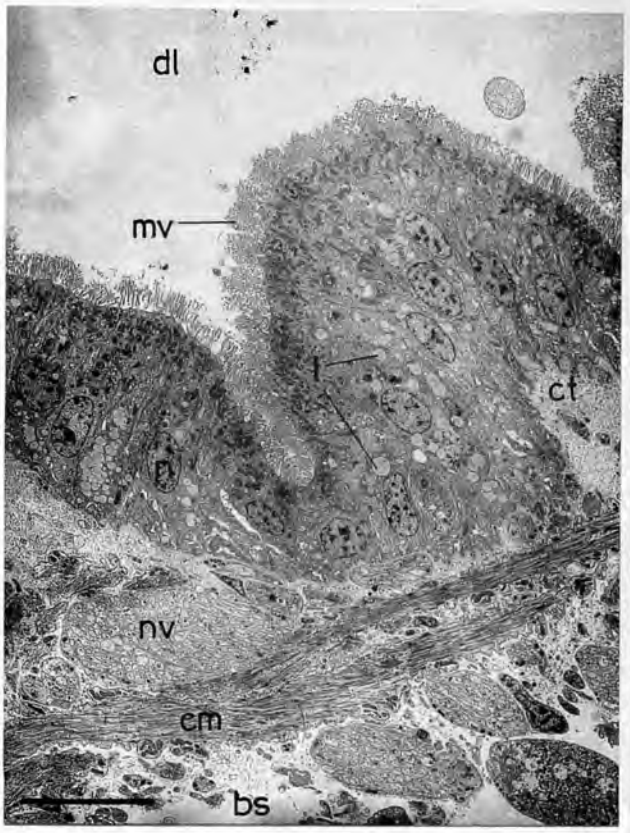
PLATE 35.

A. Starch film method, digested area of film with an overlay of the corresponding serial section. x41.

B. Stained gelatin film method, frozen section on gelatin film, clear areas indicate sites of digestion. x31.

Scale bars=1mm.

PLATE 28



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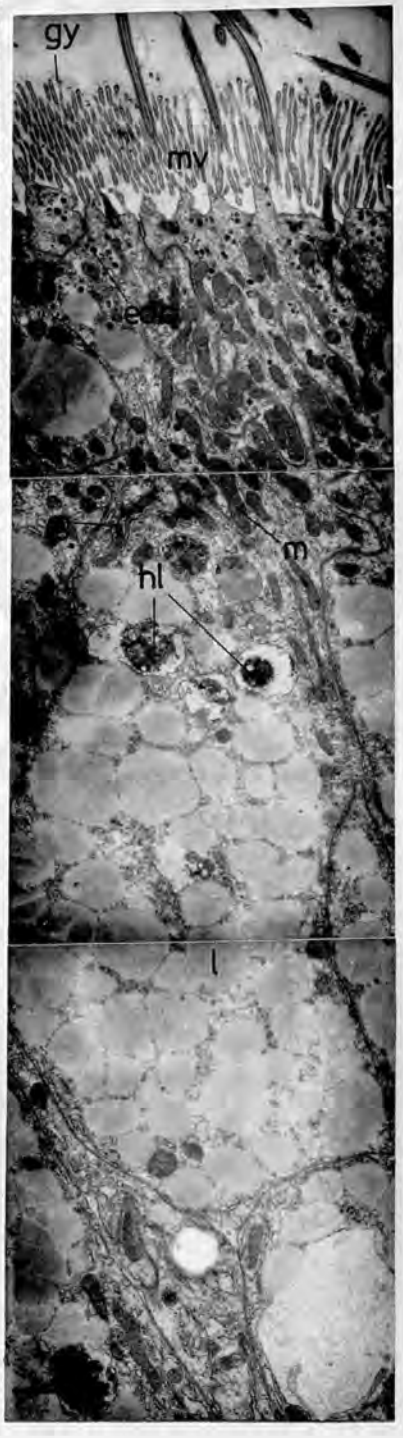
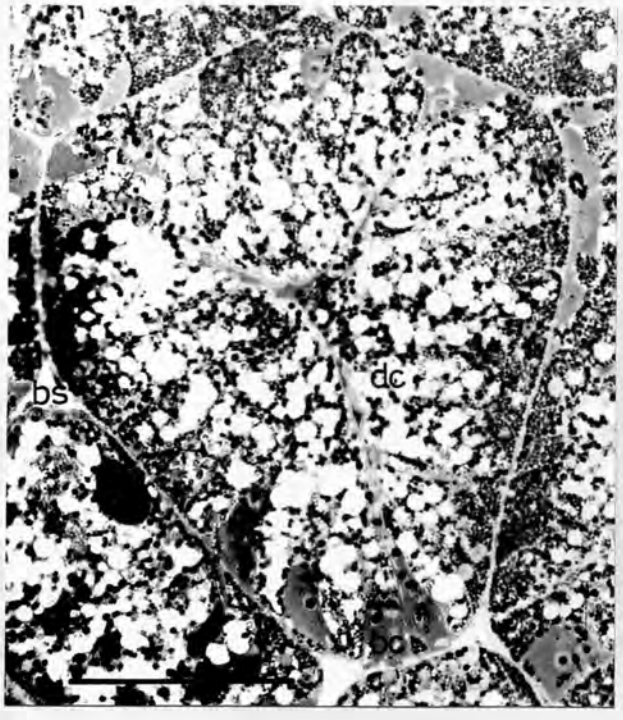


B



C

PLATE 29

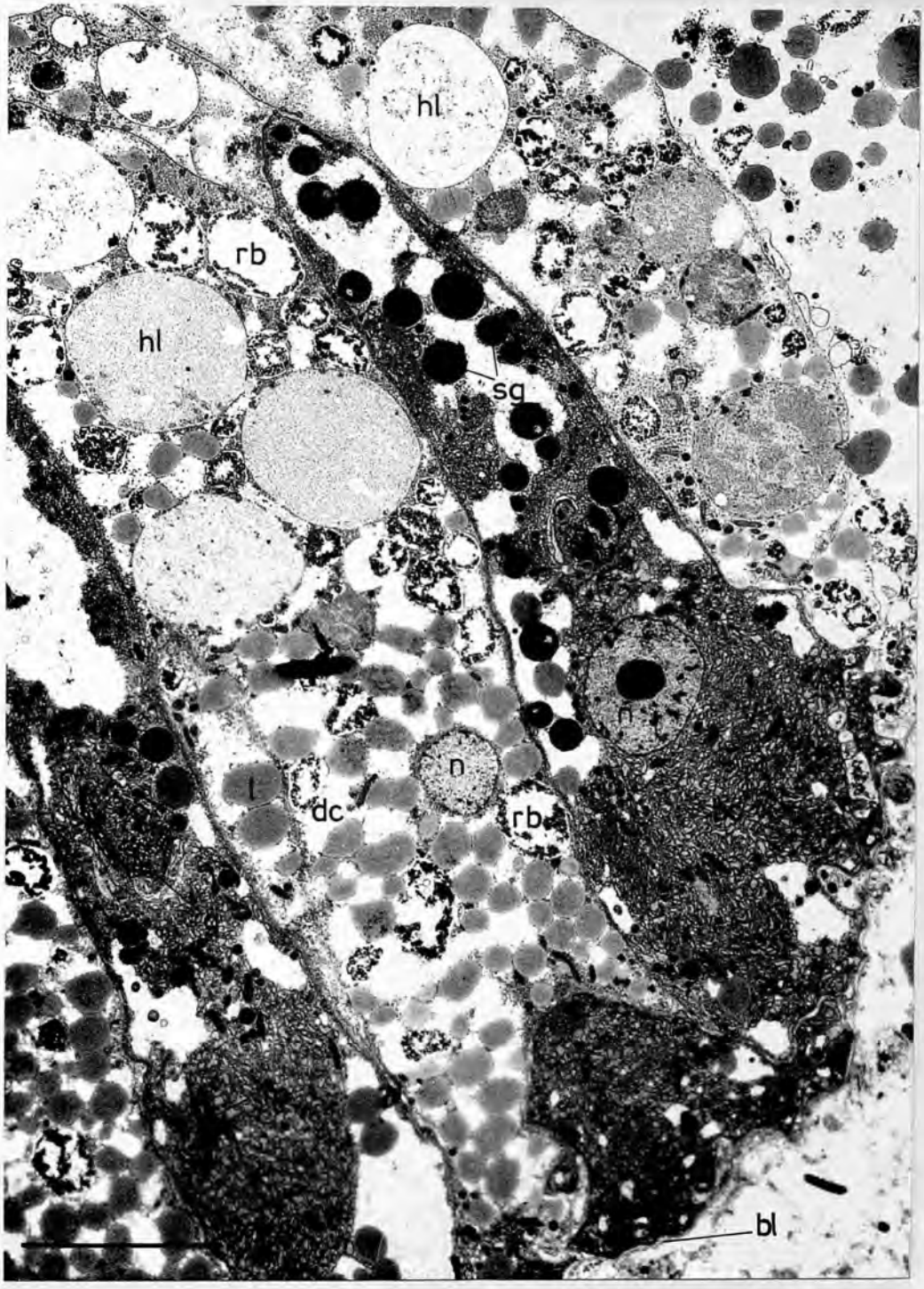
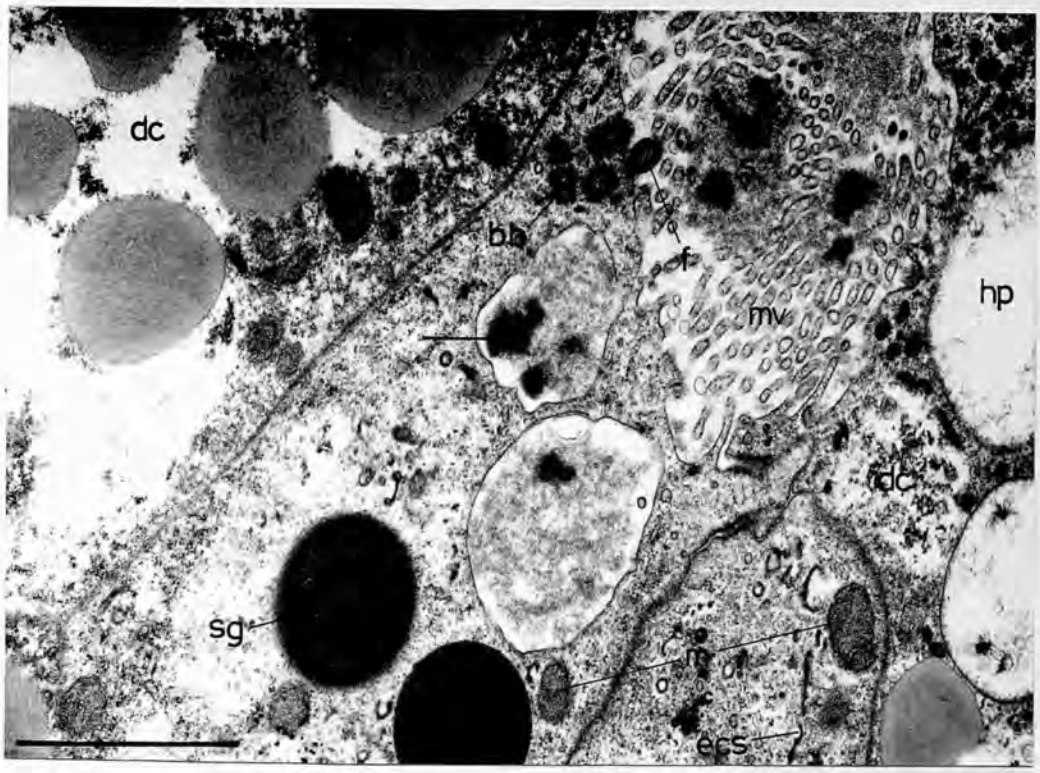
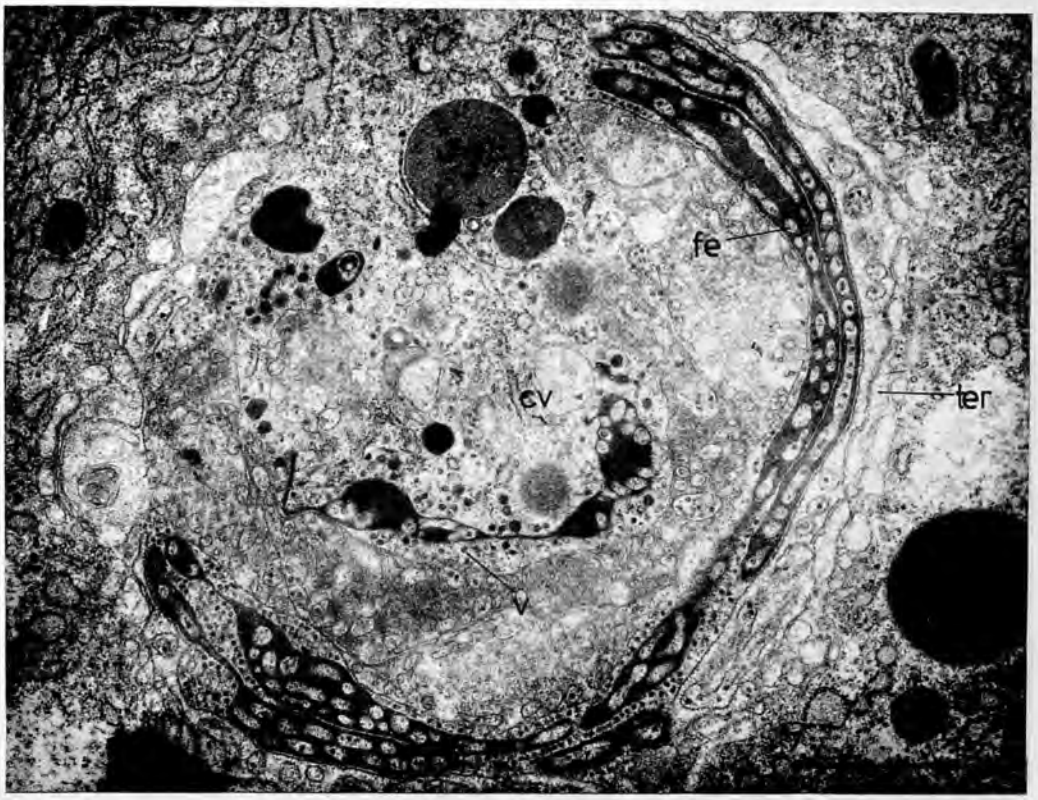


PLATE 30

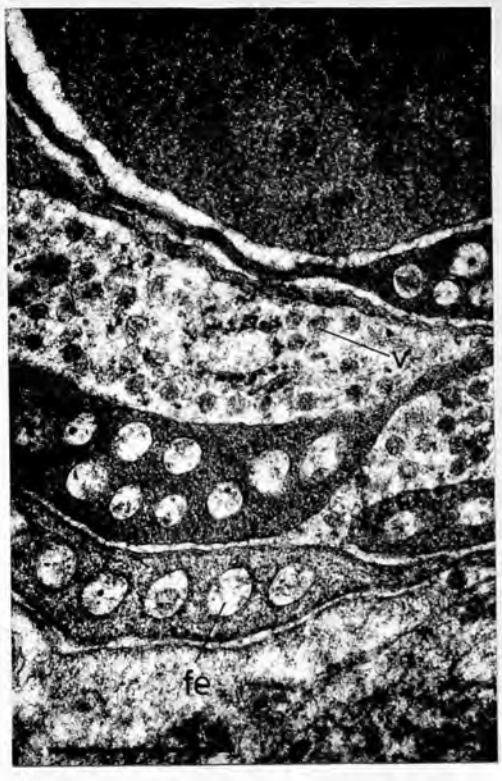


A

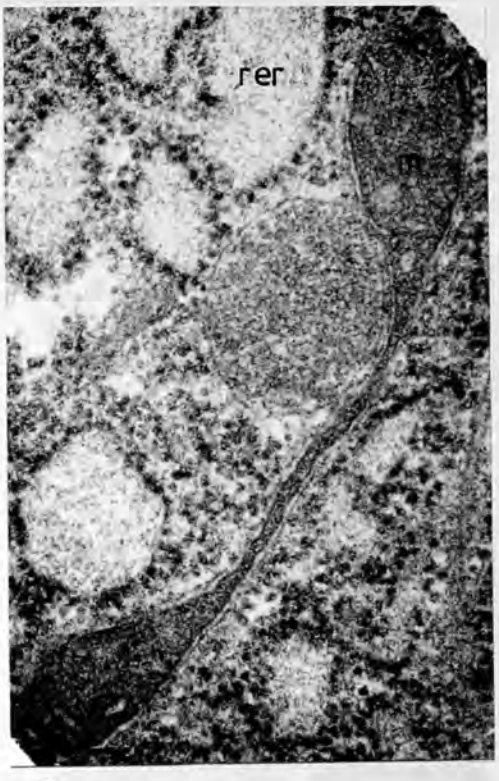


B

PLATE 31



A



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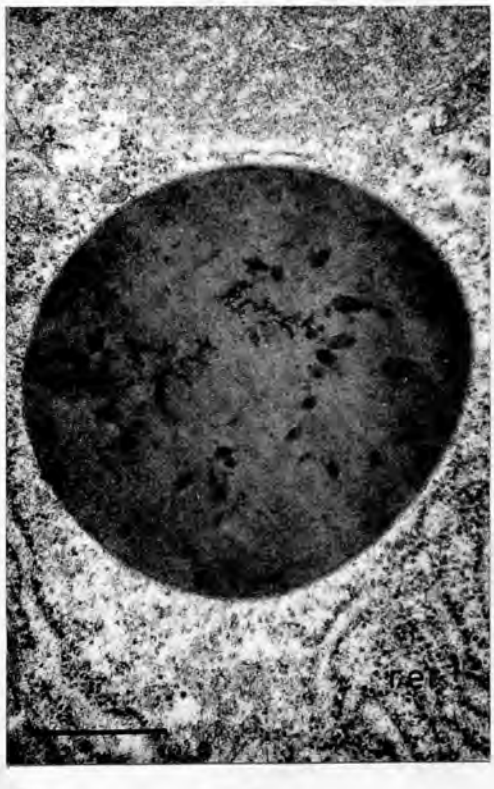

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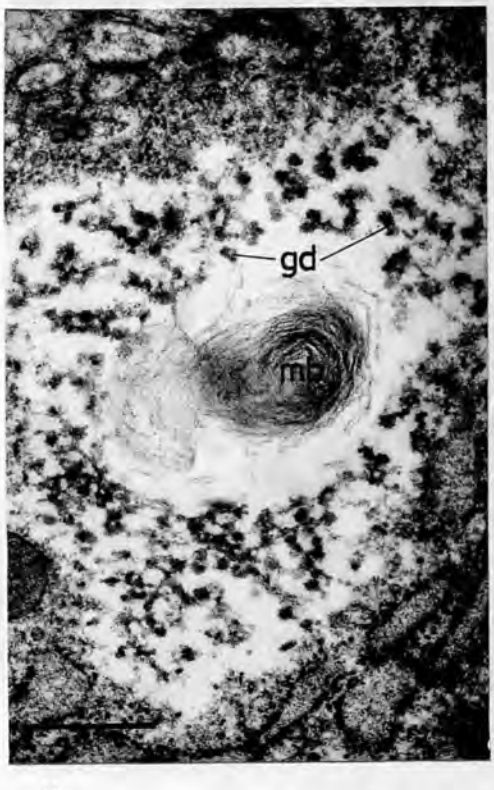
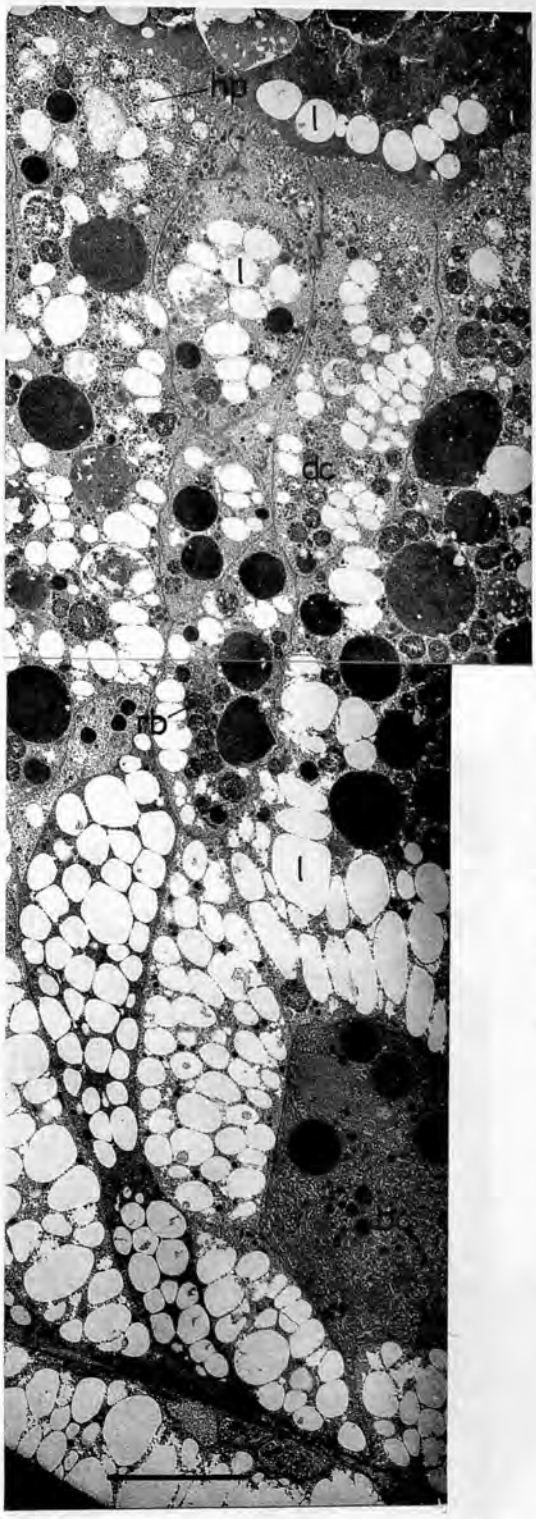
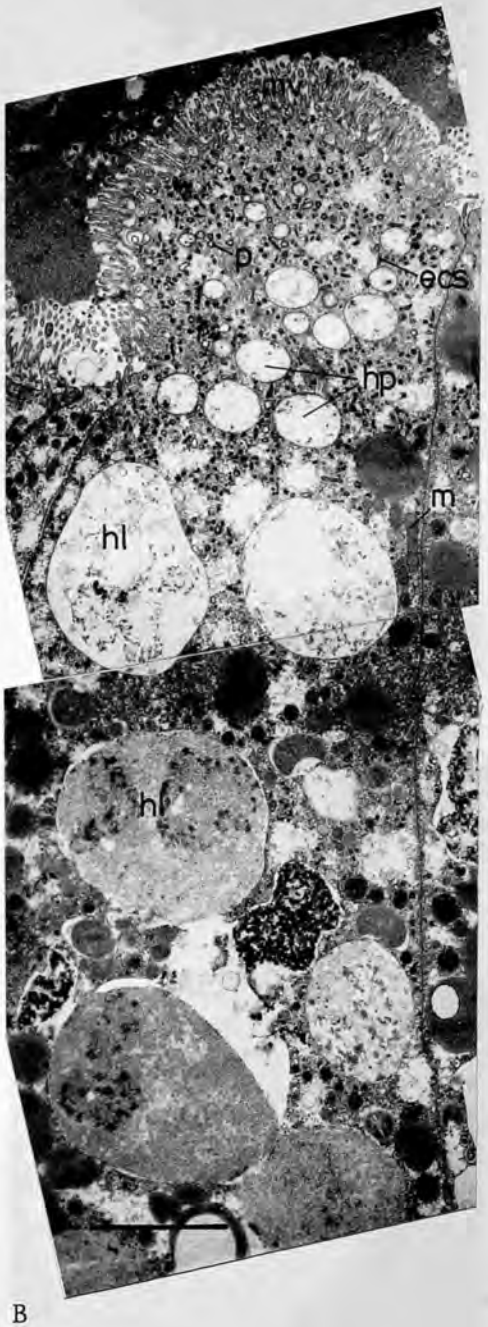


D

PLATE 32

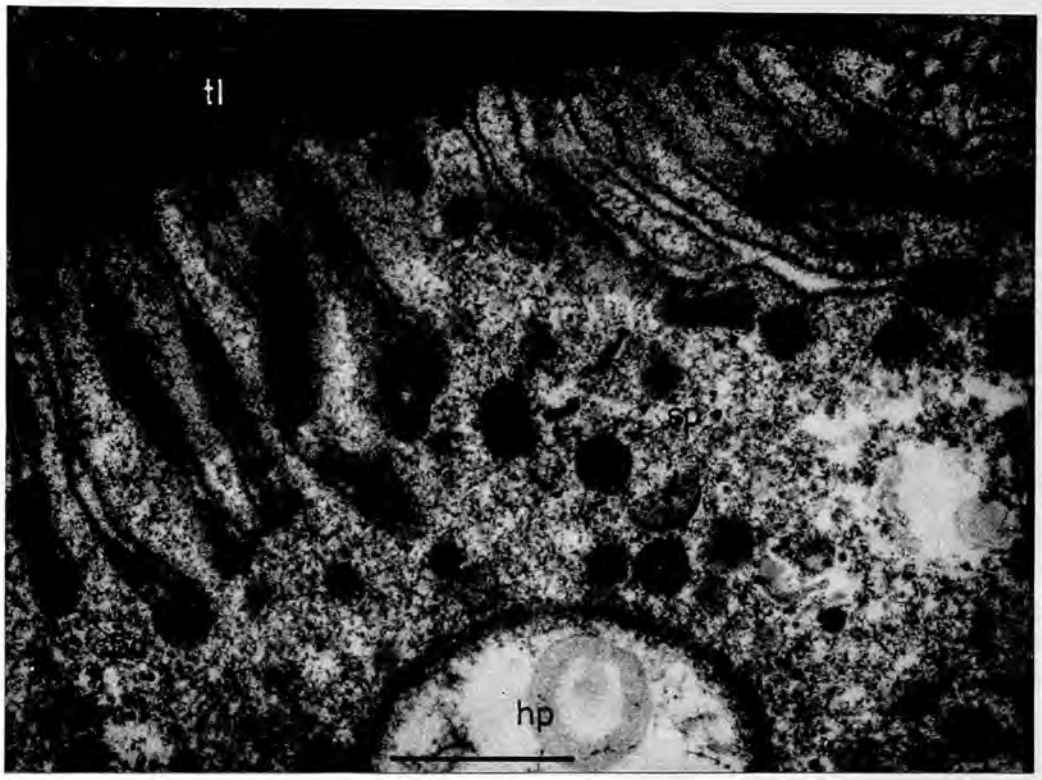


A

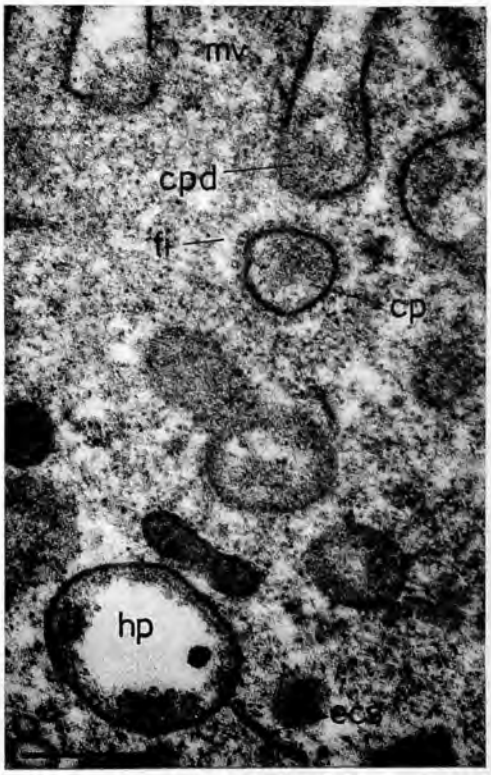


B

PLATE 33



A



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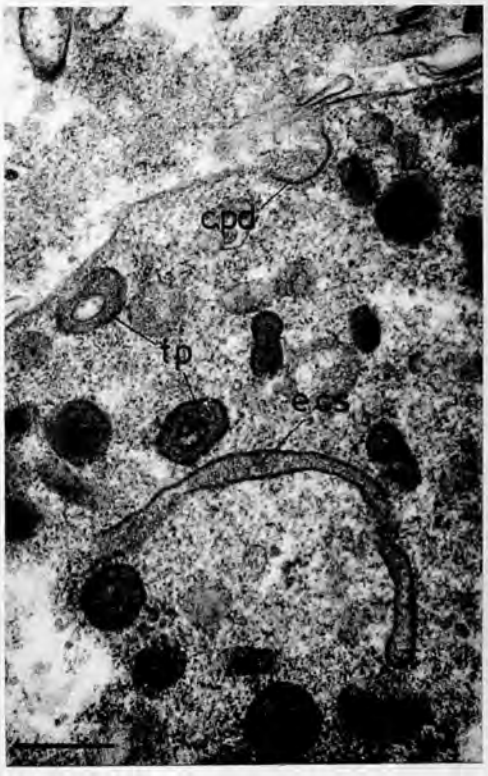

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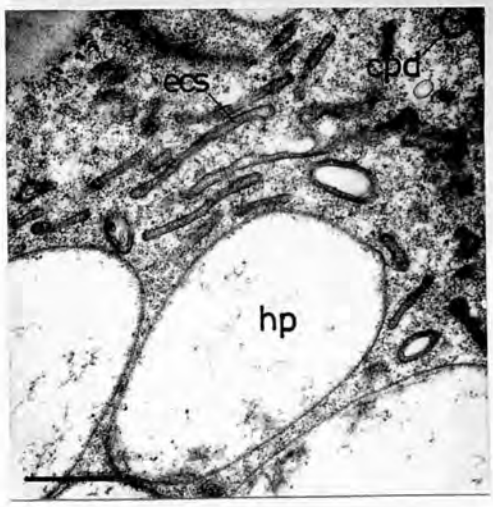
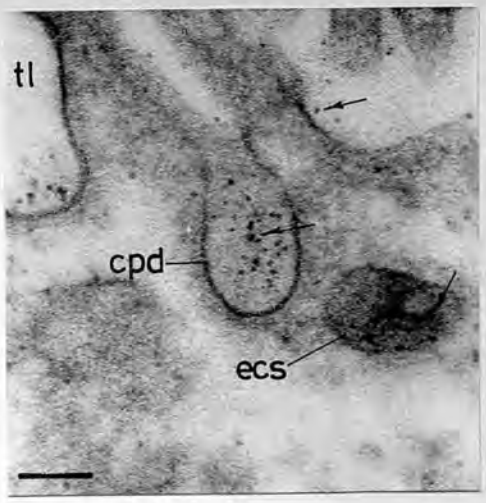
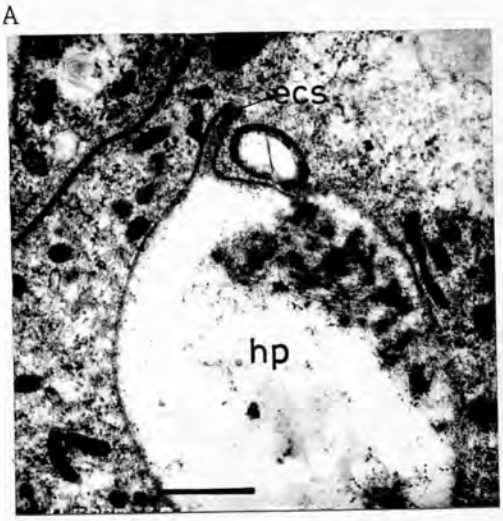
PLATE 34



A



B



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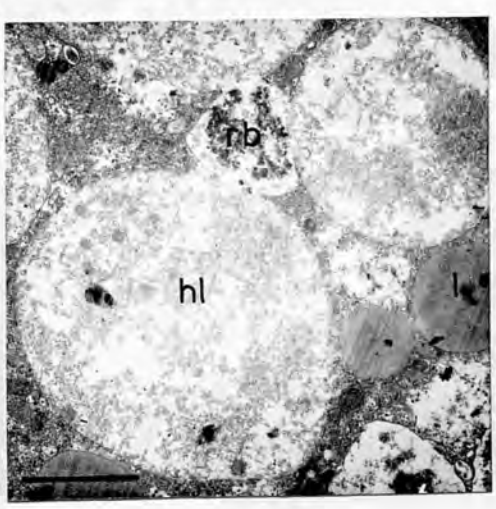
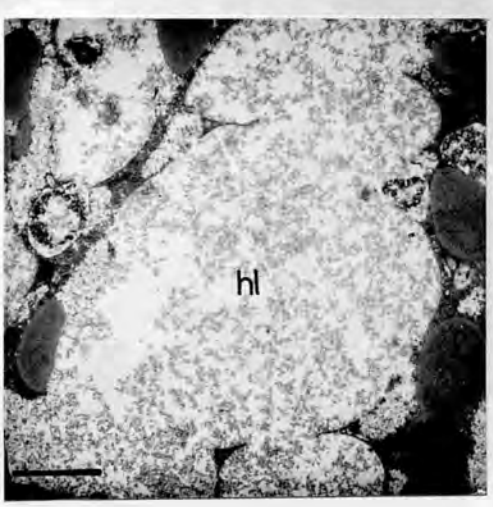
D

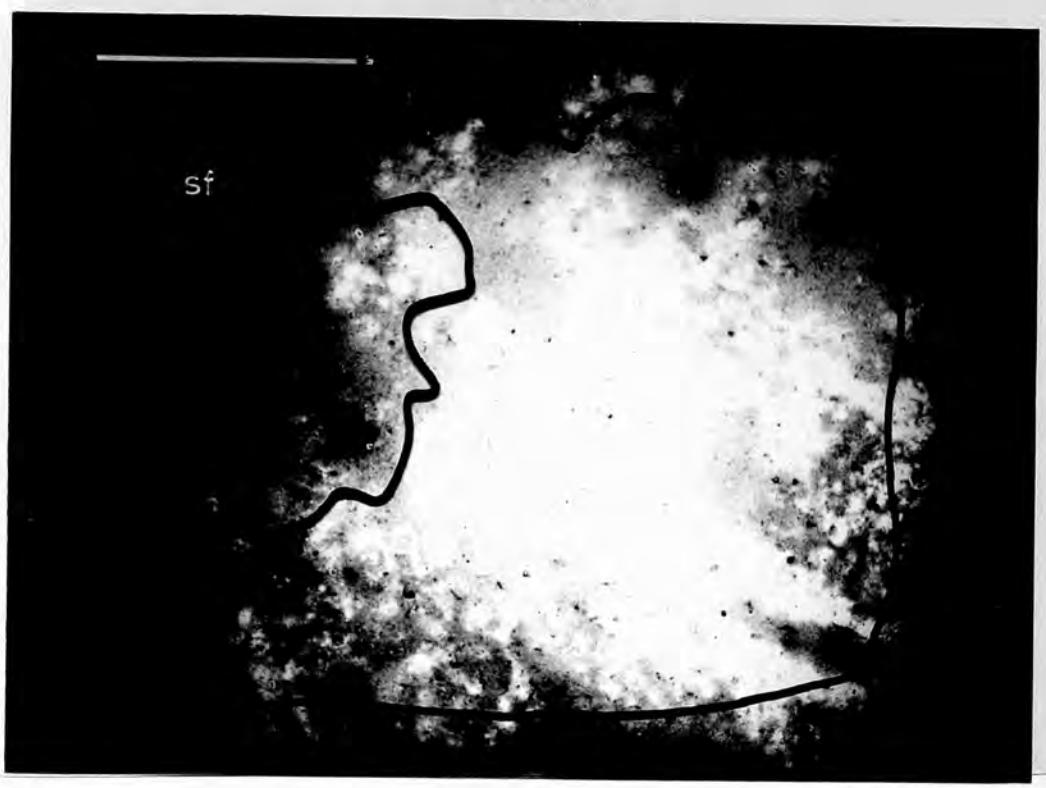


E

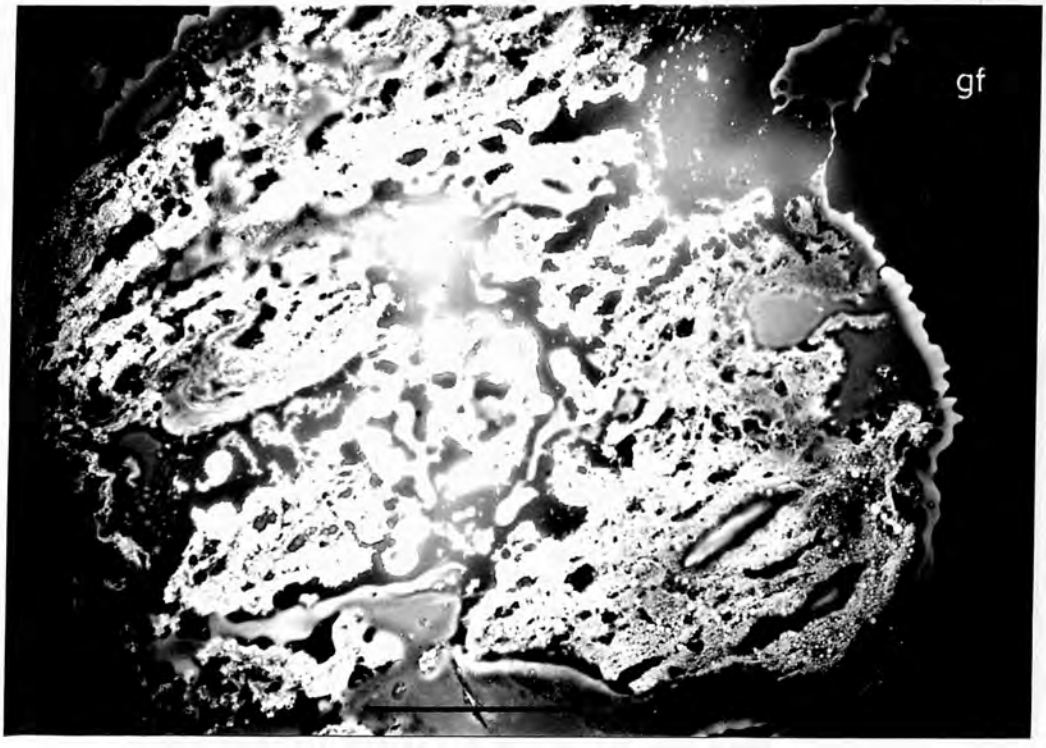


F

PLATE 35



A



B

THE INTESTINE.

The long intestine coils through and around the visceral mass in six loops measuring eight times the length of the shell (figs 2 & 6). The external appearance is that of a smooth-walled coduit connecting the style sac to the rectum. There are no ciliated cells attached to the outer surface facing the blood space. The terminology, adopted by Graham (1932), to differentiate the five distinct sections (A, B, C, D & E) of the intestine is retained. Two major divisions of the intestine can be recognised on histological grounds; an anterior portion (sections A & B) and a posterior one (C, D & E). The former is devoid of specialised gland cells, but the latter possesses basal and clavate gland cells. There is a trend towards a neutral pH along the intestine, from the anterior to the posterior portion (table 3).

1-SECTION A.

Curving anteriorly and to the right from the style sac, this section passes over the oesophagus and then loops ventrally to it, opening into section B. The two typhlosoles continue with the transverse and longitudinal folds out of the style sac into section A, which is similar to it in all respects except for the absence of the fold F3 and the secretory tracts adjacent to each typhlosole.

Histologically, the epithelium of this section resembles that of the style sac and section B (table 4, plate 36B). The outer secretory faces of the typhlosoles shed blebs which are added to the faecal rod to increase its bulk as it rotates along the intestine. Mitochondria are abundant in the basal regions of the columnar cells and the brush border gives a positive reaction for acid phosphatase with the naphthol AS- BI phosphate method.

2-SECTION B.

This length of the intestine loops to the left, then curves to the right and turns up to join the dorsal loop of the following section. All of the folds in the preceding section persist into the ventral

loop of B, then where it bends both typhlosoles terminate at the base of a transverse fold; the other folds continue unchanged past this point (plate 37A). The minor typhlosole (T2) merges into the base of the fold as the intestinal groove, still bordered by the major one (T1), runs into the groove between two adjacent transverse folds. The ciliated epithelium of T1 merges with that of the transverse fold now bordering the intestinal groove (if it can still be termed this).

The outer secretory face of T1 diverges away from the ciliated inner one and continues as a fold, F4, at the base of the series of transverse folds, separated from them by a secretory gutter lined by unciliated cells (plate 37A). Along the latter part of section B, F4 and the gutter disappear together with the longitudinal folds, so that only the transverse folds continue into the dorsal loop of section C.

The ciliated surface of this section is covered by a layer of secretion similar to that in the digestive gland duct, stomach, style sac and section A (plate 37A). Graham (1932) reported that anterior to the start of section D "the gut is full of particles, frequently white in colour"; these are clumps of free blebs circulating the lumen, destined for addition to the faecal rod. The narrower diameter (0.5mm) and muscularity of this section facilitates compaction of the faecal rod.

Graphite suspensions injected into sections A and B were traced by opening the intestine a few minutes later. Some of the particles are captured by the currents in the intestinal groove and are transferred to the transverse groove confluent with it. These are carried along the groove in a clockwise direction (as viewed from the stomach) towards the longitudinal folds and are added to other particles already embedded in the faecal rod.

The epithelium is similar to that of the previous section, the cells being slightly shorter but in other respects resembling those of the stomach and style sac (plate 37B).

3-SECTION C.

This forms two loops of the intestine, the first continuing from section B is dorsal and the other, distal loop, is ventral. It is similar in diameter to section B so faecal compaction continues along this length. The transverse folds of section B pass into the dorsal loop of C replacing the longitudinal folds on the convex wall so that they occupy the entire circumference of the intestinal wall. At the point in the dorsal loop where it bends back on itself, a few longitudinal folds reappear in their usual topographical position and continue with the interrupted transverse folds, into the ventral loop, where both diminish as they enter section D.

Both typhlosoles reappear in the ventral loop and continue uninterrupted to the rectum. T1 and T2 cannot be differentiated since they are similar in size and histologically different from those in the style sac and anterior intestine. The unciliated secretory cells forming their outer faces have been replaced with typical ciliated cells, so that the luminal surface is fully ciliated (plate 38A).

Graphite particles pipetted onto the luminal surface of section C are carried around the circumference on the transverse folds and also towards the next section by the longitudinal folds, the resultant being a spiral path. Particles injected into the previous section become embedded into the faecal rod by the time it reaches section C. Ciliary currents on the typhlosoles are directed into and along the intestinal groove and those particles adjacent to them are swept towards them and then spun away into the lumen. All these currents persist until the typhlosoles end near the rectum.

The diagnostic features of the section are the disappearance of the unciliated typhlosole epithelium and the appearance of a new cell type, the basal gland cell (Graham,1932).

A). THE BASAL GLAND CELL.

i). ULTRASTRUCTURE.

These small curious cells, restricted to the posterior intestine are located in a basal position between the ciliated columnar cells (plates 38B & 39A; fig 14). Their connection with the basal lamina is a tenuous one. Very frequently the cells rest directly upon one of the nerve fibres lying above the basal lamina or appear to "float" in the basal regions of the epithelium. The plasma membrane of the basal gland cell and the neurilemma are closely applied, separated only by a gap of 10-12nm. Small lucent (40-50nm), and granular (75-100nm) vesicles in the nerves were not seen with normal TEM preparative techniques, to fuse with the neurilemma and release their contents into the intercellular space (plate 40A). Gap-junctions are absent.

Following incubation in the TARI medium however, small areas of the intercellular space between adjacent nerves and also between nerves and gland cells, were shown to contain a material similar in appearance and staining properties to that in the granular neurovesicles. Sometimes the shape and size of the vesicle was retained after release (plate 40B). Earlier stages of the release of this material was not observed and as it is fixed by the tannic acid before any possible interaction could occur with the membrane of the gland cell, its subsequent effects cannot be determined. It seems likely, however, that these cells are controlled by a neurosecretion (=neuropeptide).

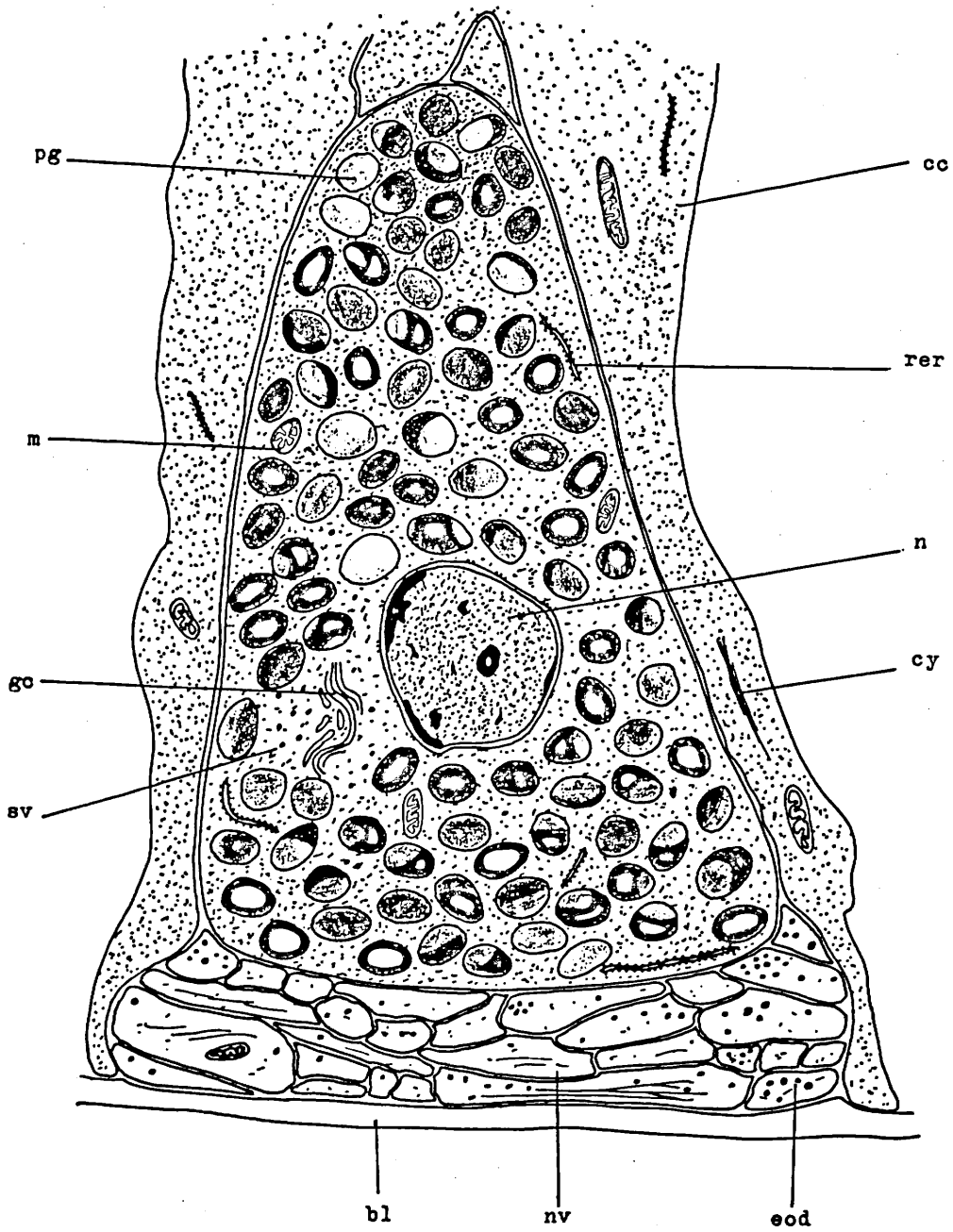
When the cut edge of the posterior intestinal epithelium was examined with the SEM, the basal gland cells were seen to be restricted to the basal half. Their apical surface is blunt, rounded and devoid of microvilli (plate 39B). The luminal surface of the intestine has a continuous covering of cilia, disrupted only in section D by the openings of the clavate gland cells.

Basal gland cells are variable in size and shape, ranging from tall, oval to short, conical, although in sections they never extend into the apical half of the epithelium. Graham's (1932) description of

ABBREVIATIONS TO FIGURES 14 AND 15.

bl	BASAL LAMINA.
c	CILIUM.
cc	CILIATED CELL.
cd	CONDENSING VACUOLE.
cy	CYTOSKELETON.
eod	ELECTRON-OPAQUE DROPLET.
gc	GOLGI COMPLEX.
m	MITOCHONDRION.
mv	MICROVILLI.
n	NUCLEUS.
ncc	NUCLEUS OF CILIATED CELL.
ncg	NUCLEUS OF CLAVATE GLAND CELL.
nv	NERVE.
pg	PROTEINACEOUS GRANULE.
rb	RESIDUAL BODY.
rer	RER.
sd	SECRETORY DROPLET.
sv	SMALL VESICLE.

FIGURE 14. A DIAGRAMMATIC REPRESENTATION OF AN INTESTINAL BASAL GLAND CELL.



fine ducts could not be substantiated. The cells could extend necks between the ciliated cells to reach the lumen, but the intercellular spaces above the cells are not enlarged, suggesting that ducts do not exist. There are no junctional complexes.

Characteristically, the cells are packed with membrane-bound granules (400-600nm) which stain distinctively with all the cytochemical stains used; some regions of individual granules stain more strongly (plates 39A, 40D, 41A & B). An irregularly shaped nucleus (2-3μm in diameter) lies in the mid-region of the coarsely granular cytoplasm; large clumps of heterochromatin occur against the inner membrane of the nuclear envelope whilst the outer one is studded with ribosomes (plate 39A). The nucleus is usually obscured by the granules in wax sections.

A small golgi complex consisting of 4-5 concave or flat cisternae lying close to the nucleus, is only rarely encountered and always has a few transitional ER elements associated with its cis face (plate 40C). The trans face usually stains more densely than the others suggesting accumulation of material, but condensing vacuoles or swellings of the margins do not occur. Small (40nm) vesicles are present in the golgi area, but their origin is unknown. Mitochondria and RER are rare, the latter normally being displaced to the peripheries of the cell.

ii). HISTOCHEMISTRY.

A strong basophilic reaction is obtained with routine histological stains, the granules stain red with Mallory and blue after toluidine blue. The histochemical results are summarised in table 15.

CARBOHYDRATES. Specific tests for carbohydrates failed to stain the granules, which react non-specifically with all the SM techniques (plates 40D & 41A), but specifically with the PTA method (plate 41B) and not at all with the PA-TSC-SP method. After treatment with SM the granules and nuclei are intensely stained, so the cells are

TABLE 15.

SUMMARISED HISTOCHEMICAL RESULTS OF THE BASAL GLAND CELL.

HISTOCHEMICAL PROCEDURES.			RESULTS	CONCLUSIONS
CARBOHYDRATES	SCHIFF		–	NO ALDEHYDE GROUPS.
	P.A.S.		–	NO 1,2-GLYCOL GROUPS.
	AB pH2.5		–	NO ACID MUCOPOLYSACCHARIDES.
	LOW pH PTA		$+^{W}$	INDICATES A POLYSACCHARIDE OR GLYCOPROTEIN CONTENT.
	PA-SM		$+^{NS}$	SUGGESTS PRESENCE OF REDUCING GROUPS OTHER THAN ALDEHYDES.
	PA-TSC-SP		–	NO 1,2-GLYCOL GROUPS.
PROTEINS	BROMOPHENOL BLUE		+	BASIC PROTEINS PRESENT.
	DE-NH_2/BROMO-PHENOL BLUE		$+^{W}$	MOIETIES OTHER THAN NH_2 REACTING.
	DNFB		+	NH_2/SH/TYROSINE PRESENT.
	DE-NH_2/DNFB		+	SH/TYROSINE REACTING.
	DIAZOTIZ'TN-COUPLING		$+^{W}$	TYROSINE PRESENT.
	IODITIZATION/ DIAZ-COUPLING		–	TYROSINE BLOCKED.
	DDD		–	NO SH GROUPS.
	THIOGLYCOLATE /DDD		–	NO SS GROUPS.
	DMAB-NITRITE		–	NO TRYPTOPHAN.
	SUDAN BLACK B		–	NO LIPIDS.
PIGMENTS	SCHMORL		–	NO MELANIN/LIPOFUCSIN.
	LONG ZIEHL-NEELSEN		–	NO LIPOFUCSIN.
	H_2O_2-NILE BLUE SULPHATE		–	NO MELANIN.
ENZYMES	NAPHTHOL AS-BI PHOSPHATE		–	NO ACID PHOSPHATASES.
	GOMORI MEDIUM pH4.9		+	ACID PHOSPHATASE LOCATED IN THE GOLGI COMPLEX.
	HUGON-BORGERS MEDIUM pH9.0		–	NO ALKALINE PHOSPHATASES.

NOTATION:

AS FOR TABLES 5 AND 6. NS.....Nonspecific reaction.

immediately obvious in the epithelium, even at low magnifications (plate 40D). Blockage of aldehydes reduces the reaction intensity (plate 41A), indicating the presence of reducing groups other than aldehydes.

PROTEINS. The granules react strongly with bromophenol blue and the DNFB test, deamination reduces the former result but not the latter. Tyrosine is demonstrated by the weak diazotization-coupling result.

ENZYMES. The golgi complex gives a positive reaction for acid phosphatase with the Gomori incubation.

4-SECTION D.

This section originates on the anterior right margin of the visceral mass then sweeps over the oesophagus, coils peripherally, adjacent and ventral to the style sac and section A. It is histologically distinct from the previous section due to the presence there of a new type of cell, called the clavate gland cell by Graham (1932) (plates 41C & 43A). The ciliated epithelium is slightly taller than that of the former three sections and the cilia are longer (table 4), but is otherwise similar.

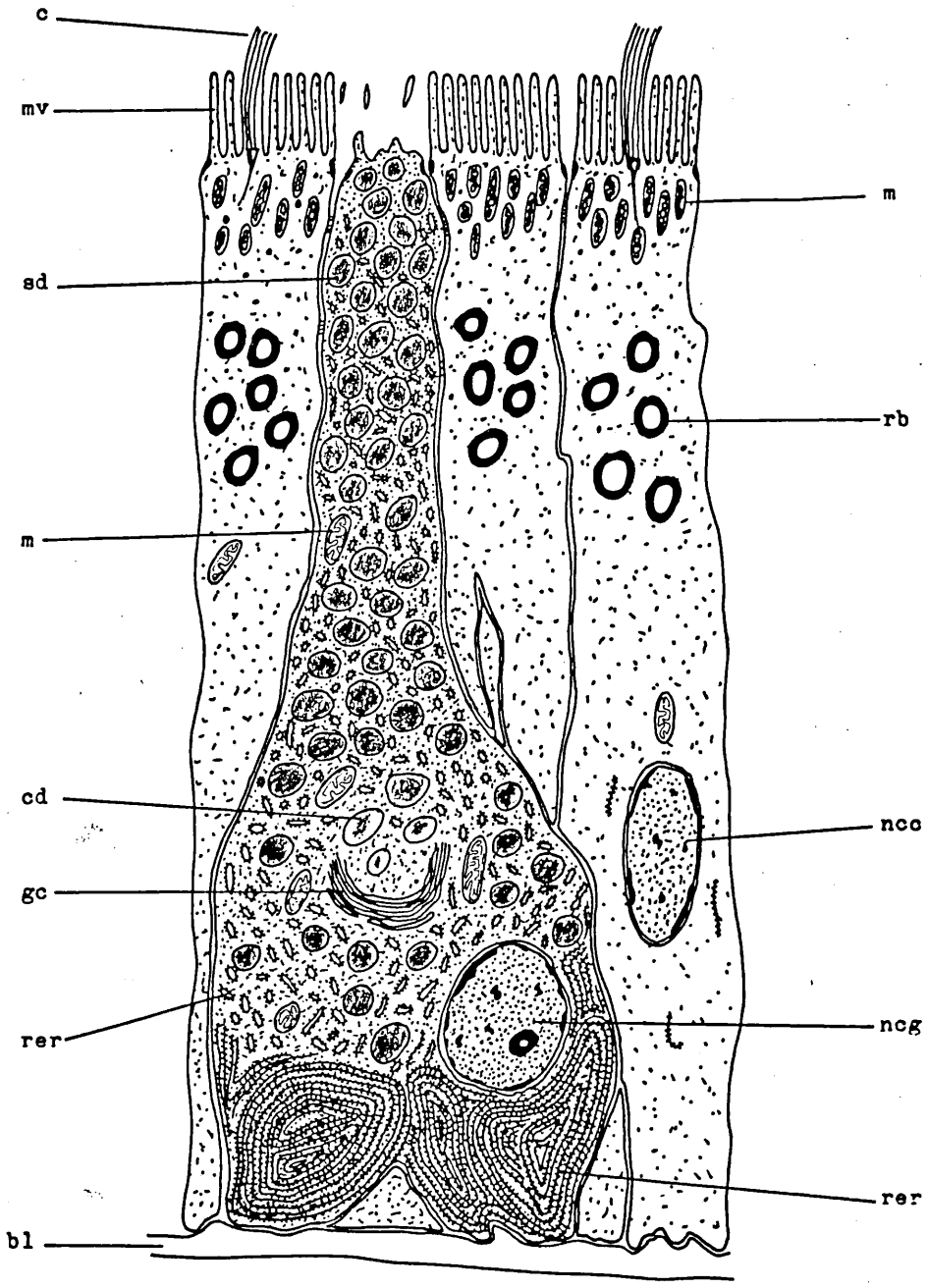
A). THE CLAVATE GLAND CELL.

i). ULTRASTRUCTURE.

These are club-shaped cells resting upon the basal lamina and showing no close association with the nerves (plate 42); their wide necks open to the intestinal lumen (plate 43A). They are joined to adjacent cells by typical junctional complexes. A characteristic feature of these cells is a moderately opaque cytoplasm containing secretory vesicles of similar opacity and abundant RER (fig 15).

Curvilinear whorls of RER are present in the nuclear regions of cells (plate 43B), whilst a tubular form is common elsewhere (plate 43C). Both varieties contain a flocculant, thread-like material that is attached to the inner face of the cisternal membrane. The homogeneous, membrane-bound secretory droplets are somewhat oval in

FIGURE 15. A DIAGRAMMATIC REPRESENTATION OF AN INTESTINAL CLAVATE GLAND CELL.



shape and between 0.7-2.0μm in diameter; the finely granular matrix (plate 43C) is only moderately electron-opaque, so the vesicles are inconspicuous at low magnifications (plates 41C & 42).

A large (3-4μm), spherical nucleus containing a moderate amount of heterochromatin and sometimes a nucleolus (1-2μm), lies in the basal portions of the cell. Mitochondria are numerous especially in the mid-region where the golgi is usually found (plate 44A). It consists of 6-7 cisternae stacked in a cup-shaped profile with elements of TER associated with the cis face and various vacuoles in the area enclosed by the cisternae (plate 44B). The larger condensing vacuoles (300-400nm) contain, within an electron-lucent peripheral halo, material similar to the matrix of the secretory droplet. As they increase in size so the halo diminishes (plate 44A).

Exocytosis of the secretory matrix was never observed in sections, but examination of the luminal surface with the SEM revealed large (4μm), wrinkled blebs. These were not present in the other regions of the posterior intestine so were considered to be the secretion of the clavate cells and not blebs of cytoplasm released from the ciliated cells, which they do not resemble.

ii). HISTOCHEMISTRY.

The cells are strongly basophilic and the vesicles stain red with Mallory and deep blue after toluidine blue. Summarised results are presented in table 16.

CARBOHYDRATES. Carbohydrates are absent from the cell and its secretion.

PROTEINS. Routine histological staining indicated that the secretory vesicles are proteinaceous, this is confirmed by the strongly positive results with bromophenol blue and the diazotization-coupling method for tyrosine. Tryptophan was demonstrated by the DMAB-nitrite test.

ENZYMES. Incubation in the naphthol AS-BI phosphate reagents gives a positive result for acid phosphatase in the mid-region of the cell

TABLE 16.

SUMMARISED HISTOCHEMICAL RESULTS OF THE CLAVATE GLAND CELL.

HISTOCHEMICAL PROCEDURES			RESULTS	CONCLUSIONS
CARBOHYDRATES	SCHIFF		–	NO ALDEHYDE GROUPS.
	P.A.S.		–	NO 1,2-GLYCOL GROUPS.
	AB pH2.5		–	NO ACID MUCOPOLYSACCHARIDES
	LOW pH PTA		–	NO POLYSACCHARIDES OR GLYCOPROTEINS.
	PA-SM		–	NO 1,2-GLYCOL GROUPS OR OTHER REDUCING GROUPS.
	PA-TSC-SP		–	NO 1,2-GLYCOL GROUPS.
PROTEINS	BROMOPHENOL BLUE		+	BASIC PROTEINS PRESENT.
	DE-NH_2/BROMO-PHENOL BLUE		$+^W$	MOIETIES OTHER THAN NH_2 GROUPS REACTING.
	DNFB		$+^W$	NH_2/SH/TYROSINE PRESENT.
	DE-NH_2/DNFB		–	NH_2 BLOCKED.
	DIAZOTIZ'TN-COUPLING		+	TYROSINE PRESENT.
	IODITIZATION/ DIAZ-COUPLING		–	TYROSINE BLOCKED.
	DDD		$+^W$	SH GROUPS PRESENT.
	THIOGLYCOLATE /DDD		$+^W$	SIMILAR REACTION TO DDD INDICATES ABSENCE OF SS GROUPS.
	DMAB-NITRITE		+	TRYPTOPHAN PRESENT.
	PERSULPHATE/ DMAB-NITRITE		–	TRYPTOPHAN BLOCKED.
	SUDAN BLACK B		–	NO LIPIDS.
PIGMENTS	SCHMORL		–	NO MELANIN/LIPOFUCSIN.
	LONG ZIEHL-NEELSEN		–	NO LIPOFUCSIN.
	H_2O_2-NILE BLUE SULPHATE		–	NO MELANIN.
ENZYMES	NAPHTHOL AS-BI PHOSPHATE		+	ENZYME ACTIVITY RESTRICTED TO GOLGI REGION OF CELL.
	GOMORI MEDIUM pH4.9		+	ACID PHOSPHATASE ACTIVE IN CIS FACE OF GOLGI.
	HUGON-BORGERS MEDIUM pH9.0		–	NO ALKALINE PHOSPHATASE.

NOTATION:

AS FOR TABLES 5 AND 6.

where the golgi complex is located. The Gomori incubation indicates activity in the cisternae and the smaller condensing vesicles enclosed within the golgi (plates 44A & B).

5-SECTION E.

This section retraces the course of section D to a dorsal position above the oesophagus where it joins the rectum. As it approaches the rectum, both typhlosoles gradually diminish, so the intestinal groove subsequently becomes shallower until it disappears as the large rectal folds arise. It resembles section C histologically.

It was suggested by Graham (1932), that the secretions of the gland cells in the posterior intestine wrap the faecal rod "like a cigarette paper round the tobacco inside"; this is a very apt analogy as plate 45B illustrates, the coarse-textured centre is surrounded by a smooth continuous layer of secretion.

ABBREVIATIONS USED IN PLATES 36-45.

b....Cytoplasmic bleb.

bgc..Basal gland cell.

bl...Basal lamina.

bm...Basal membrane of bgc.

bs...Blood space.

c....Cilia.

cc...Ciliated columnar cells.

ce..."Cement" secretion of cgc.

cgc..Clavate gland cell.

cm...Circular muscle.

ct...Connective tissue.

cv...Condensing vacuole.

es...External surface of intestine.

F4...Large ciliated fold.

fr...Faecal rod.

g....Golgi complex.

gl...Gut lumen.

is...Intercellular space.

l....Lipid.

lf...Longitudinal fold.

lm...Longitudinal muscle.

ls...Luminal surface of intestine.

m....Mitochondrion.

ms...Muscle.

mv...Microvilli.

n....Nucleus.

nl...Neurilemma.

ns...Neurosecretory vesicle.

nv...Nerve.

rer..Rough ER.

rb....Residual body.

s.....White secretion.

sg....Secretory granule of bgc.

sgt...Secretory gutter.

sv....Secretory vesicle of cgc.

T1,2..Major & minor typhlosoles.

ter...Transitional ER.

tf....Transverse folds.

v.....Unspecified vesicle.

LEGENDS TO PLATES 36-41.

THE INTESTINE.

PLATE 36.

A. SEM, section A opened by a longitudinal dorsal incision. x58.

B. The ciliated epithelium of section A. 2.5K.

Scale bars: A=0.5mm; B=8μm.

PLATE 37.

A. SEM, section B, the region where both typhlosoles disappear. x143.

B. The ciliated epithelium of section B. 2.5K.

Scale bars: A=140μm; B=8μm.

PLATE 38.

A. SEM, the ciliated typhlosoles of the posterior intestine. x300.

B. The ciliated epithelium of section C. 3K.

Scale bars: A=66μm; B=10μm.

PLATE 39 Basal gland cell.

A. Typical cell resting on a nerve. 8.8K

B. SEM, cut edge of the epithelium lining section C. 3K.

Scale bars: A=2μm; B=5μm.

PLATE 40 Basal gland cell.

A. Junction between nerve and gland cell. 84K.

B. As A, but after incubation in TARI medium, arrows indicate exocytosed neurosecretory material. 153K.

C. Golgi complex of cell. 144K.

D. Cell stained by the PA-CrA-SM method. 11K.

Scale bars: A=250nm; B,C=100nm; D=2μm.

PLATE 41 Basal gland cell.

A. Cell stained by the PA-SB-SM method. 8K.

B. Cell stained by the PTA method. 8K.

C. The ciliated epithelium of section D. 3K.

Scale bars: A,B=2μm; C=10μm.

LEGENDS TO PLATES 42-45.

THE INTESTINE.

PLATE 42.

A montage of a typical clavate gland cell. 7K.

Scale bar=3μm.

PLATE 43 Clavate gland cell.

A. SEM, cut edge of the epithelium lining section D. 3K.

B. Curvilinear whorls of RER. 22K.

C. Tubular form of RER. 91K.

Scale bars: A=7μm; B=1μm; C=250nm.

PLATE 44 Clavate gland cell.

A. Acid phosphatase localised in the golgi complex. 11K.

B. Enlargement of plate 44A. 35K.

C. Blebs of secretion at the luminal surface of section D. 7.5K

Scale bars: A,C=2μm; B=500nm.

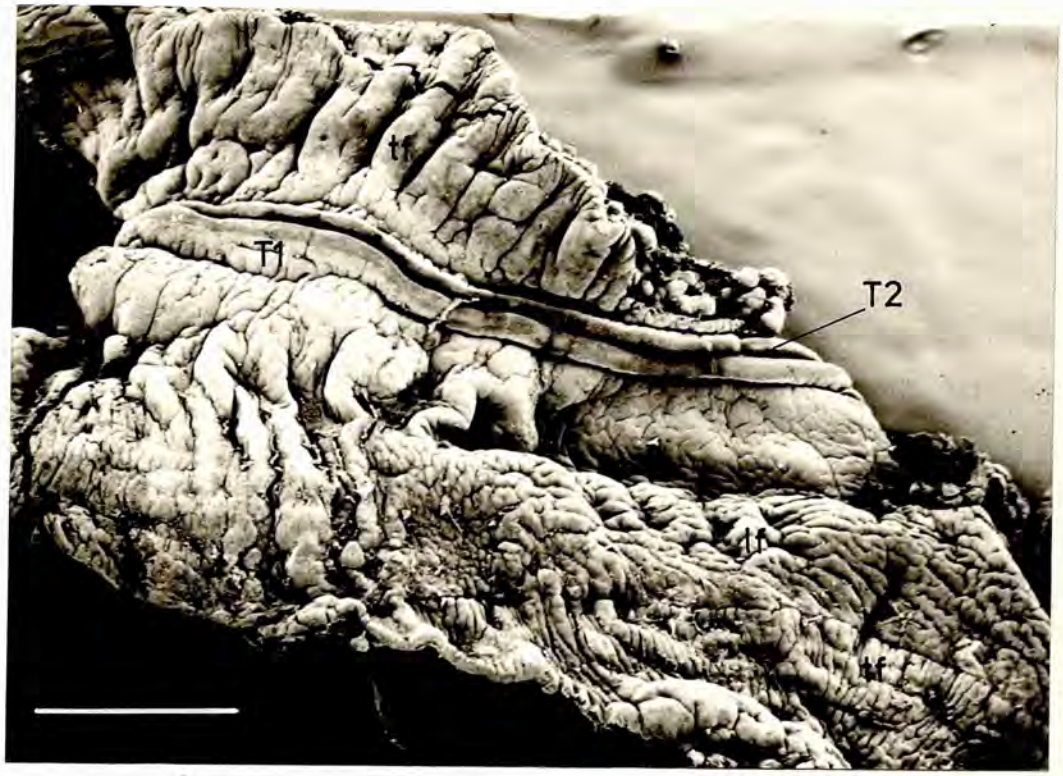
PLATE 45.

A. The ciliated epithelium of section E. 3K.

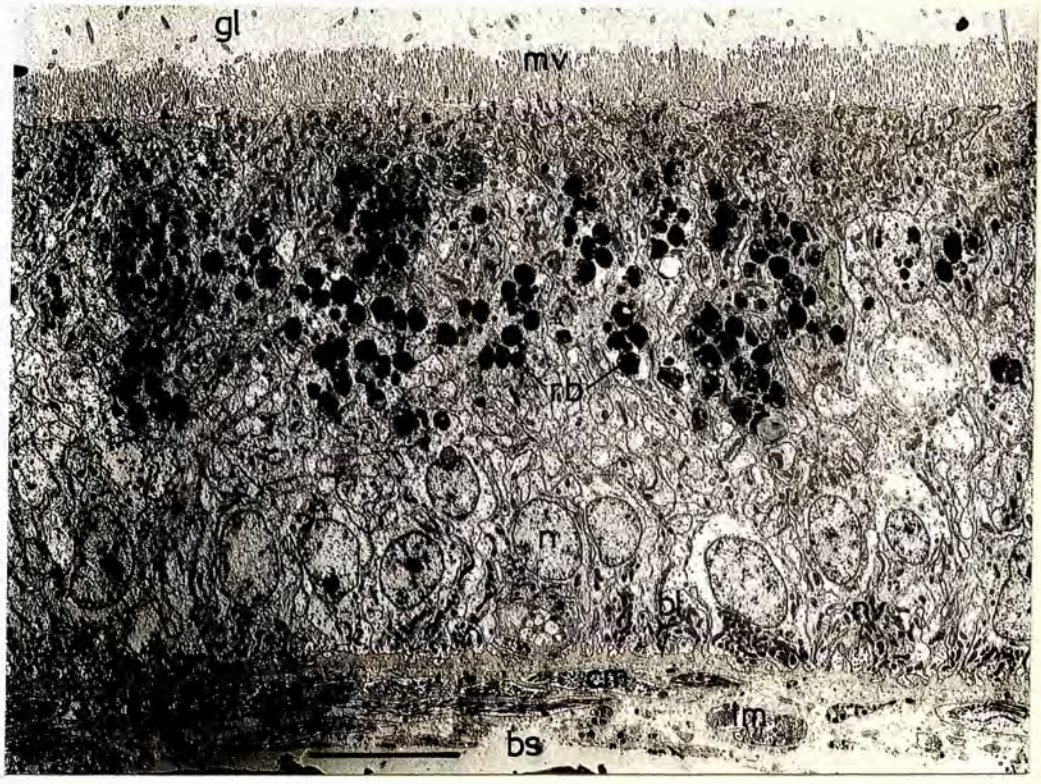
B. SEM, the cut end of section E. x161.

Scale bars: A=7μm; B=125μm.

PLATE 36



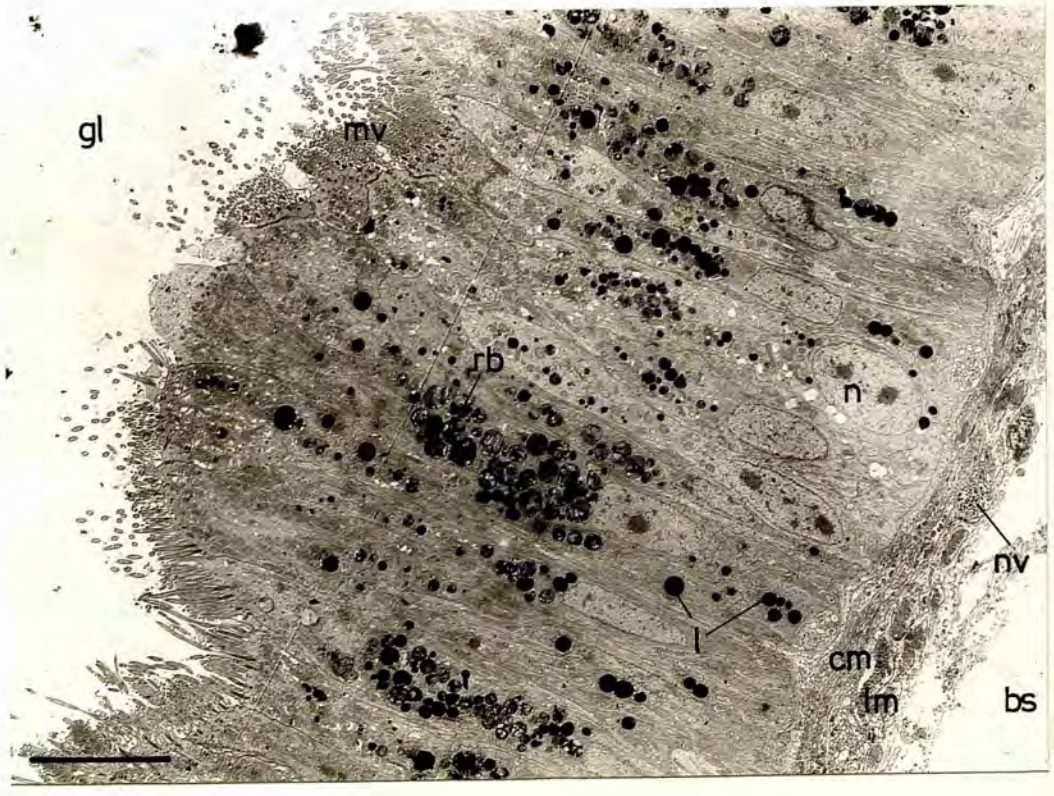
A



B

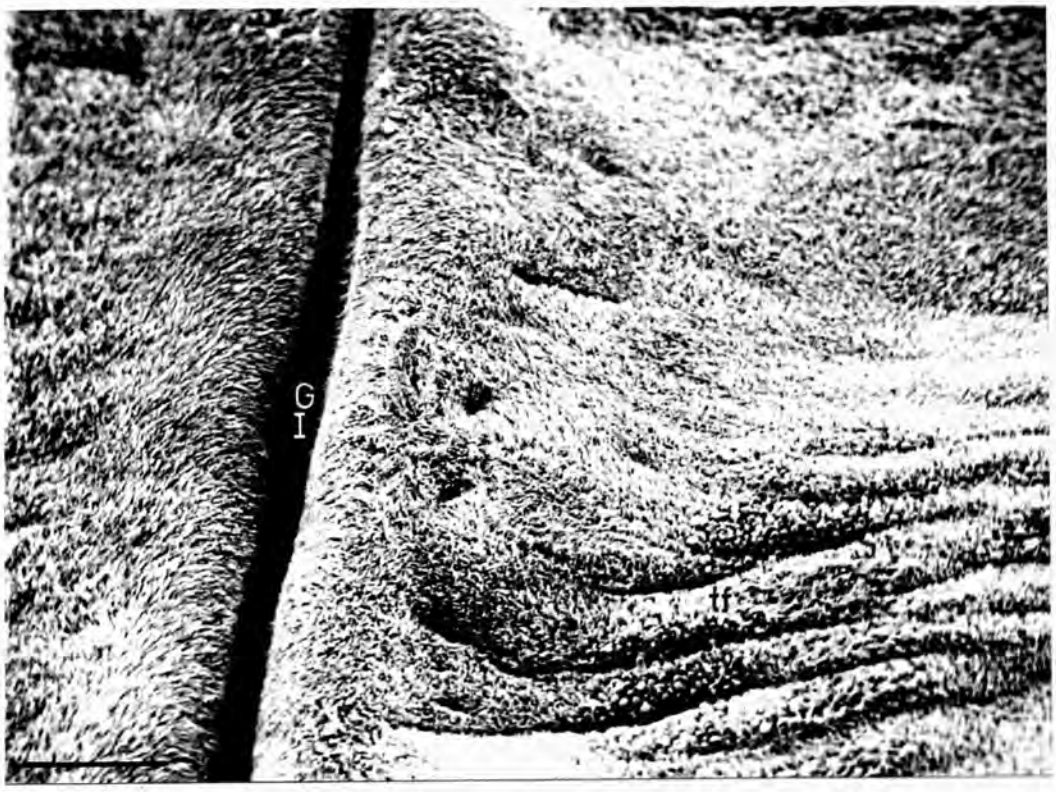
PLATE 37

A



B

PLATE 38



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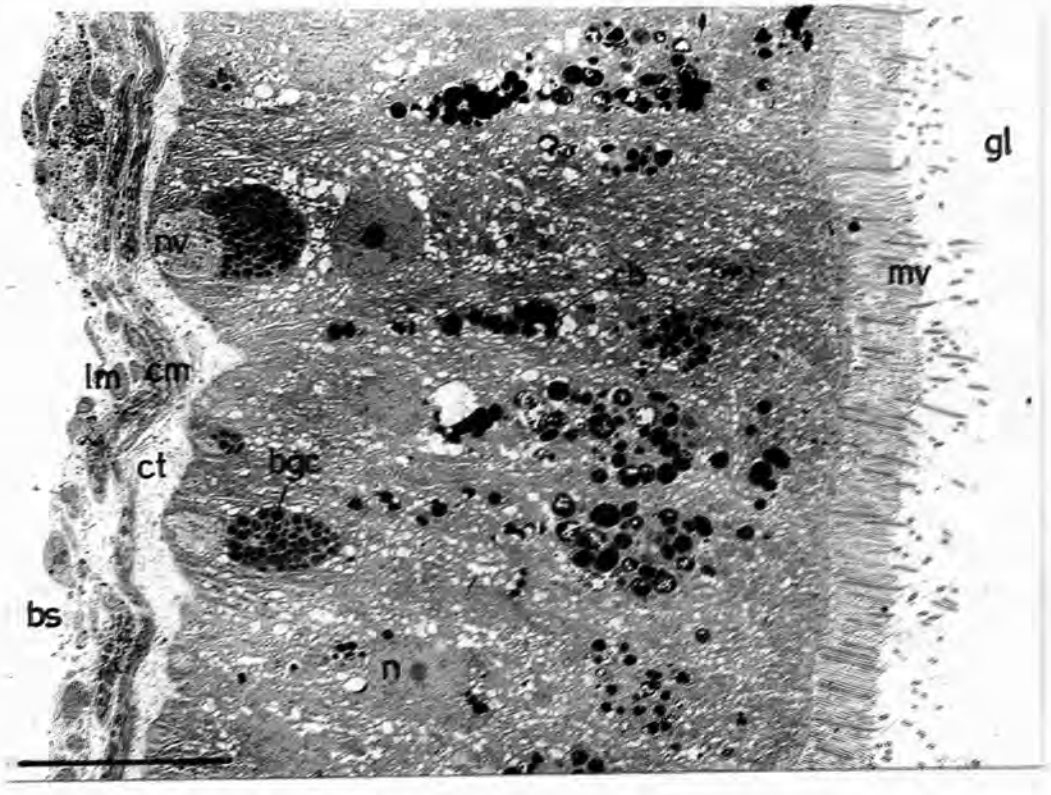
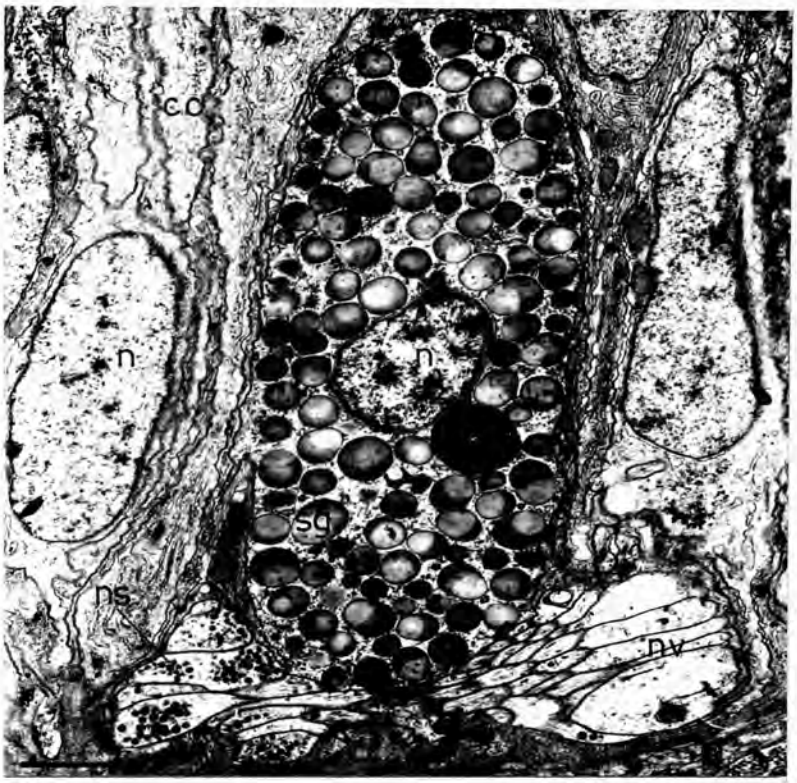


B

PLATE 39



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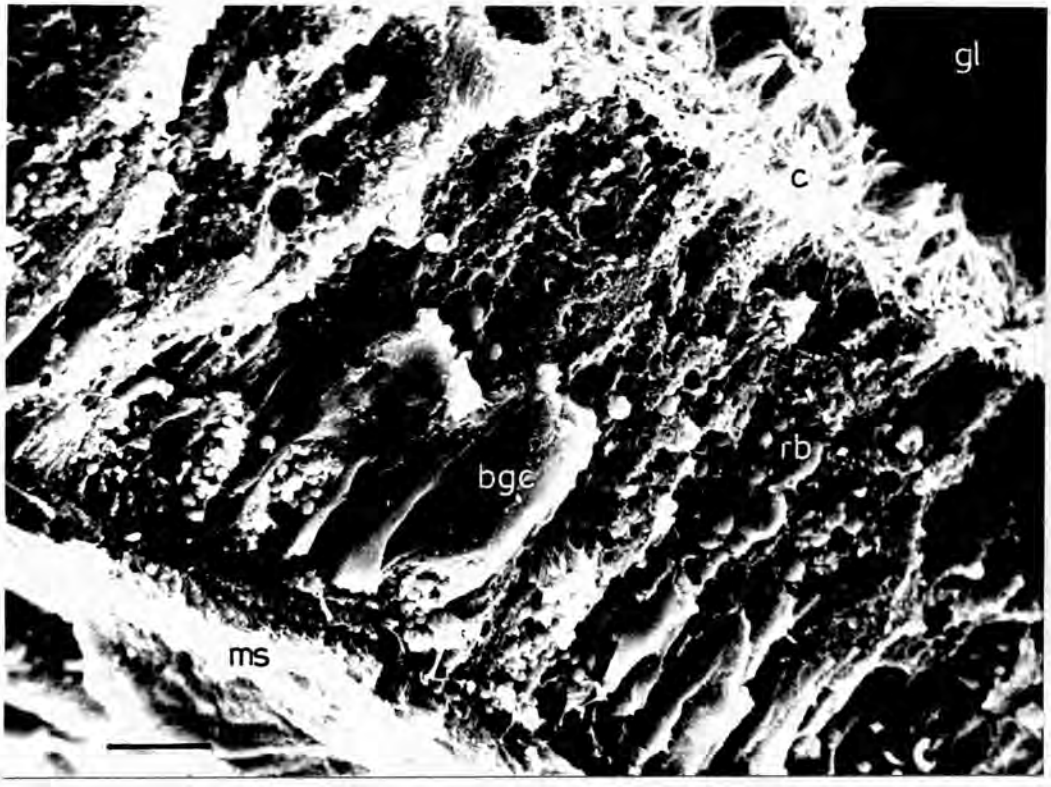
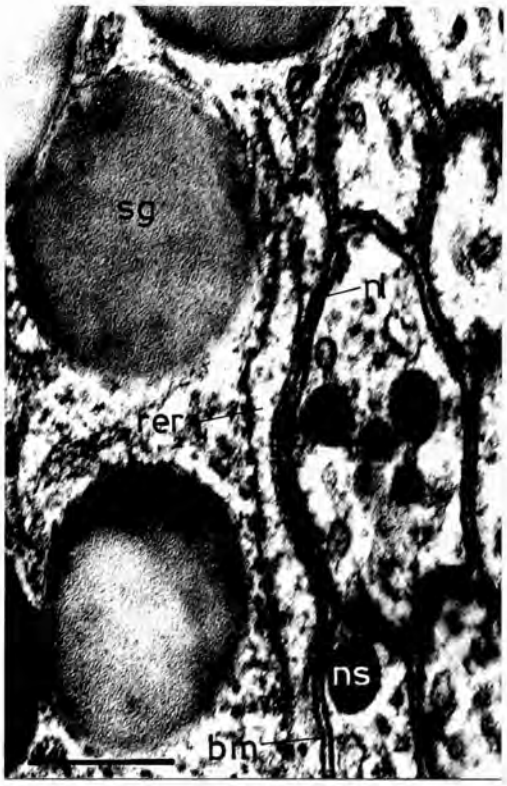


B

PLATE 40



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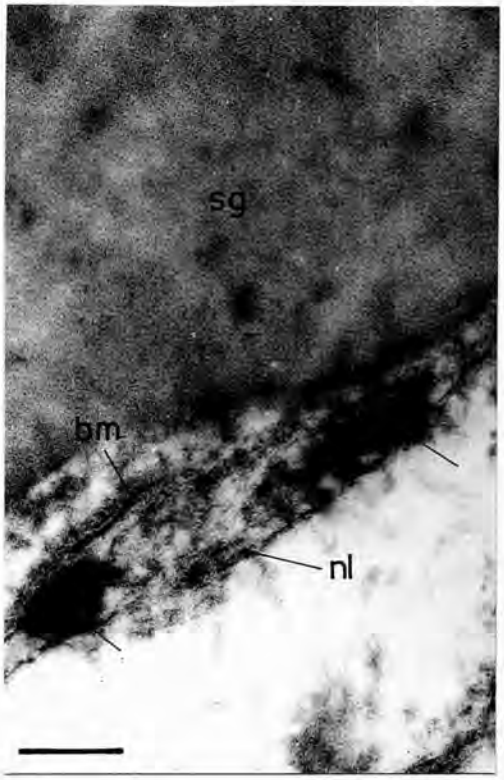

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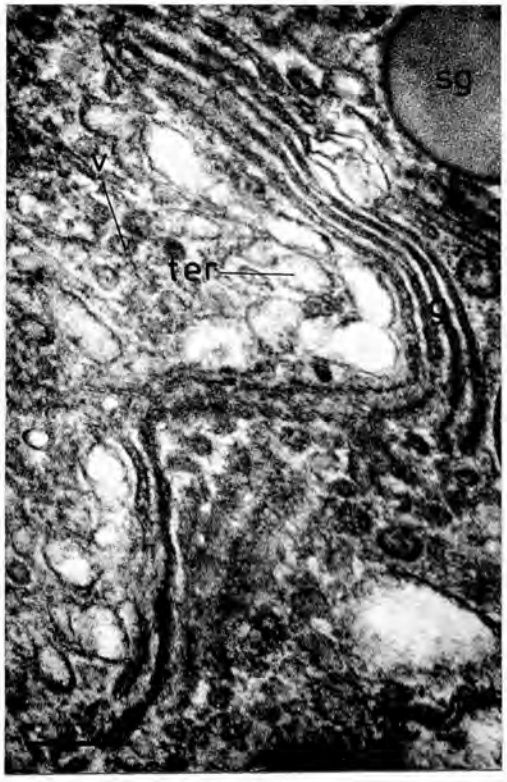
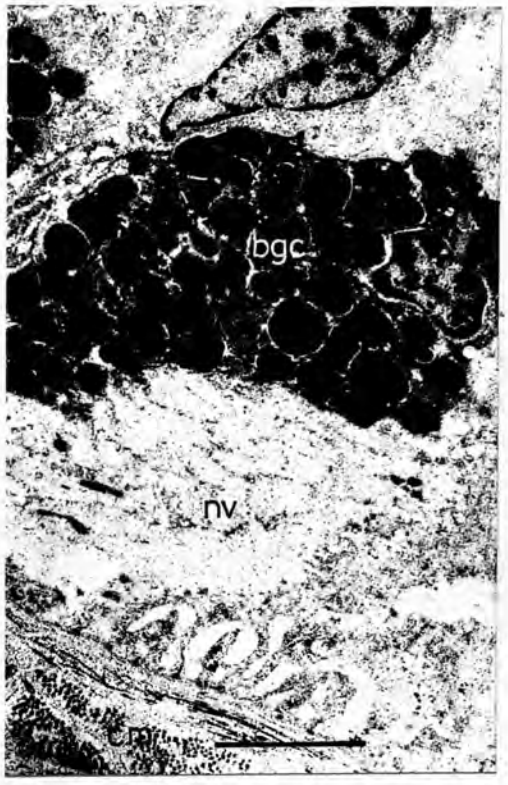
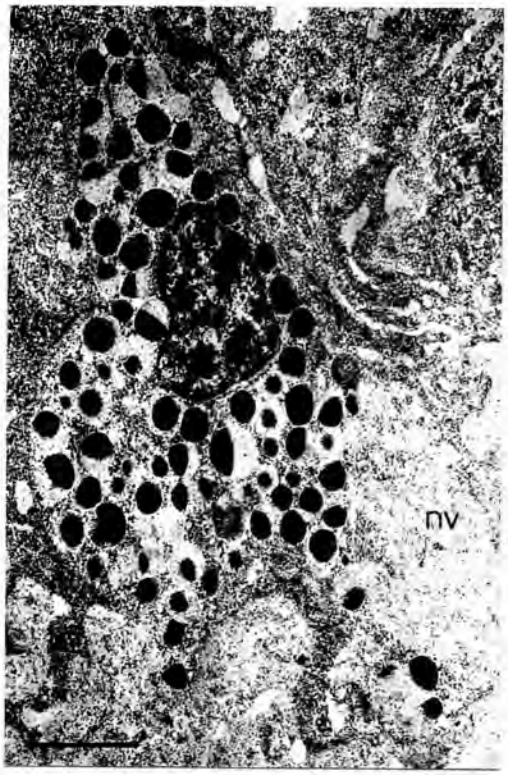


C

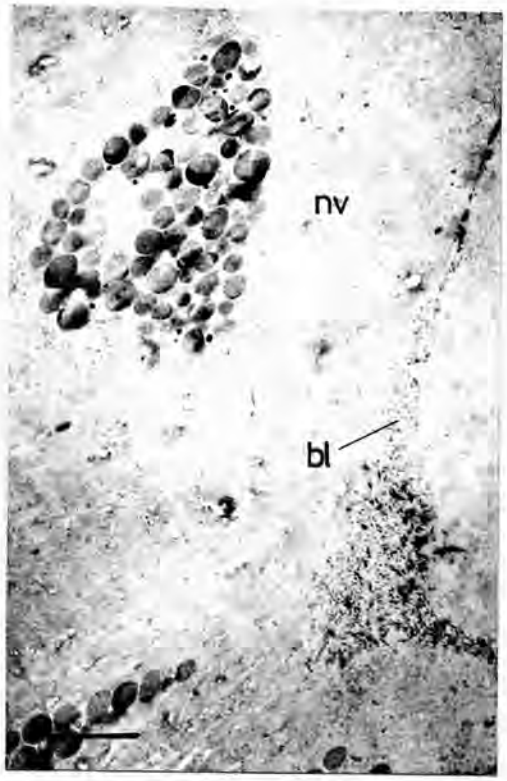


D

PLATE 41



A



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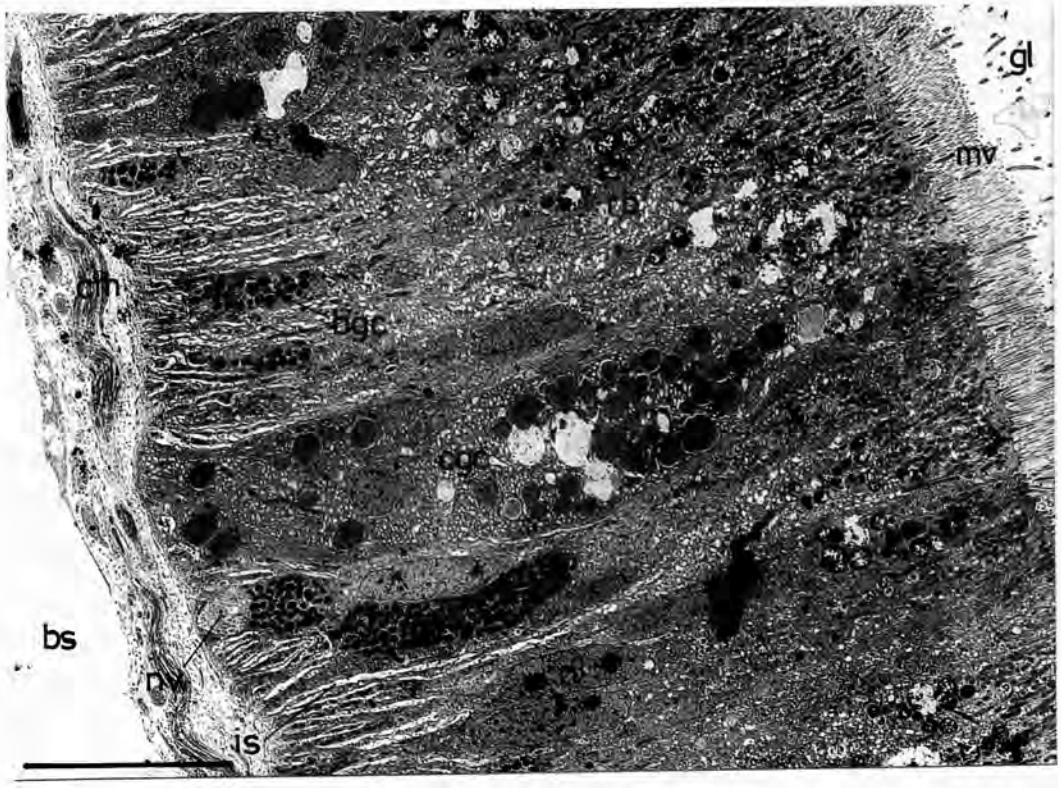
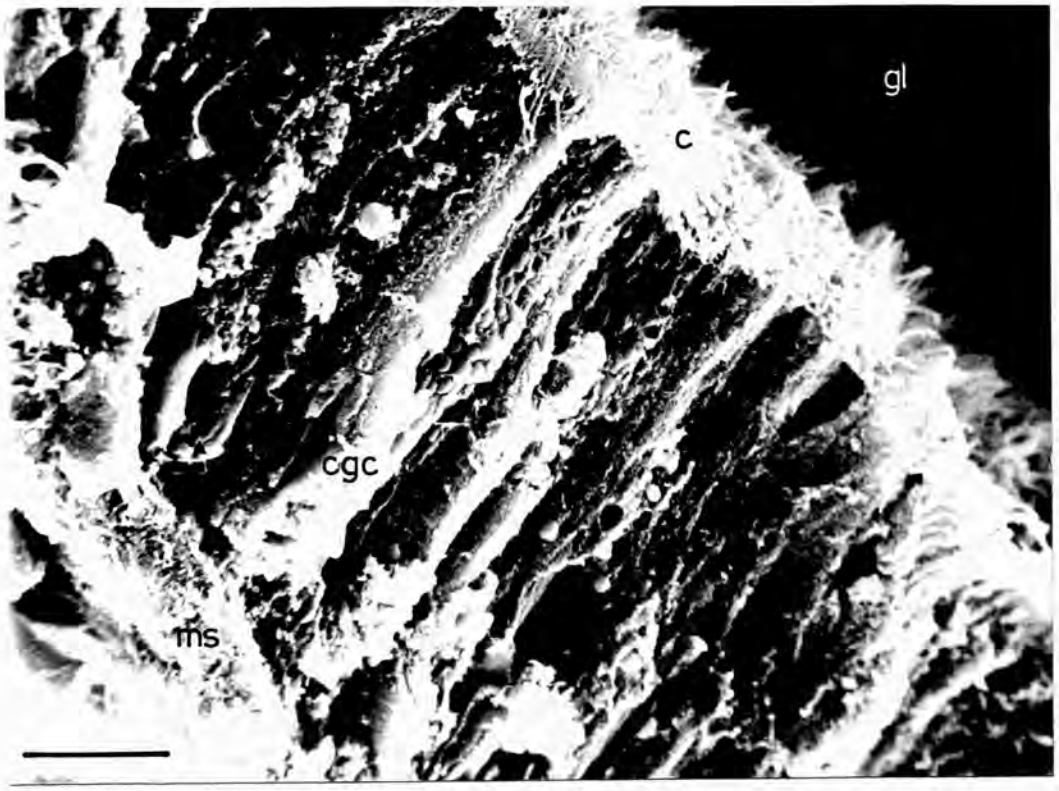


C

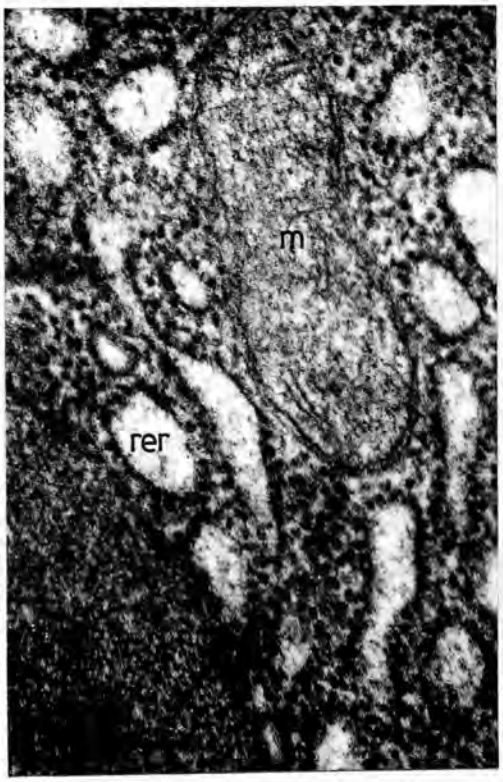
PLATE 42

PLATE 43



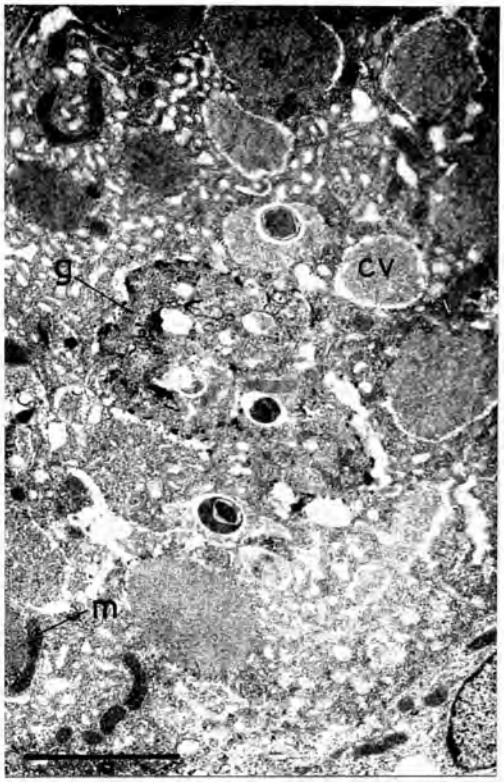
A

B

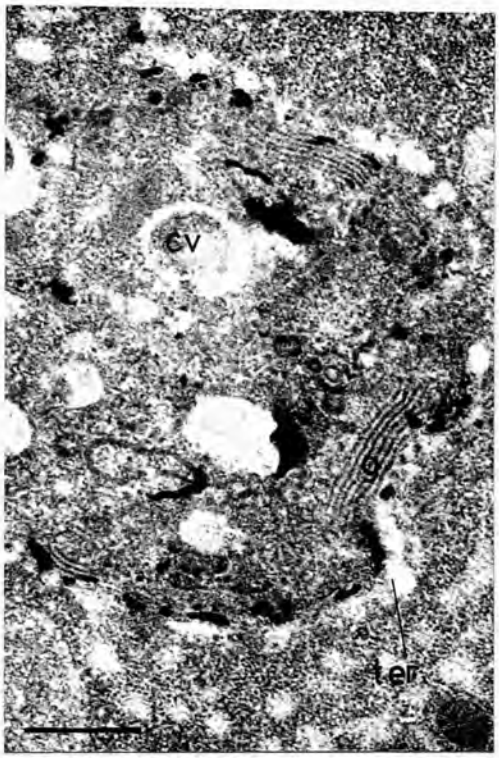


C

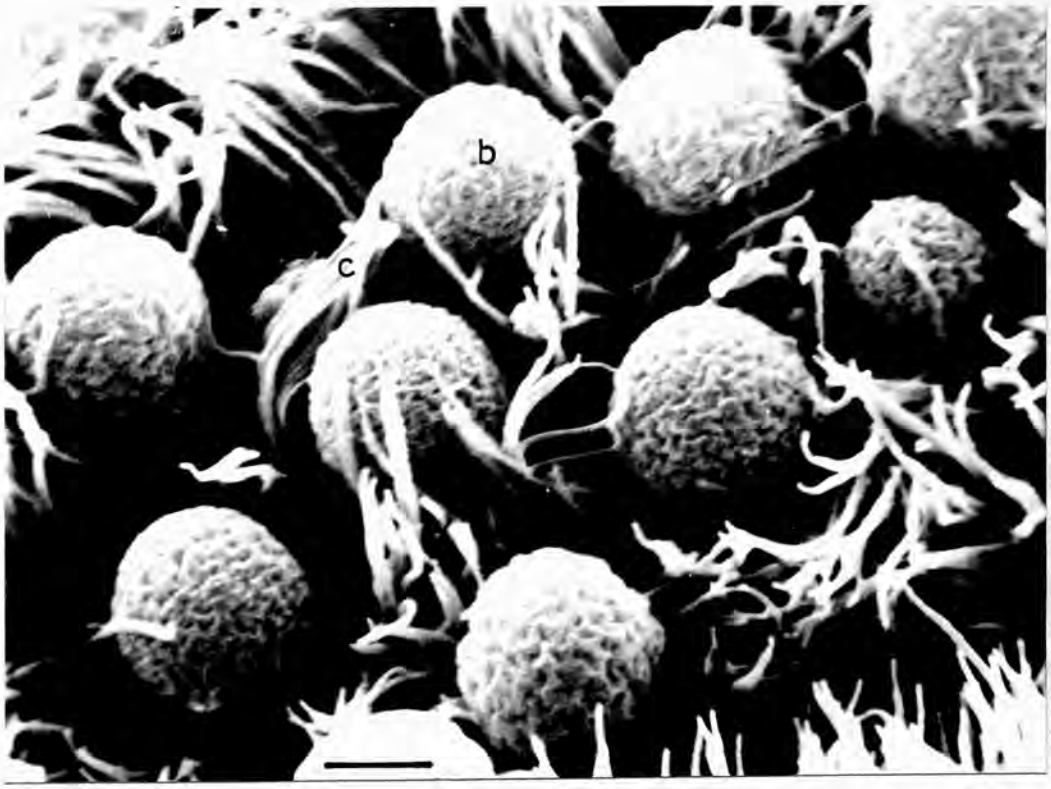
PLATE 44



A

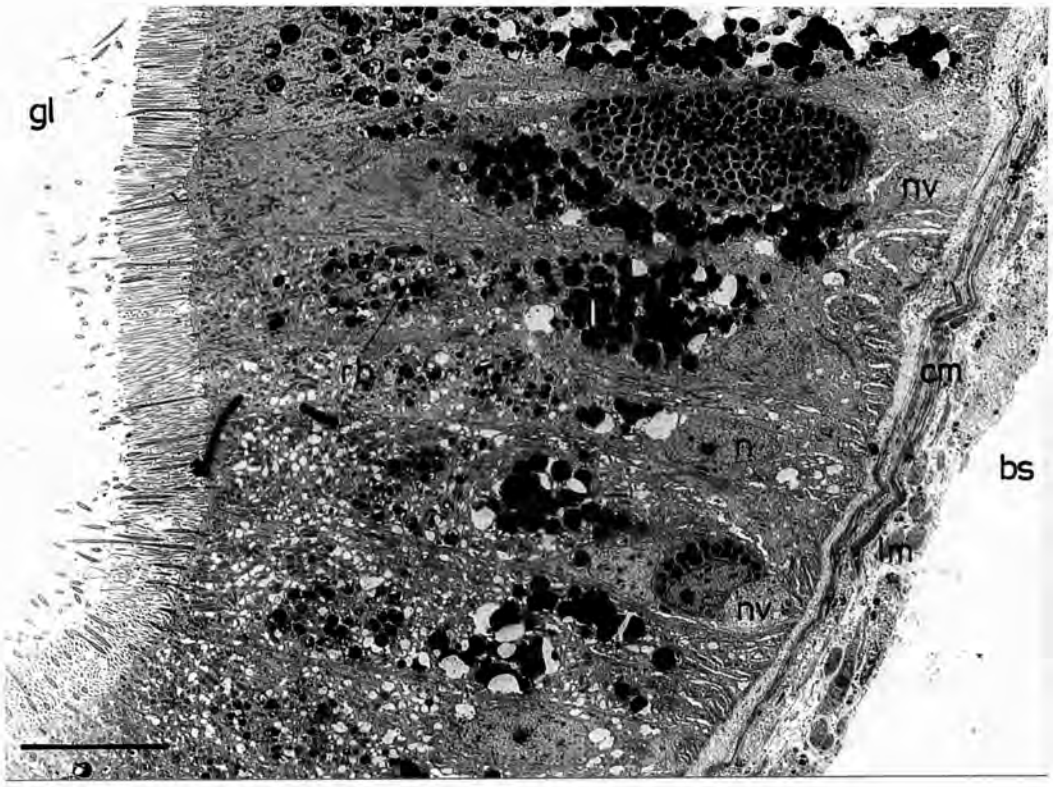


B

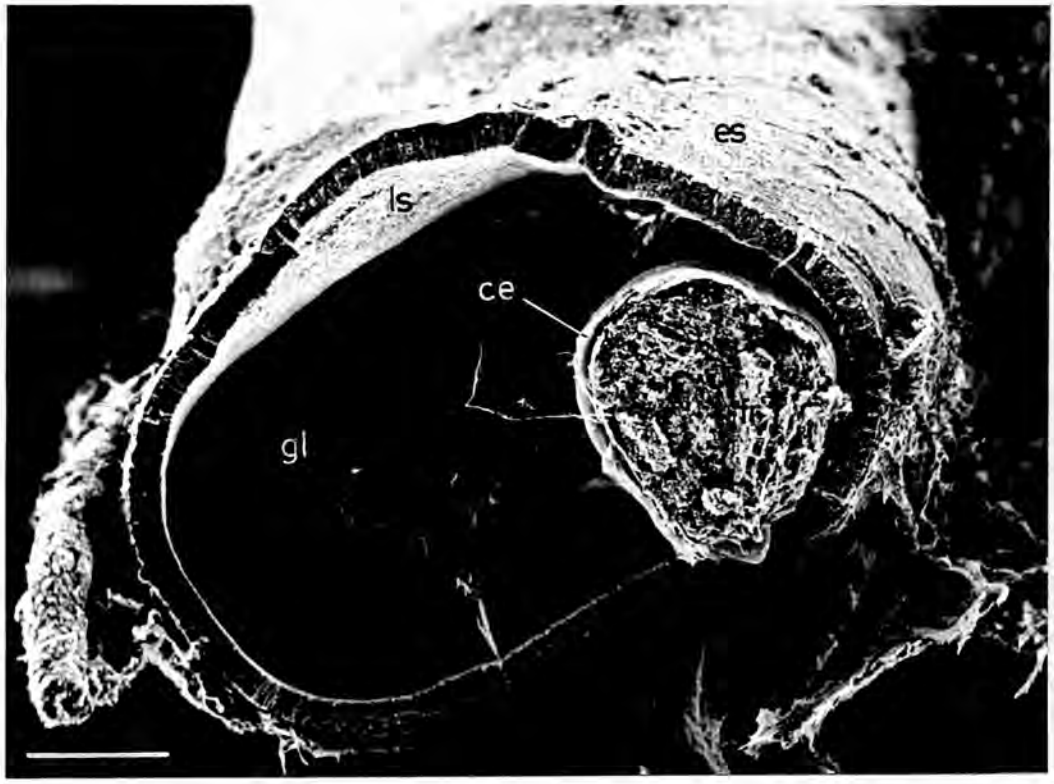


C

PLATE 45



A



B

THE RECTUM.

The pink-tinted rectum projects into the mantle cavity to the right of the mid-line and dorsal to the rest of the gut; left and right kidney openings lie on either side (figs 2 & 6). The posterior limit of the intestine is that region where the intestinal groove diminishes and a series of longitudinal folds arise from the intestinal wall and continue along the rectum to the anus. Two of these folds, lateral in position, divide the lumen into dorsal and ventral channels, the latter being the larger of the two (fig 16; plate 46); the folds can attain a height of 400-450μm). The epithelium lining both channels is thrown into numerous longitudinal folds which increase in height as they approach the anus. Those in the ventral channel are slightly shorter (140-200μm) than those in the dorsal portion (250μm) that practically occlude its lumen. All the ciliary currents on these folds are directed towards the anus.

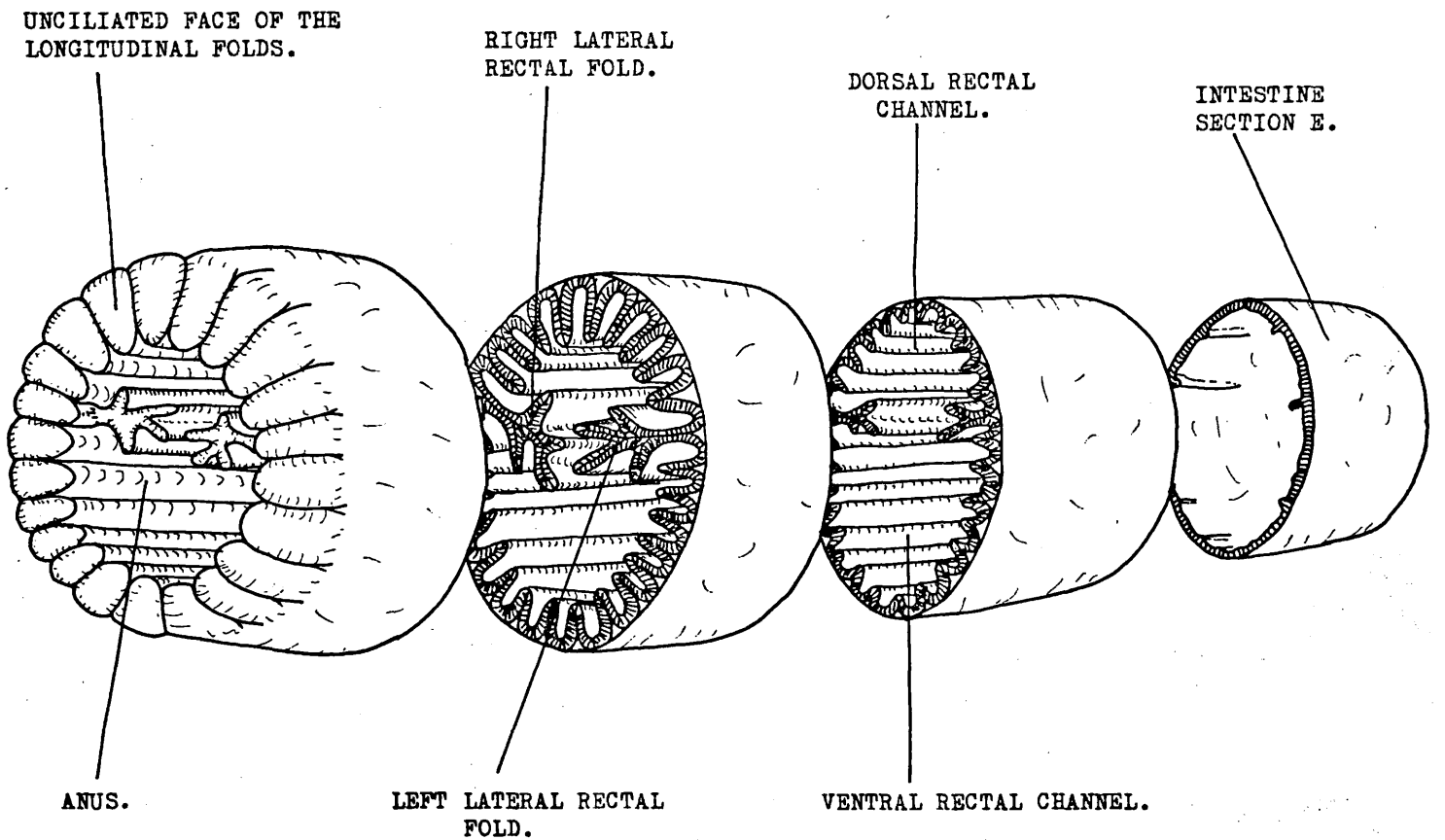
ULTRASTRUCTURE. The two channels are morphologically and histologically distinct from one another. The dorsal channel is lined by an epithelium composed of ciliated and unciliated cells arranged alternately, some are 9-12μm tall with cilia 15-17μm long (plate 47A). On the crests of the lateral folds this epithelium changes to taller (15-25μm) ciliated cells with shorter cilia (5-8μm) that line the ventral channel (plate 47B). The ciliated cells are absent from the ends of the folds that face into the mantle cavity. Sparse subepithelial mucous gland cells similar to those of the oesophagus occur in the ventral channel.

The ciliated cells differ from those described in section 1 by lacking ciliary rootlets, intercellular channels, a cytoskeleton and glycogen deposits. Electron-opaque bodies, multivesicular bodies and profiles of the endocytotic canal system are rare, though small dense residual bodies are much more numerous in the cells lining the ventral channel (table 4). A well developed muscle layer of circular fibres

encircles the rectum.

HISTOCHEMISTRY. The brush border reacts weakly to P.A.S. but not with alcian blue at pH 2.5. The residual bodies and basal lamina reacts with both these methods. The mucous cells are histochemically similar to those of the oesophagus, but do not stain with the PA-TSC-SP method.

FIGURE 16. A DIAGRAMMATIC STEREOGRAM OF THE RECTUM.



TO FACILITATE THIS VIEW OF THE RECTUM, BOTH OF THE KIDNEY OPENINGS, THE LEFT KIDNEY AND THE MANTLE EPITHELIUM HAVE BEEN REMOVED. THE RECTUM HAS BEEN SECTIONED TO DISPLAY THE INTERNAL FOLDS.

LEGENDS AND ABBREVIATIONS USED IN PLATES 46-47.

THE RECTUM.

PLATE 46.

A. SEM, rectum opened by a longitudinal dorsal incision. x140.

B. Light micrograph of a lateral rectal fold stained with toluidine blue. x480.

Scale bars: A=140μm; B=42μm.

PLATE 47.

A. The epithelium lining the dorsal rectal channel. 3K.

B. The epithelium lining the ventral rectal channel. 3K.

Scale bars=5μm.

a....Anus.

c....Cilia.

cm...Circular muscle.

ct...Connective tissue.

dc...Dorsal channel of rectum.

edc..Epithelium lining the dorsal channel.

evc..Epithelium lining the ventral channel.

gm...Gland cells of mantle epithelium.

lf...Lateral rectal folds.

lgt..Longitudinal folds.

m....Mantle epithelium.

mc...Mantle cavity.

n....Nucleus.

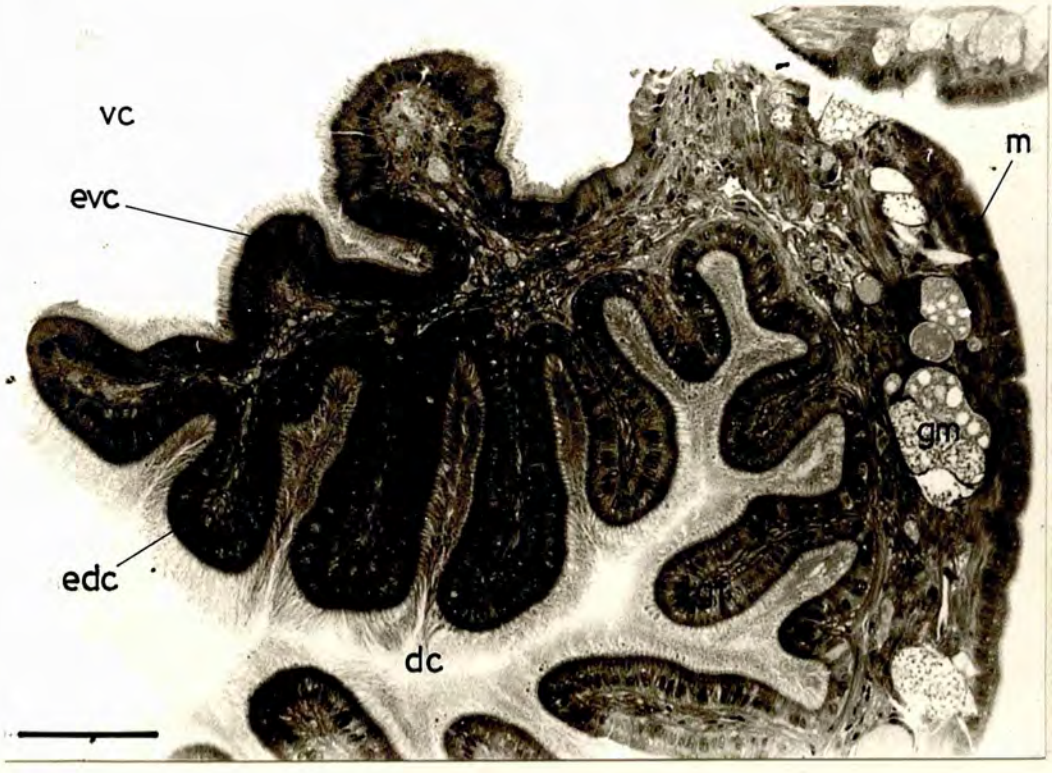
nv...Nerve.

rb...Residual body.

vc...Ventral channel.

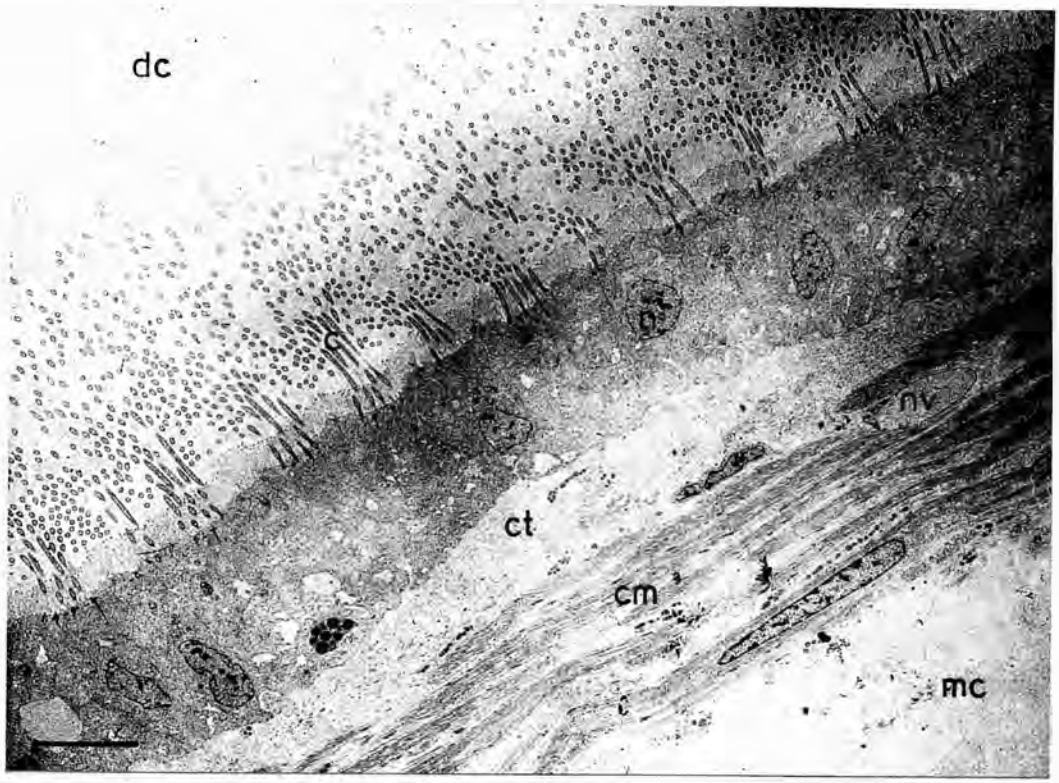
PLATE 46

A

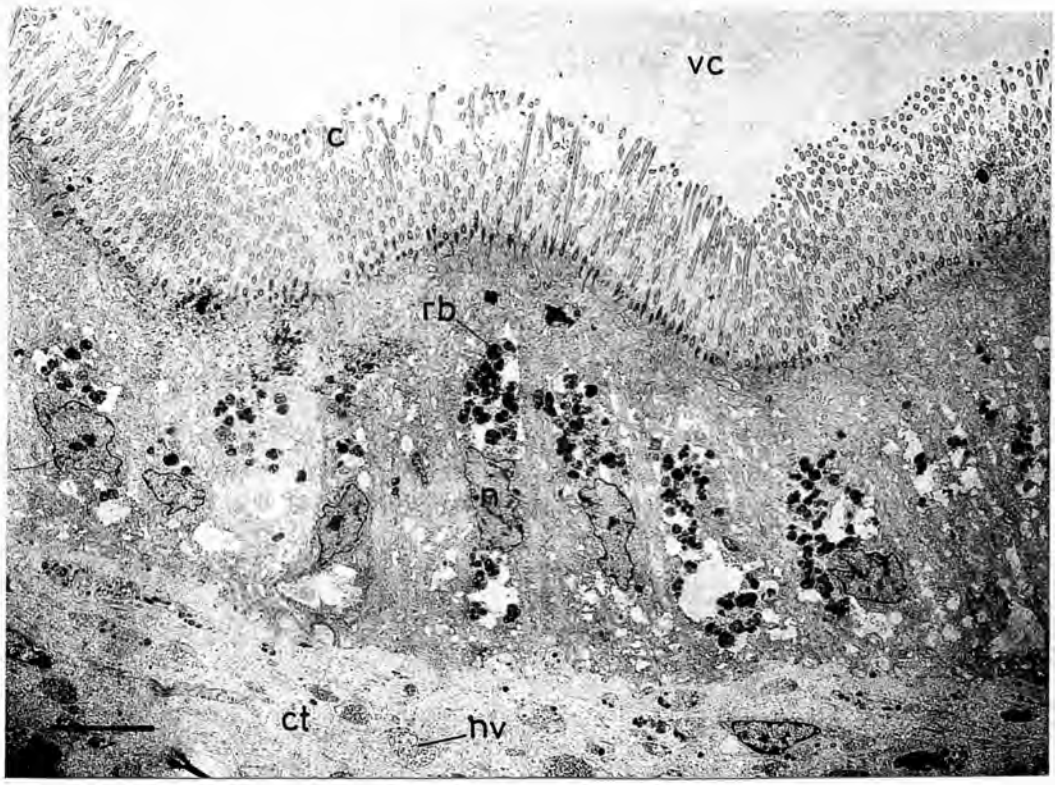


B

PLATE 47



A



B

THE EFFECTS OF STARVATION ON THE GUT.

The gross differences observed between starved and healthy limpets are the shrinkage of the digestive gland and gonad which consequently reduces the size of the visceral mass, the colour change of the salivary and digestive glands and the emptiness of the alimentary tract which retains its normal pigmentation. Cilia remain active along the gut. Histological differences, however, are more pronounced.

The ciliated epithelium shows few changes; the sparse lipid droplets in the intestinal columnar cells are even rarer and cytoplasmic blebs fewer. Both lysosomes and the golgi complex react weakly to acid phosphatase activity. When opened, the intestine contains a thin, yellowish string consisting of fragmentation spherules from the digestive cells and cytoplasmic blebs from the columnar cells. It lacks the proteinaceous cement normally secreted in section D although the basal and clavate gland cells contain their typical secretory granules.

Salivary glands from starved specimens assume a cream colour corresponding to a reduction in the number of type 1 secretory vesicles. The majority of the droplets present are of the lucent type 2 variety. The RER, nucleus and mitochondria retain their normal ultrastructure and distribution; the golgi complex is not apparent. A few cells contain one or two small, dense autophagic bodies lacking acid phosphatase activity; fragments of mitochondria occur in the granular matrix.

Drastic histological changes are evident in the oesophagus and digestive gland. The mucous cells of the food channel do not stain blue with Mallory, only the nuclei are stained; mucus is absent from the channel lumen. The secretory cells of the oesophageal gland lack both secretory granules and residual bodies and are consequently difficult to distinquish at low magnifications. Instead the cells contain vacuoles staining a very pale blue with Mallory. At the

ultrastructural level they resemble digestive cell heterophagosomes, empty except for a peripherally distributed granular material. The endocytotic canal system remains unaltered, but ribosomes detach from the empty RER cisternae in the peripheral cytoplasm and blebs of cytoplasm are infrequently released.

The digestive gland is yellow and sections reveal the epithelium to be in the "holding phase" described by Owen (1972) (plates 54A & B). The tubules are uniformly circular in outline and the low epithelium (20-35μm tall) surrounds a dilated lumen. The ducts retain their normal ultrastructure and their lumina contain fragmentation spherules.

Basophilic secretory cells are extremely difficult to distinquish in Mallory-stained sections, the intense basophilia characteristic of "healthy" cells is lost; even the nucleus is inconspicuous. In thin resin sections these cells adopt squat triangular shapes without a neck. Apart from the nucleus with its nucleolus, organelles and inclusions are rarely encountered. Although condensing vesicles are present, the cis and trans cisternae of the hypotrophied golgi complex are empty (plate 48B), only small whorls of RER remain. The electron-lucent cytoplasm contains numerous free ribosomes and mitochondria with weakly staining matrices, some cristae are slightly swollen. Hypertrophied mitochondria are not present. Autophagic vacuoles of a size similar to secretory granules are encountered only rarely, they contain membranous whorls and a matrix of varying granularity. In one section such a body was surrounded in section by a mitochondrion (plate 48A), intimate association of this kind suggests active processes, but acid phosphatase activity was not displayed by the vacuoles.

The apical surface of the digestive cells is slightly domed and short (<1μm), non-uniform microvilli lacking a glycocalyx form an indistinct brush border. The pale endocytotic canal system is

hypotrophied and there is little evidence of pinocytosis. Heterophagosomes are absent and only a few heterolysosomes (4-5μm) together with typical residual bodies occur (plate 54B). The heterolysosomes are full of a finely granular electron-opaque material that stains pale blue with Mallory, the residual bodies bodies appear similar to those found in "healthy" cells. Large "apical granules", staining greenish with Mallory, contain materialsimilar to that of the residual bodies.

A result of the reduction in vesicle numbers is that more of the agranular, moderately electron-opaque cytoplasm is visible. Throughout the cytoplasm are lipid droplets and mitochondria, the rarely encountered, inconspicuous golgi complex characteristic of "healthy" cells remains unchanged, however, and displays a weak acid phosphatase activity (plate 48C).

Necrotic cells, presumably digestive cells because of the numerous lipid droplets in the cytoplasm, with a vacuolated, electron-opaque granular cytoplasm are observed infrequently. At high magnifications the small vacuoles are resolved as distended RER cisternae containing a finely granular material. Also present are a few dense heterolysosomes but mitochondria are uncommon.

LEGENDS AND ABBREVIATIONS USED IN PLATE 48.

THE EFFECTS OF STARVATION ON THE GUT.

PLATE 48.

A. Hypotrophied golgi complex of basophilic cell. 21K.

B. Mitochondrion surrounding an autophagic body in a basophilic cell. 55K.

C. Inconspicuous golgi complex of a digestive cell reacts for acid phosphatase. 70K.

Scale bars: A=1μm; B,C=250nm.

ap...Autophagic body.

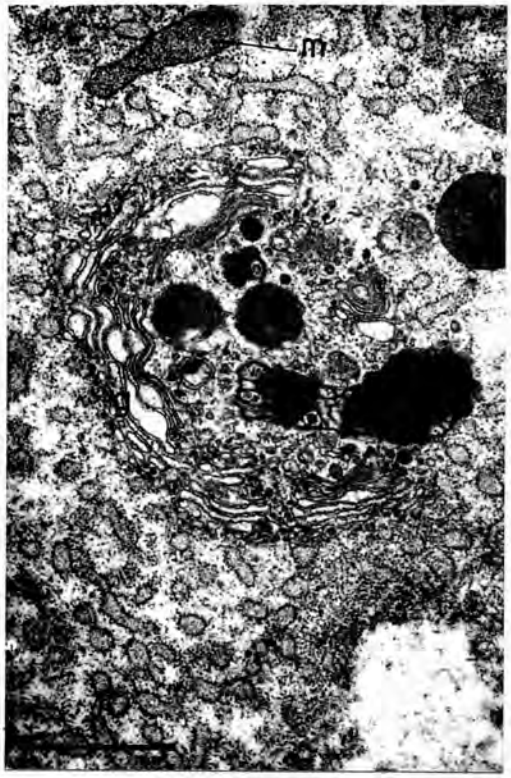
cv...Condensing vacuole.

g....Golgi complex.

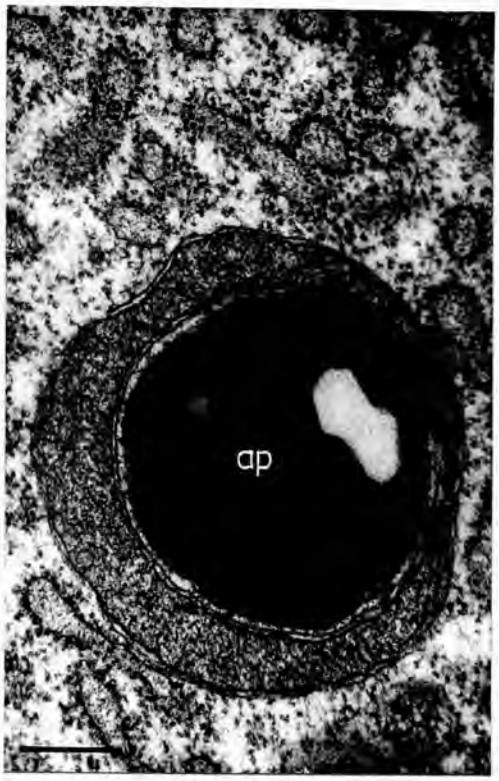
m....Mitochondrion.

sv...Secretory vesicle.

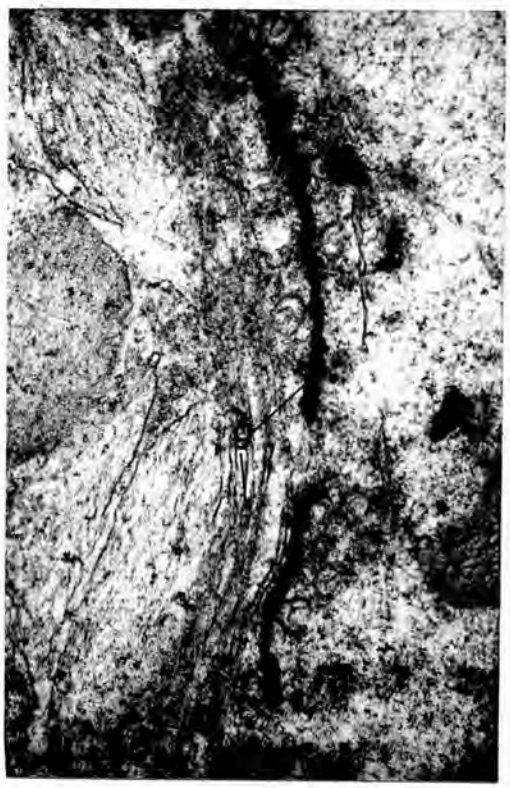
PLATE 48



A



B



C

PHASIC ACTIVITY.

The results presented in this section should be regarded with some caution; such a small sample number (one limpet per hour) may not represent the typical condition of the oesophageal and digestive glands in the local population. This preliminary study has produced interesting results from which rather tentative conclusions have been drawn, they may be confirmed by a future comprehensive study.

1-THE OESOPHAGEAL GLAND.

Two different phases of activity have been demonstrated in the amylase-secreting cell by histological, ultrastructural and histochemical methods. A secretory phase results in the production of an extracellular amylase in the form of proteinaceous granules, that are released by apocrine secretion (plate 49A). The other phase, probably absorptive and digestive results in the production of a large residual body in each cell, this is released similarly to the granules (plate 49B). This section examines the ultrastructural characteristics of both phases with respect to function and explores the synchrony of the two phases with each other and with the tidal cycle.

A). ULTRASTRUCTURAL CHARACTERISTICS.

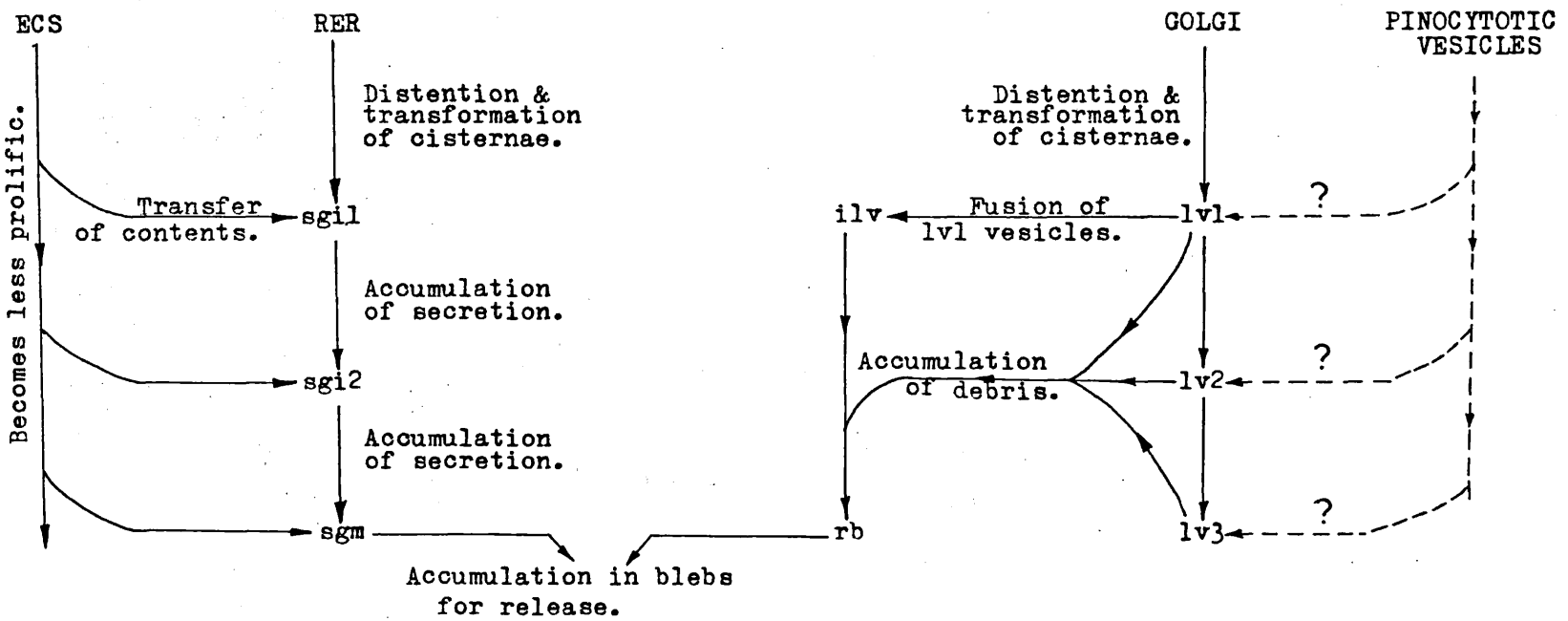
The following descriptions represent separate static stages of a dynamic process, the characteristics change with time and thus trends in changes rather than distinct changes are seen. Figure 17 displays the sequence and interrelationships of the organelles from the two phases.

During the secretory phase the cells are most conspicuous due to the production and release of the secretory granules from the swollen apical regions of the cells (fig 18, plates 18B & D). Early on, the cells do not normally contain many mature secretory granules (sgm) (plate 50), though some may remain from a previous secretory phase. As the phase proceeds, a series of small (1.5-3.0μm), oval,

FIGURE 17 . PHASIC ACTIVITY;SUGGESTED SEQUENCES AND INTERRELATIONSHIPS OF THE ORGANELLES CHARACTERISTIC OF THE TWO PHASES DISPLAYED BY THE AMYLASE-SECRETING CELL.

A).SECRETORY PHASE.

B).ABSORPTIVE/HETEROPHAGIC PHASE.



NOTATION:

ECS...Endocytotic canal system.
RER...Rough endoplasmic reticulum.
sgi1/sgi2...Immature secretory granules.
sgm...Mature secretory granules.

ilv...Interconnected large vesicles.
lvl/lv2/lv3...Types of large vesicles.
rb....Residual body.

membrane-bound vesicles appear (fig 18, plate 51A); these are immature secretory granules and they stain yellow, orange and red after Mallory and shades of purple with toluidine blue. Two types can be distinguished using ultrastructural criteria.

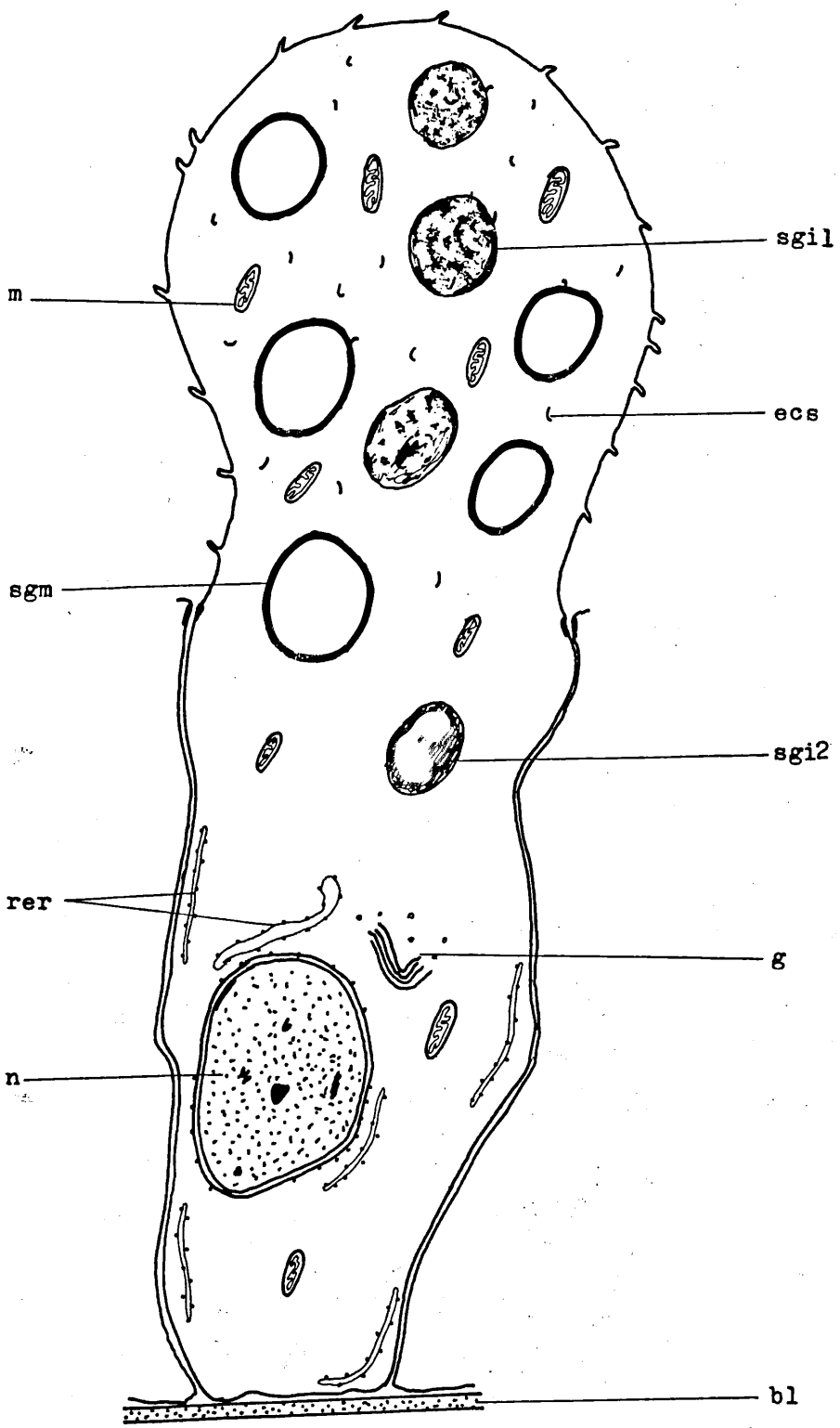
The earliest, sgi1, contains clumps of irregular electron-opaque, granular deposits separated from one another by lucent areas. A later form, sgi2, is characterised by various amounts of a moderately electron-opaque, granular-filamentous matrix interupted by small (300-500nm) lucent areas (plate 51A). Mature granules (2-3μm) appear later and are very characteristic (plates 51A & C); after Mallory staining they are red, red-purple and deep purple and with toluidine blue intense purple. This intense basophilia is due to their electron-opaque matrix that stains specifically for proteins (table 9).

Numerous intermediate stages are apparent in the maturation process and as the phase proceeds the greater proportion of the granules in a cell mature, a process which can be seen as an increase in staining intensity and electron-opacity. A distinct secretory pathway is not obvious and the second phase confuses the issue, but the following idea is presented to explain their derivation.

The normal cisternal space of the RER (20-35nm) is sometimes increased to 90-450nm. Normal and distended RER together with the immature granules all contain a similar matrix, which in the latter occurs in higher densities. Connections between the RER and granules were not seen. The distended RER may be pinched off to form an initial stage of the granule, whilst the ribosomes fall off during this transformation (plate 51B). Accumulation of the proteinaceous material in the lumen transforms this form into the mature one. How this may occur is speculated below.

The initial form cannot synthesise the proteinaceous matrix because the ribosomes are no longer attached, so an indirect method has been

FIGURE 18 . PHASIC ACTIVITY;AMYLASE-SECRETING CELL-SECRETORY PHASE.



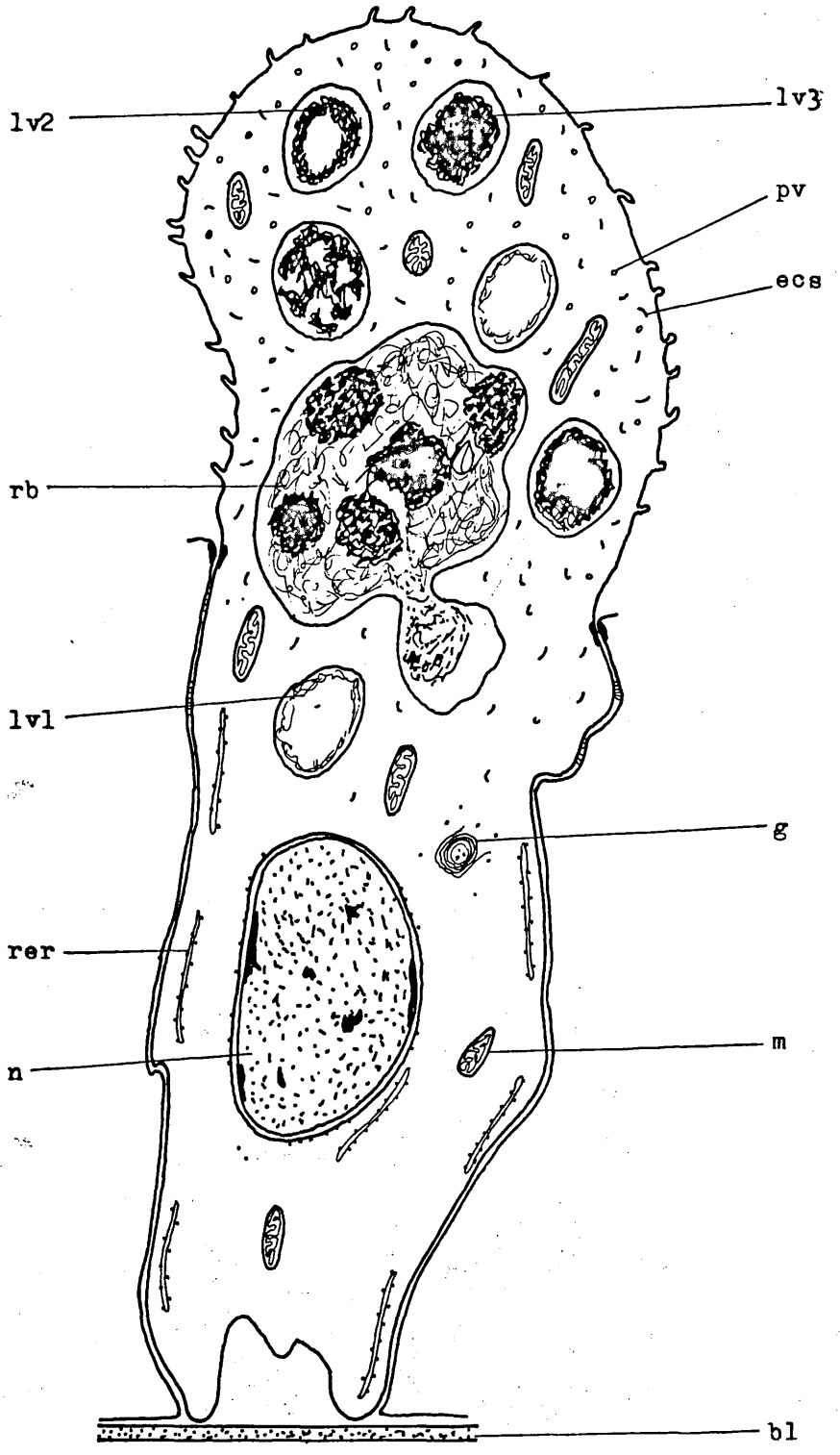
inferred from the following evidence. Closely associated with the secretory granules and distended RER are profiles of the endocytotic canal system (plates 51B & C), which fuse with and empty their contents into the granules. It is not known if these connections are permanent or temporary. As the phase proceeds and the mature granules accumulate in the apical regions of the cell, the canal system becomes less prolific (plate 51A). The canal system absorbs proteinaceous material (see page 117 for evidence of ferritin uptake) but it is not known how this is converted into the secretory product.

The final stage is reached when cytoplasmic blebs are abstricted from the cell (plate 18D). There is no differential segregation of mature granules into the blebs and the continued association of the canal system with the granules suggests that maturation continues even as the blebs are transported to the food channel.

During the absorptive phase numerous types of vesicles and a residual body form (fig 19). The first vesicles (lv1) to appear, stain pinkish or blue green after Mallory and pale blue with toluidine blue; they are 1.5-3.0µm in diameter and may be empty or contain a finely granular peripheral halo. Some of these may fuse with others to form an irregularly shaped vesicle (ilv) with a common lumen. Pinocytotic vesicles are present early in this phase (plate 20A). Later the "lv" vesicles can be distinguished into different types on the basis of their content. One type (lv2) contains material varying from moderately to very opaque, distributed in ring-shapes or clumps (plate 52A). Another form (lv3) resembles a mature secretory granule with a lucent halo surrounding a matrix similar to that in the lv2 vesicles (plate 52B).

All of these forms represent stages of an heterophagic process which results in the formation of a residual body as proposed below. The "ilv" vesicle is the first stage of the residual body and is basically just an empty vesicle. Any of the "lv" vesicles (1, 2 or 3)

FIGURE 19 . PHASIC ACTIVITY;AMYLASE-SECRETING CELL-HETEROPHAGIC PHASE.



may fuse with and eject their contents into the residual body (plate 21A), which consequently increases in size. The more electron-opaque material sometimes retains its shape and size, but the less opaque matrices tend to flow into the residual body. Maturation occurs as more material is sequestered.

Mature residual bodies are the largest organelles found in the cells (plate 52C). After staining in Mallory they appear as a cluster of yellow refractile granules (1-2μm) within a clearly defined vesicle, after toluidine blue the granules are stained blue (plate 49B). The contents consist of a moderate to coarse granular material and membranous bodies, the former existing as oval and spherical masses corresponding in size to the granules seen with optical microscopy.

During this phase the golgi complexes commonly exist as concentric whorls surrounding a central area of cytoplasm, containing one or two small (40-60nm) vesicles that may be primary lysosomes (plate 52D). Similar moderately electron-opaque vesicles occur around the golgi and are probably derived from the distal margins of the cisternae. Whilst in this form the cis cisternae may dilate up to 100-150nm wide.

As the golgi complex appears to play no significant role during the secretory phase, it is suggested that it is responsible for the formation of the "lv" vesicles from the distended cisternae. The primary lysosomes lie close to the secretory granules (plate 52E), lucent vesicles and residual bodies (plate 52F); they may fuse with them to release digestive enzymes, though phosphatase activity was absent. Fusion with the secretory granules indicates lysis of the secretory product to recover useful components.

The few necrotic cells encountered showed ultrastructural similarities with this phase but were undergoing autolysis (plate 53). Their cytoplasm and endocytotic canal system stains more intensely

than normal, but the cytoplasm does not have an increased granularity; the profiles of the canal system are curled or ring-shaped. Vesicles resembling the "lv" vesicles in size, accumulate and discharge (by exocytosis) small vesicular elements of unknown origin and function. The nuclear membrane and RER cisternae are distended (150-200nm wide) and the golgi complex adopts a cup-shape or concentric whorl.

B-SYNCHRONY OF PHASES THROUGHOUT THE GLAND.

The histological evidence indicates that in any one secretory cell there are two phases. This means that residual bodies and mature secretory granules do not coincide in any one cell; however there may be a slight overlap of phases. It is now pertinant to ask if all the population of amylase-secreting cells of the oesophageal gland are in synchrony? Are they all in a certain phase simultaneously? To determine this, a survey of serial wax sections of oesophagus was undertaken at the different times of the tidal cycle. The results of this are presented in figures 20 and 21.

Figure 20 shows that the amylase-secreting cells are not synchronous, for example at t= 06, 07, 08, 11, 15, 16 and 03 hours, some cells and blebs contained residual bodies and others secretory granules. Large numbers of either of these organelles were rarely encountered in the blebs.

The degree of asynchrony can be expressed as a percentage (table 17A) as outlined below; the "very few" marks in figures 20 and 21 are considered insignificant. From figure 20, some cells contained only residual bodies on 16 occasions (t=06, 07, 08, 11, 13, 15, 16, 17, 18, 21, 22, 01, 02, 03, 04 & 05 hours) and also at 6 of these times, other cells contained only secretory granules. So 6/16 . 100 = 37.5%. Thus, 37.5% of the time over the 24 hour period, the two phases were occuring in the gland simultaneously. One would expect this figure to be reflected by a similar analysis of the bleb contents. On 5

FIGURE 20. PHASIC ACTIVITY;AMYLASE-SECRETING CELL-CHART DISPLAYING THE FREQUENCY OF CERTAIN HISTOLOGICAL FEATURES IN RELATION TO THE TIDAL CYCLE(FIRST SERIES TISSUE).

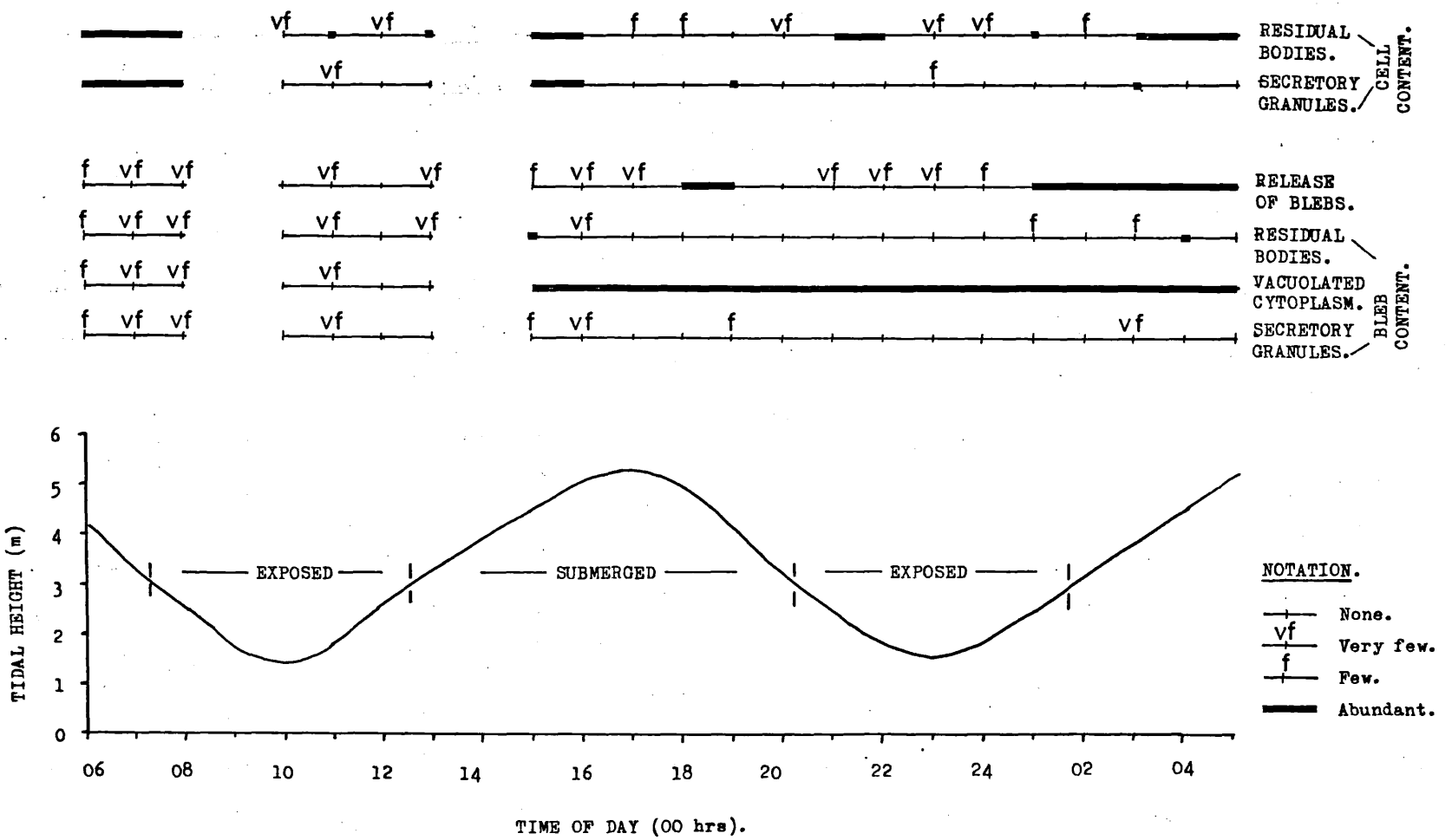


FIGURE 21 . PHASIC ACTIVITY:AMYLASE-SECRETING CELL-CHART DISPLAYING THE FREQUENCY OF CERTAIN HISTOLOGICAL FEATURES IN RELATION TO THE TIDAL CYCLE (SECOND SERIES).

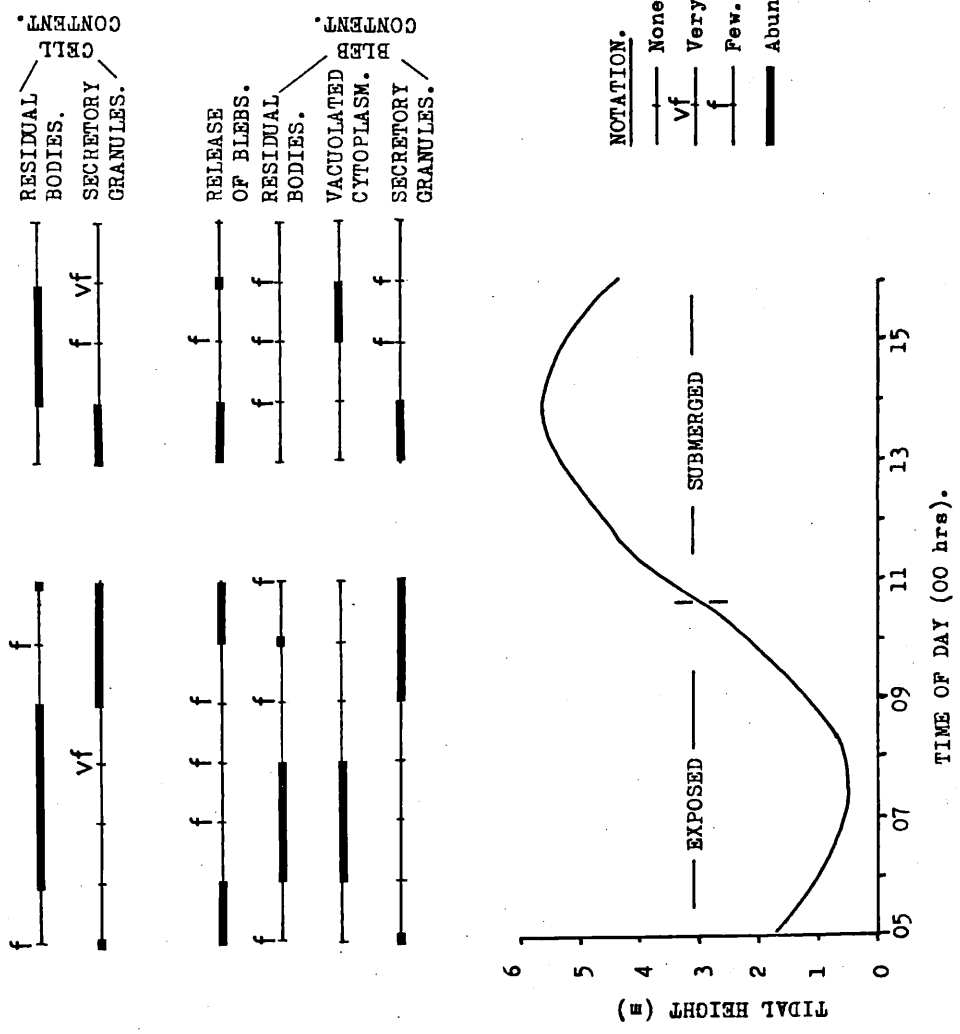


TABLE 17 .

PERCENTAGE SYNCHRONICITY OF THE TWO PHASES OF THE AMYLASE-SECRETING CELL.

A).FIRST SERIES TISSUES.

VESICLES PRESENT AT ANY ONE TIME.	VESICLE LOCATION	
	CELLS	BLEBS
RESIDUAL BODIES AND ALSO SECRETORY GRANULES	37.5	40
SECRETORY GRANULES AND ALSO RESIDUAL BODIES	75	66.6
SECRETORY GRANULES	25	33.3
RESIDUAL BODIES	62.5	60

B).SECOND SERIES TISSUES.

RESIDUAL BODIES AND ALSO SECRETORY GRANULES	60	70
SECRETORY GRANULES AND ALSO RESIDUAL BODIES	85.7	87.5
SECRETORY GRANULES	14.3	12.5
RESIDUAL BODIES	40	30

instances (t= 06, 15, 01, 01 & 03 hours) blebs contained only residual bodies and on two of these (t= 06 & 15 hours), some blebs contained secretory granules. 2/5 . 100 = 40%.

The degree of synchrony can be expressed similarly on occasions when residual bodies and secretory granules do not coincide at a certain time (table 17A).

Figure 21 indicates that the cells from the second experimental series were more active, because abundant residual bodies and secretory granules were encountered frequently. Futhermore, the results confirm more strongly that the cells are asynchronous (table 17B)

C-SYNCHRONY OF THE PHASES WITH THE TIDAL CYCLE.

Two relevant questions can be asked. Are all the secretory cells in phase with the tidal cycle and does the release of blebs containing mature secretory granules (amylase) coincide with feeding opportunities (submergence)? In view of the asynchrony demonstrated above, the answer to the first question must be no. A modified question can be asked: are the majority of the cells in phase with the tidal cycle?

Figure 20 indicates that mature secretory granules are present in cells on 7 occasions (t= 06, 07, 08, 15, 16, 19 & 03 hours) when the limpets would be submerged and this coincided with the release of a few blebs containing secretory granules at t= 06, 15 & 19 hours. Significant bleb release occured only in submerged limpets (t= 18, 19, 01, 02, 03, 04 & 05 hours), but these blebs did not contain secretory granules.

Synchrony of the phases with the tidal cycle is clearer in the second series (figure 21). Significant bleb release coincided with submergence and they contained secretory granules on all but one occasion (t=06 hours). The presence of granules in the cells also coincided with their release in blebs. During emersion, the cells

release fewer blebs that contain residual bodies. So there is some evidence to suggest that the release of the amylase coincides with submergence.

2-THE DIGESTIVE GLAND.

The digestive gland tubules exhibit five histological conditions brought about by quite profound changes in the digestive cells. Changes in the secretory cells are less obvious. The five conditions are; holding, absorbing, digesting, fragmenting and disintegrating phases. Figure 22 shows their probable sequence and interrelations.

It must be remembered that the tubule epithelium is dynamic and that the numerous intermediate tubule forms blur the distinction of the five phases mentioned. There is a gradual transformation from one phase to the next as intracellular digestion proceeds. Spatial-temporal factors may cause the regions of a tubule nearest to its connection with a duct, to receive food before those regions at the furthest end. This may be responsible for the slight asynchrony of phases in some sections; some tubules can be at a later phase than others.

The ultrastructural characteristics of the digestive and basophilic cells during each phase will be examined, followed by the synchronisation of the phases with the tidal cycle.

A). THE DIGESTIVE CELL.

i). HOLDING PHASE. Tubules in this phase were encountered rarely in wax sections and once at the ultrastructural level (plates 54A & B). The wide tubule lumen (100µm) may be empty or contain a granular material, crypts are absent and the low (30µm) epithelium is composed of cuboidal digestive and squat, pyramidal basophilic cells. A conspicuous brush border is absent and the flat apical membranes of the cells gives the epithelium a smooth border with the lumen.

The slightly vacuolated apical cytoplasm of the digestive cells stains pale purple or pink after Mallory. Small numbers of blue

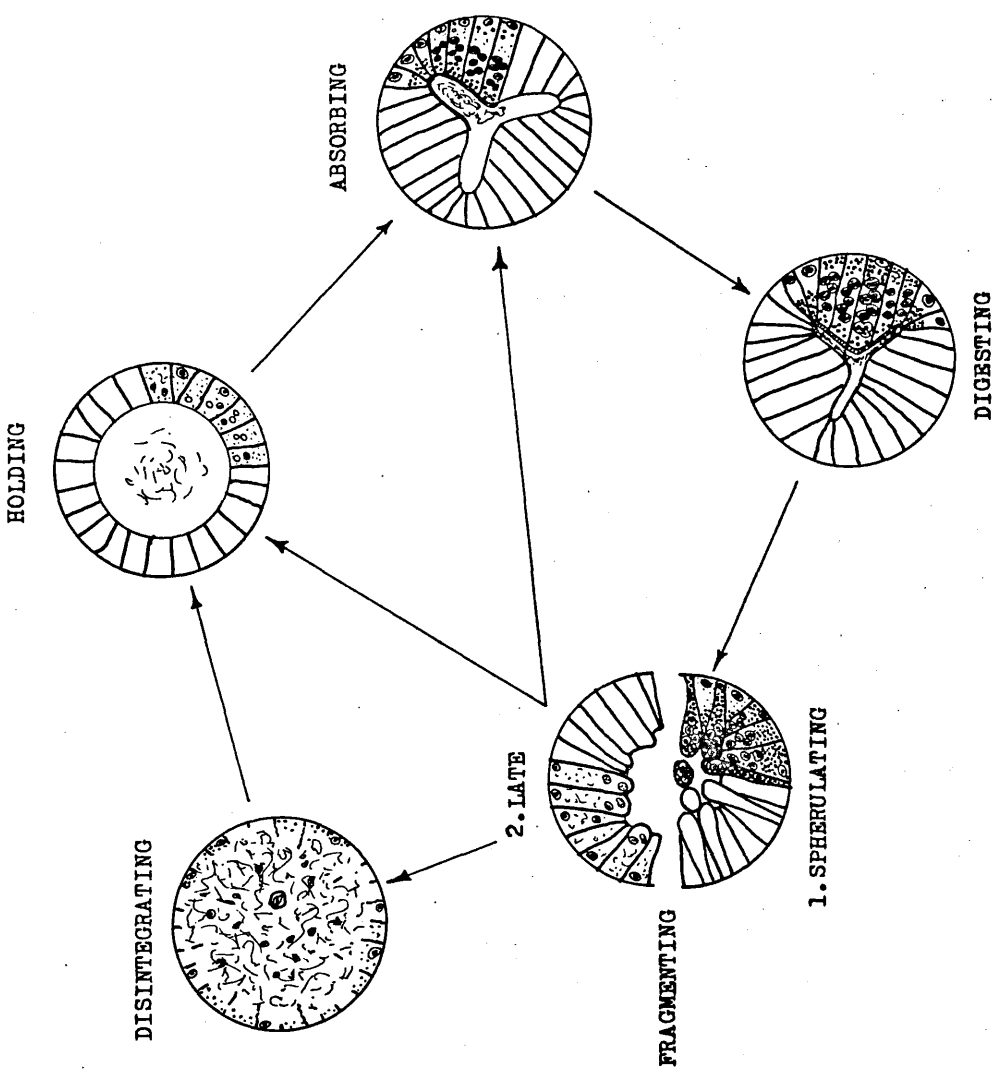


FIGURE 22. PHASIC ACTIVITY;DIGESTIVE GLAND TUBULES-PROPOSED SEQUENCES OF PHASES.

heterolysomes occur throughout the cell; residual bodies and lipid droplets can not be distinguished. Some tubules contain digestive cells that appear in a state of late fragmentation, suggesting that the tubules enter the holding phase once the fragmenting stage has ended. Dilation of the lumen will facilitate entry of food into the tubules, during the next feeding phase.

Basophilic cells stain dull purple with Mallory, this reduced basophilia suggests that the cells are not actively synthesising protein. Secretory granules are absent, but vacuoles with a central mass (1μm) of material occur; these could not be correlated with the ultrastructure.

ii). ABSORBING PHASE. Distinct crypts appear at this stage as the lumen is reduced by the incresing height of the epithelium; those cells mid-way between crypts are tallest (plate 32A). As the lumen shrinks the food is concentrated above the brush border, composed of distinct microvilli (1μm long). Endocytosis is prolific and pinosomes are abundant.

Heterophagosomes and heterolysosomes are present from the onset. As the phase progresses the pinosomes cluster around the heterophagosomes to which the endocytotic canal system may connect. Heterophagosomes frequently contain slight peripheral deposits of food material. By the start of the digestive phase the heterolysosomes are full of material similar to that in the lumen. As food is sequestered, emptier heterolysosomes tend to occur above those which are full. Some residual bodies may be present in the mid- and basal regions, they contain peripheral deposits of material. Most lipid droplets are situated basally, but spread through the cell as the phase proceeds.

iii). DIGESTIVE PHASE. There are no distinct differences between this and the preceding phase. The lumen becomes obliterated, so that the brush borders of opposing cells almost touch (plate 55). As intracellular digestion proceeds, the contents of the heterolysosomes

become more lucent and diffuse, when in this form they tend to fuse to form "apical granules" (plate 56). Residual bodies become more numerous and electron-opaque as they fill with a coarse granular material; lipid droplets accumulate throughout the cell. Once food has disappeared from the lumen, endocytosis is reduced. Well into this phase the microvilli become reduced, lipid droplets and residual bodies increase in numbers, the latter may connect with heterolysosomes or "apical granules" and heterophagosomes become rare. iv). FRAGMENTING PHASE. This can be divided into an early spherulating stage, in which apical blebs are abstricted from the digestive cells (plate 57B) and a later stage in which the tubules appear in cellular disarray (plate 57A). Digestive cells approaching the first stage increase in height, the apical portions dilate and bulge into the lumen whilst the basal regions are seen as a narrow slip of cytoplasm. The brush border is poorly developed and the microvilli are lost, as the spherules are abstricted from the cell.

By this stage, endocytosis has ceased and both the sparse pinosomes and canal system are empty. The few remaining heterophagosomes contain clumps of granular material instead of the normal fine peripheral deposits. Heterolysosomes and residual bodies accumulate apically and the cells are nearly full of lipid droplets.

As the apical portions bulge further outwards, the lumen becomes reduced and then full of released spherules containing micro- and macrovesicles, lipid droplets and mitochondria (plate 57A). The nucleus remains in the remnants of the cell. The number of spherules released by each cell is unknown.

Once the later stage is reached, the cells lose their basophilia and the chaotic epithelium acquires an overall blue hue (with Mallory) due to the residues in the heterolysosomes. The digestive cells produce small apical swellings and contain one or two partially empty heterolysosomes that were not ejected. Presumably these remain in the

cell until presented with a new supply of food.

v). DISINTEGRATING PHASE. The only recognisable portion of tubules that have disintegrated is the connective tissue layer and the very basal regions of the tubule cells, suggesting that the cells rupture rather than detaching from the basal lamina (plate 57C). The lumen of such tubules is full of cellular debris.

B). THE BASOPHILIC CELL.

The most conspicuous change in the basophilic cell is its variable basophilia, ranging from dull purple to bright red/purple or orange with Mallory. Few ultrastructural changes were observed. On only one occasion was the release of the secretory material noticed and that coincided with a digesting-fragmentation phase, when the limpet was submerged (plate 30A).

Hypertrophied mitochondria, an active golgi complex and distended RER full of a material resembling that in the golgi, occur in cells from tubules containing basophilic cells staining intensely with Mallory. Cells with a reduced basophilia are ultrastructurally similar but have normal mitochondria. The reduced basophilia may be indicative of a less active phase of the secretory cycle during which protein synthesis is diminished.

C). SYNCHRONY OF THE PHASES WITH THE TIDAL CYCLE.

Figures 23 and 24 display the phases encountered together with the staining reactions and abundance of the basophilic secretory cells in relation to the tidal cycle. Examination of the first series of tissues (fig 23) reveals that the most frequently encountered phases are digestion and fragmentation, but that the occurence of these phases can not be related to the tidal cycle. The two plots of the abundance of the basophilic cell show the same overall trend; a sudden drop at t= 06, 07 and 08 hours (low tide) followed by an increase and then fluctuations over the remainder of the cycle. Statistical analyses of these plots is not possible because of the small sample

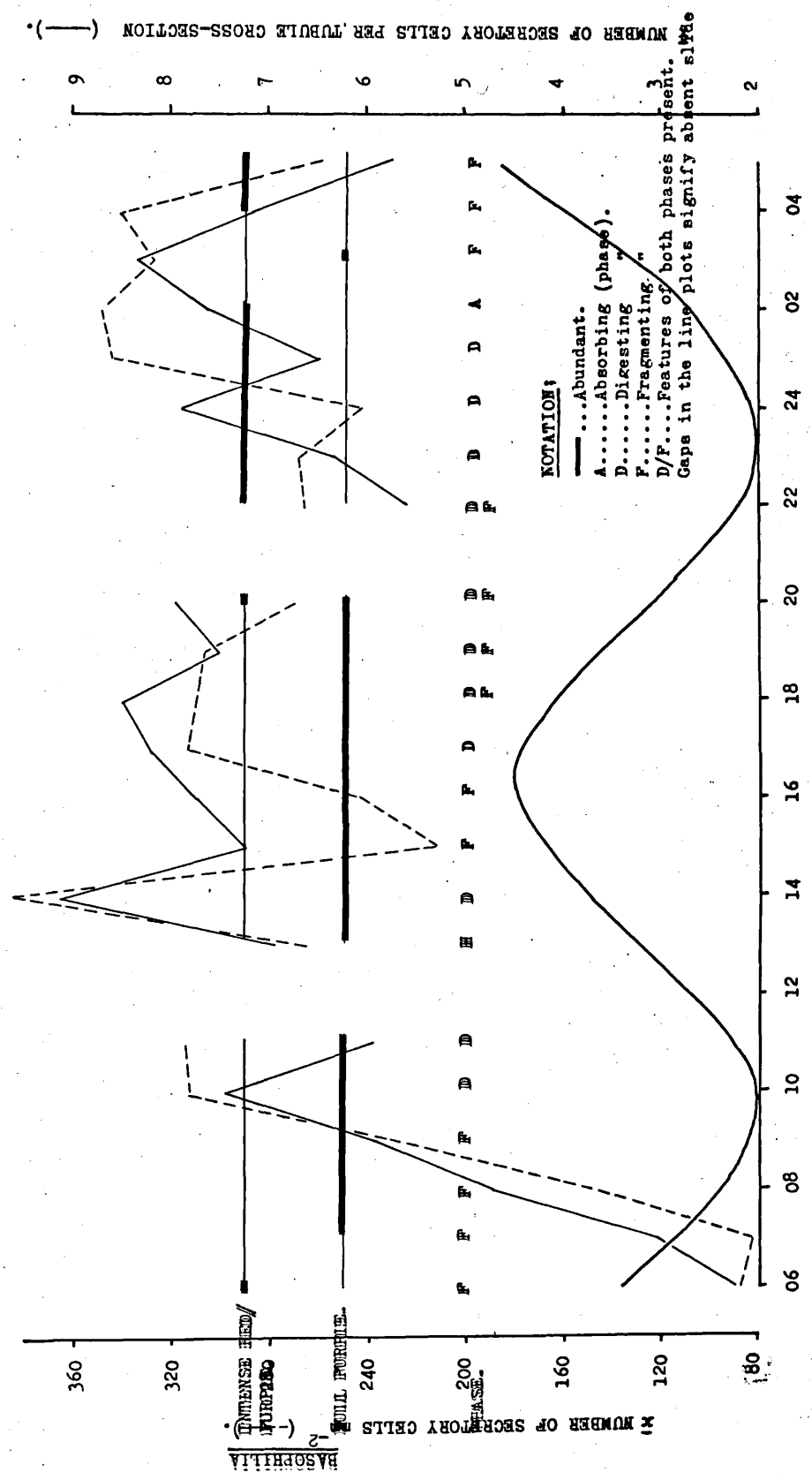


FIGURE 23. PHASIC ACTIVITY; CHART DISPLAYING NUMBERS AND BASOPHILIA OF THE SECRETORY CELL IN RELATION TO THE TUBULE PHASE AND TIDAL CYCLE (FIRST SERIES TISSUES).

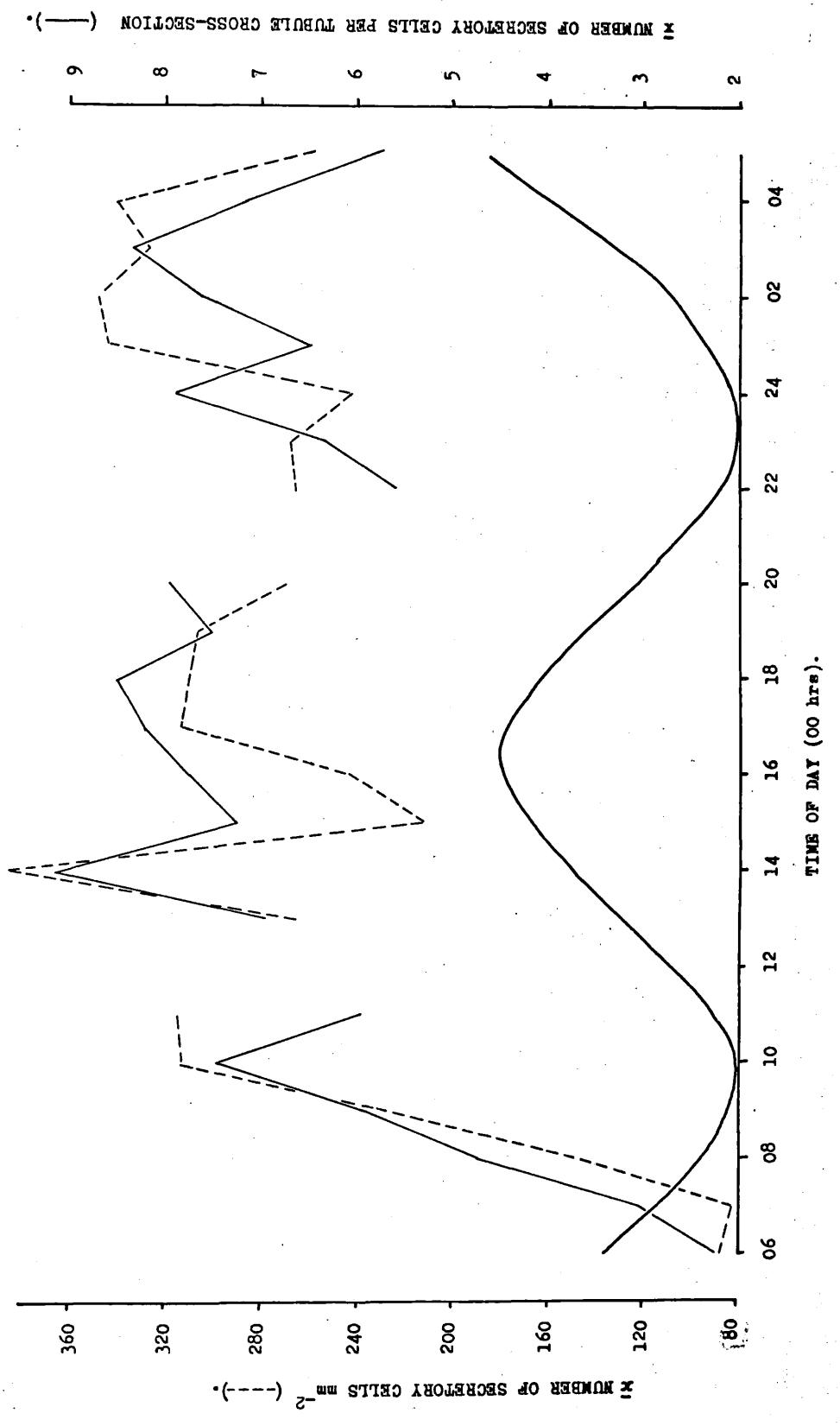


FIGURE 23. PHASIC ACTIVITY: CHART DISPLAYING NUMBERS AND BASOPHILIA OF THE SECRETORY CELL IN RELATION TO THE TUBULE PHASE AND TIDAL CYCLE (FIRST SERIES TISSUES).

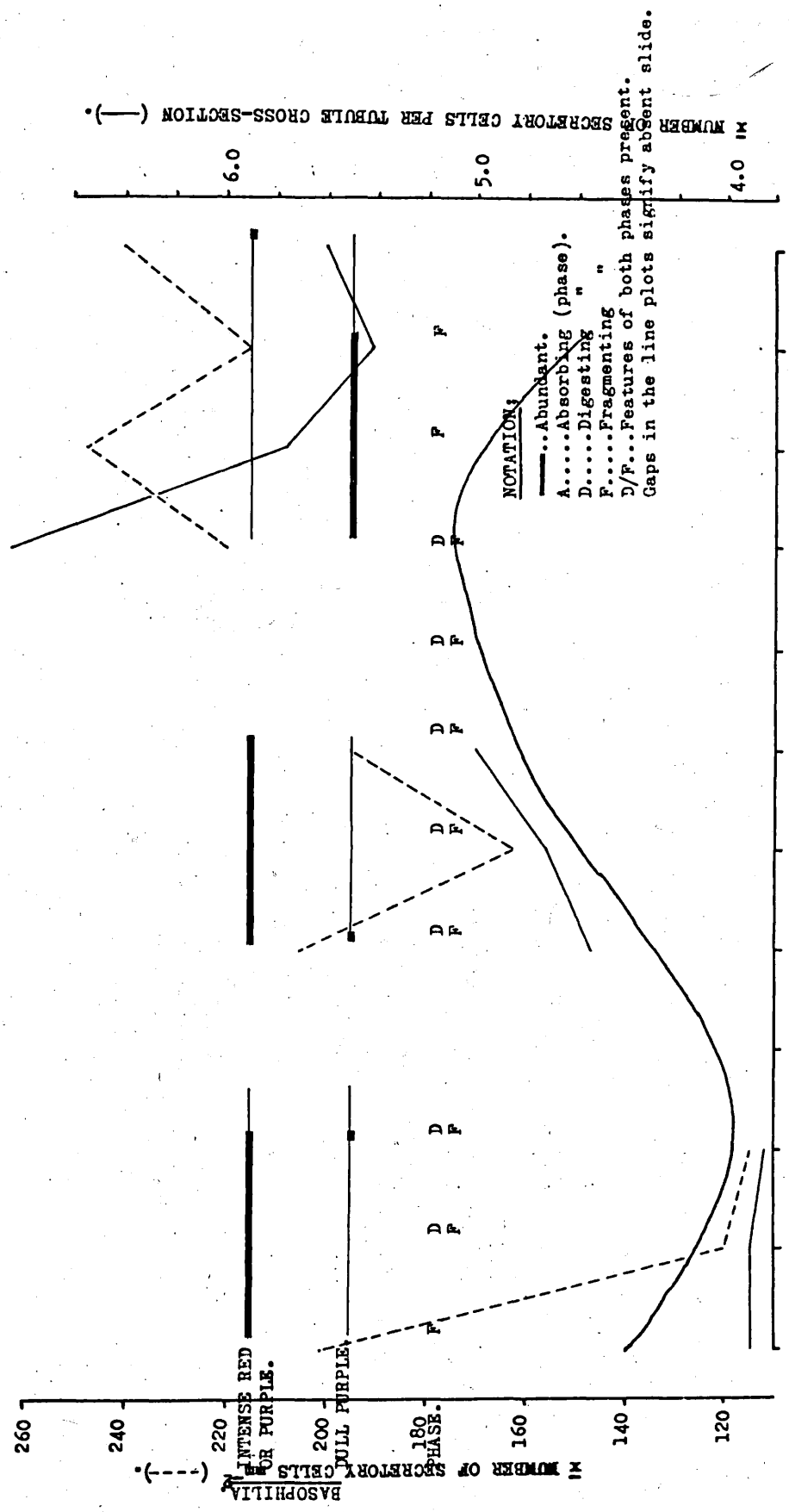


FIGURE 24. PHASIC ACTIVITY CHART DISPLAYING NUMBERS AND BASOPHILIA OF THE SECRETORY CELL IN RELATION TO THE TUBULE PHASE AND TIDAL CYCLE (SECOND SERIES TISSUES).

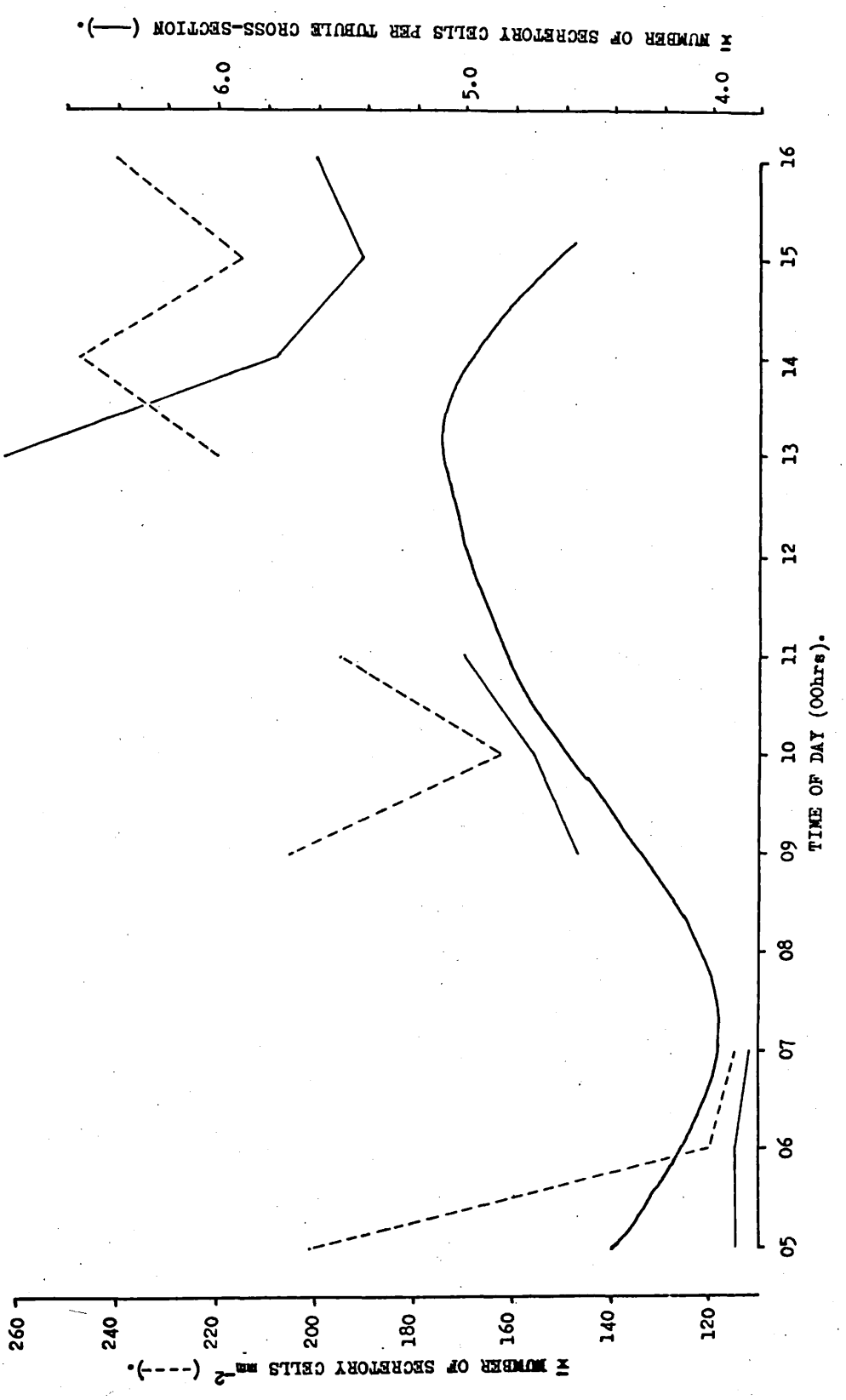


FIGURE 24. PHASIC ACTIVITY CHART DISPLAYING NUMBERS AND BASOPHILIA OF THE SECRETORY CELL IN RELATION TO THE TUBULE PHASE AND TIDAL CYCLE (SECOND SERIES TISSUES).

(one limpet examined each hour) and as a result little positive information can be extracted from them.

Dull purple cells were abundant for approximately one tidal cycle, from the time just after emersion (t=07 hours) to the next point of emersion (t=20 hours). Before and after these times the intensely basophilic cells were abundant. The change in basophilia does not relate to the overall abundance of the cells.

Examination of figure 24 shows similar trends. The digestive and fragmentation phases are the most frequently encountered and they are not related to the tidal cycle. The overall numbers of basophilic cells is low at low tide (t=05, 06 and 07 hours), but rises as the tide returns. Intensely basophilic cells occur approximately during emersion (t=05-11 hours), a similar result to that in figure 23.

ABBREVIATIONS USED IN PLATES 49-57.

ab.....Autophagic body.

ap....."Apical granule".

asc.....Amylase-secreting cell.

b.....Bleb.

bc.....Basophilic cell.

bl.....Basal lamina.

bs.....Blood space.

cc.....Ciliated columnar cell.

cd.....Cellular debris.

dc.....Digestive cell.

der.....Distended ER.

ecs.....Endocytotic canal system.

g.....Golgi complex.

hl.....Heterolysosome.

hp.....Heterophagosome.

jc.....Junctional complex.

l.....Lipid.

lf.....Lateral fold.

lp.....Lateral pouch.

lv1,2,3..Types of lucent vesicle.

m.....Mitochondrion.

mv.....Microvilli.

n.....Nucleus.

pl.....Primary lysosome.

rb.....Residual body.

rer.....Rough ER.

sgi1,2...Immature secretory granules.

sgm.....Mature secretory granules.

sp.....Spherule of digestive cell.

tl.....Lumen of tubule.

LEGENDS TO PLATES 49-53.

PHASIC ACTIVITY-THE OESOPHAGEAL GLAND.

PLATE 49.

A. Epithelium of the lateral folds during the secretory phase. x540.

B. As A, but during the heterophagic phase. x540.

Scale bars=37μm.

PLATE 50.

An amylase-secreting cell during the early secretory phase. 12K.

Scale bar=2μm.

PLATE 51 Secretory phase.

A. Formation of a bleb containing secretory granules. 7.25K.

B. Endocytotic canal system connected to distended RER. 86K.

C. Canal system connected to (arrows) mature secretory granules. 37K.

Scale bars: A=2μm; B=250nm; C=500nm.

PLATE 52 Heterophagic phase.

A. Type "1v1 and 2" vesicles. 4K.

B. Type "1v3" vesicles. 5.7K.

C. Residual body. 5.7K.

D. Golgi complex. 42K.

E. Primary lysosomes fusing with secretory granules. 65K.

F. Primary lysosomes fusing with a residual body. 42K.

Scale bars: A,B,C=4μm; D,F=500nm; E=250nm.

PLATE 53.

A necrotic amylase-secreting cell exhibiting an extreme condition of autophagy. 12K.

Scale bar=2μm.

LEGENDS TO PLATES 54-57.

PHASIC ACTIVITY-THE DIGESTIVE GLAND.

PLATE 54 Holding phase.

A. Light micrograph of a tubule stained with Mallory. x480.

B. Typical digestive cells.

Scale bars: A=42μm; B=10μm.

PLATE 55.

Digestive cells during the digesting phase. 3.1K.

Scale bar=6μm.

PLATE 56.

Digestive cells during a later stage of the digesting phase. 2.8K.

Scale bar=7μm.

PLATE 57.

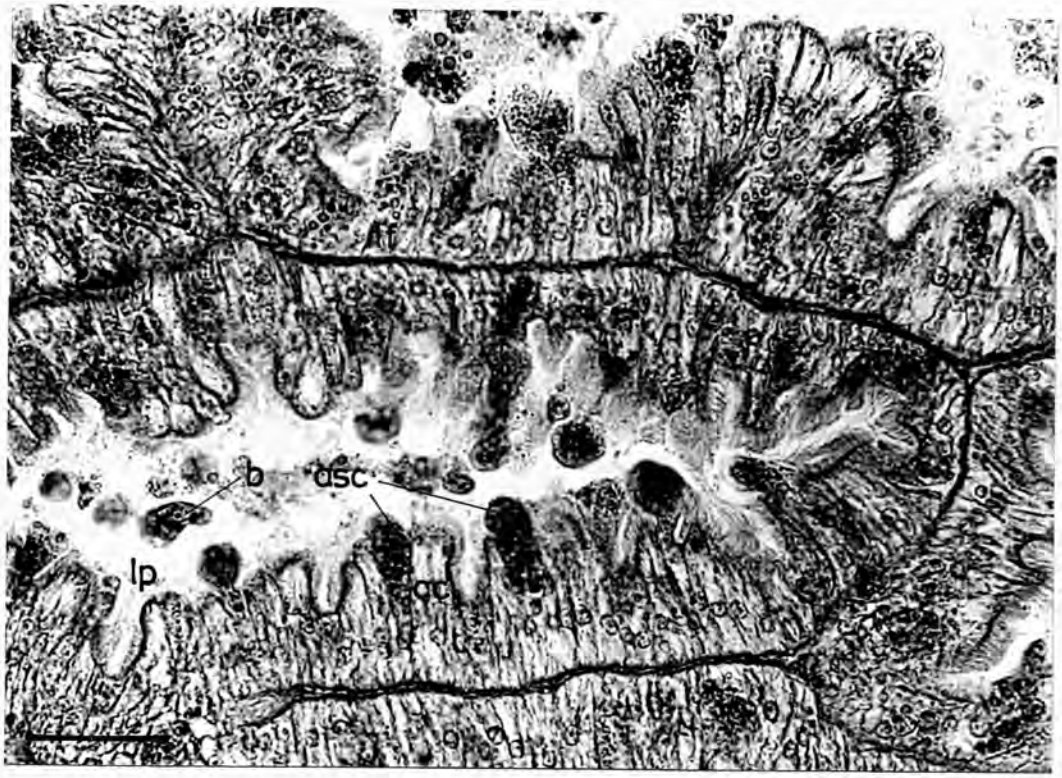
A. Light micrograph of a tubule during the late fragmentation phase. x550.

B. Abstriction of spherules from digestive cells during the spherulating phase. 5.3K.

C. A disintegrated tubule. 1.75K.

Scale bars: A=100μm; B=4μm; C=10μm.

PLATE 49



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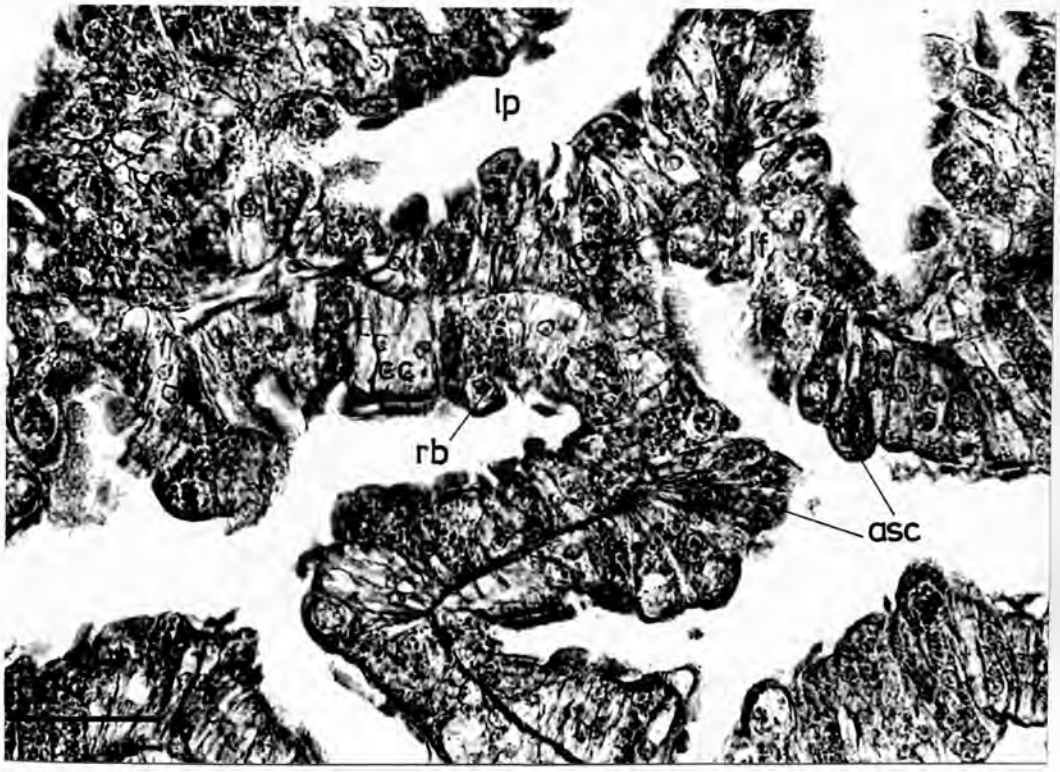


B

PLATE 50

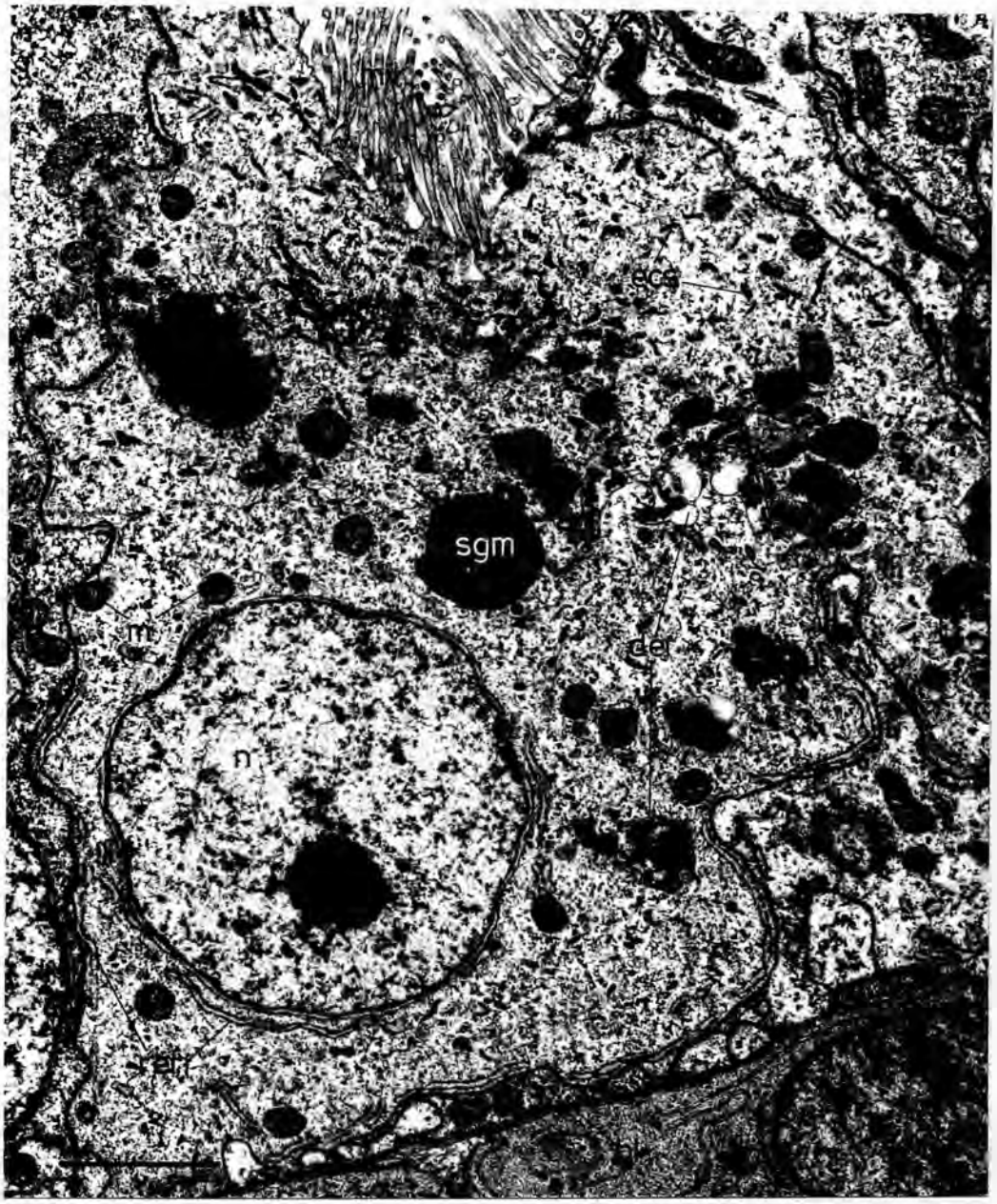
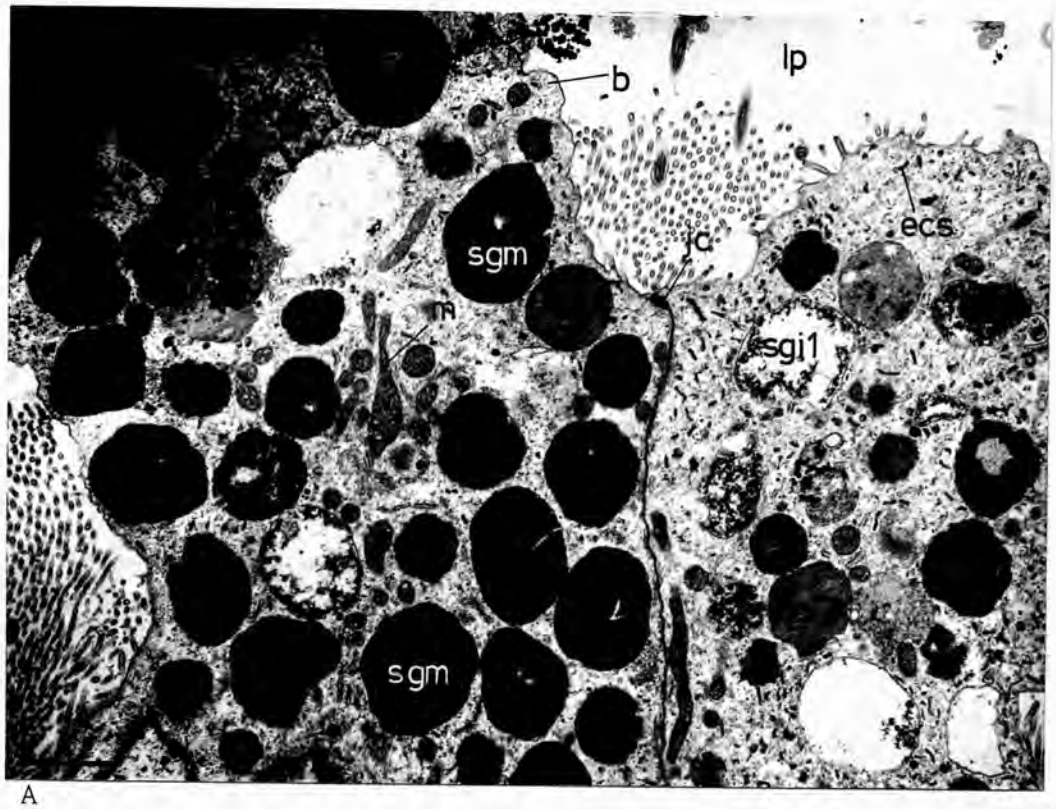


PLATE 51



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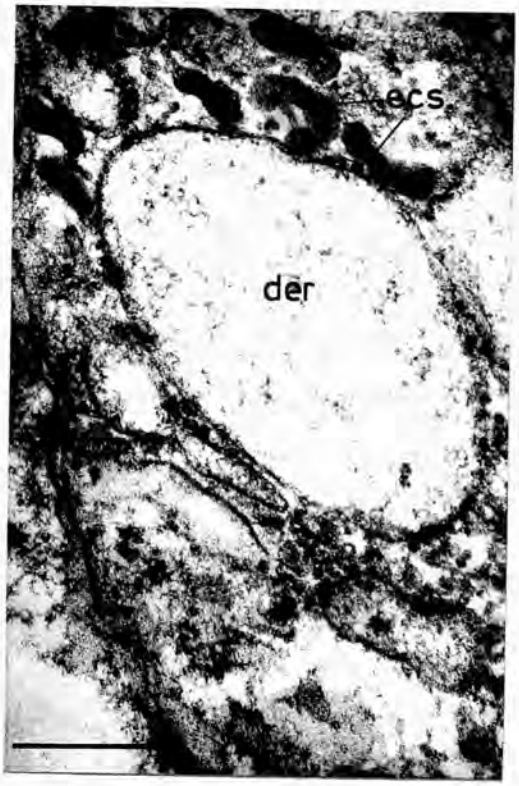

B

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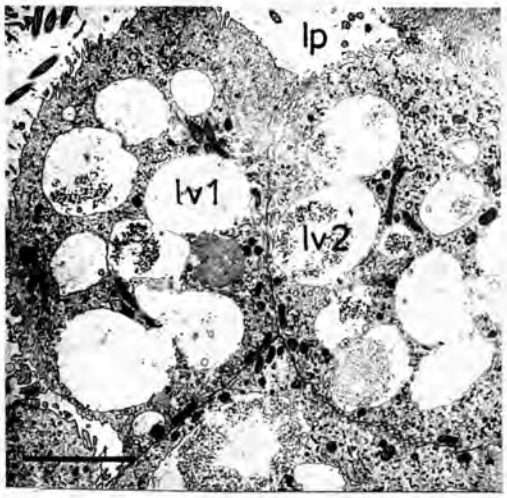
PLATE 52



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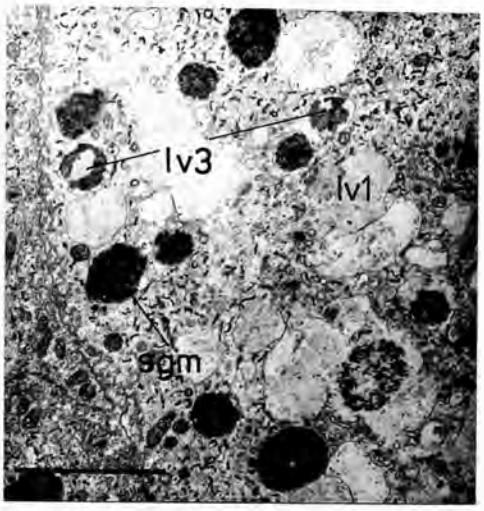

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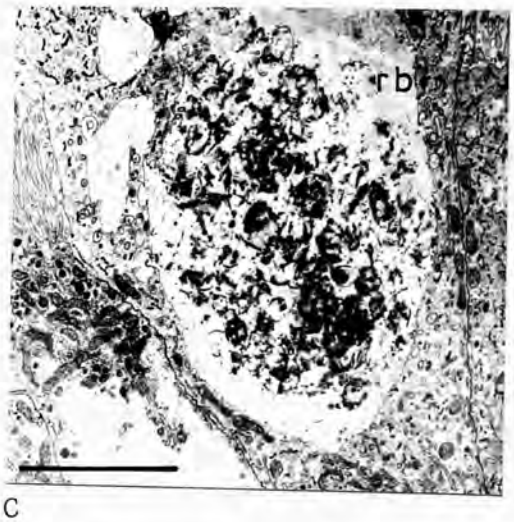

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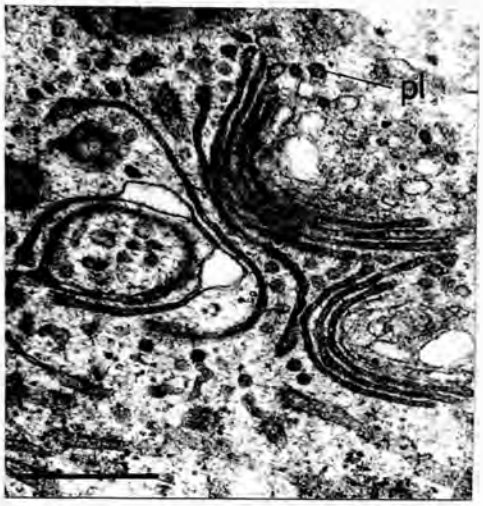

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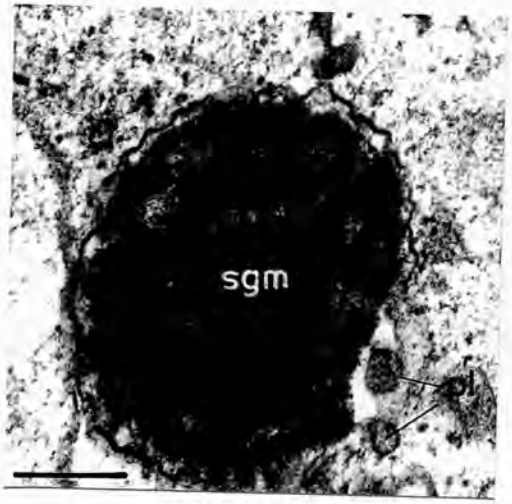

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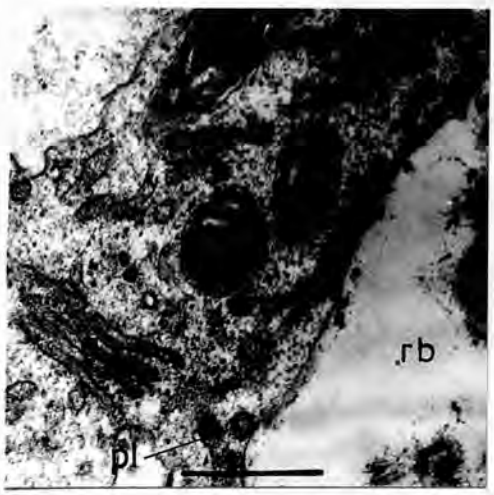

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PLATE 53

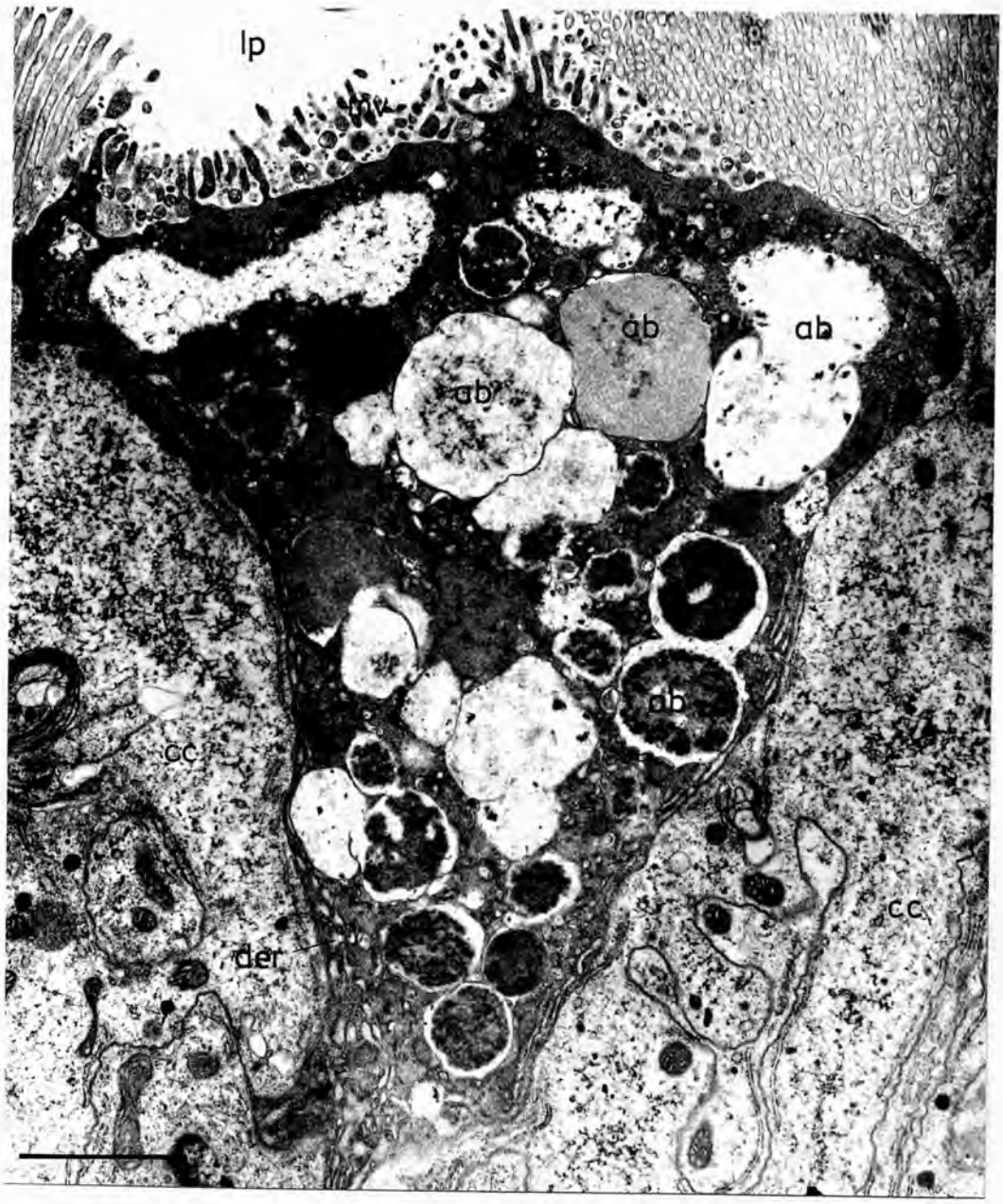
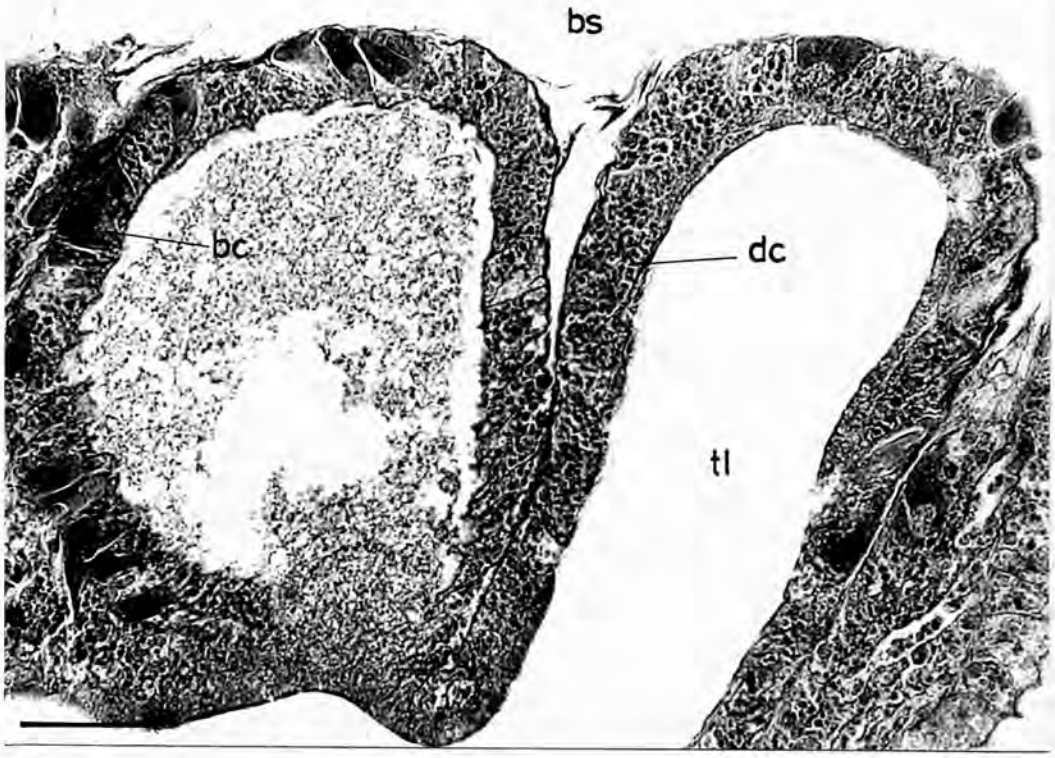


PLATE 54



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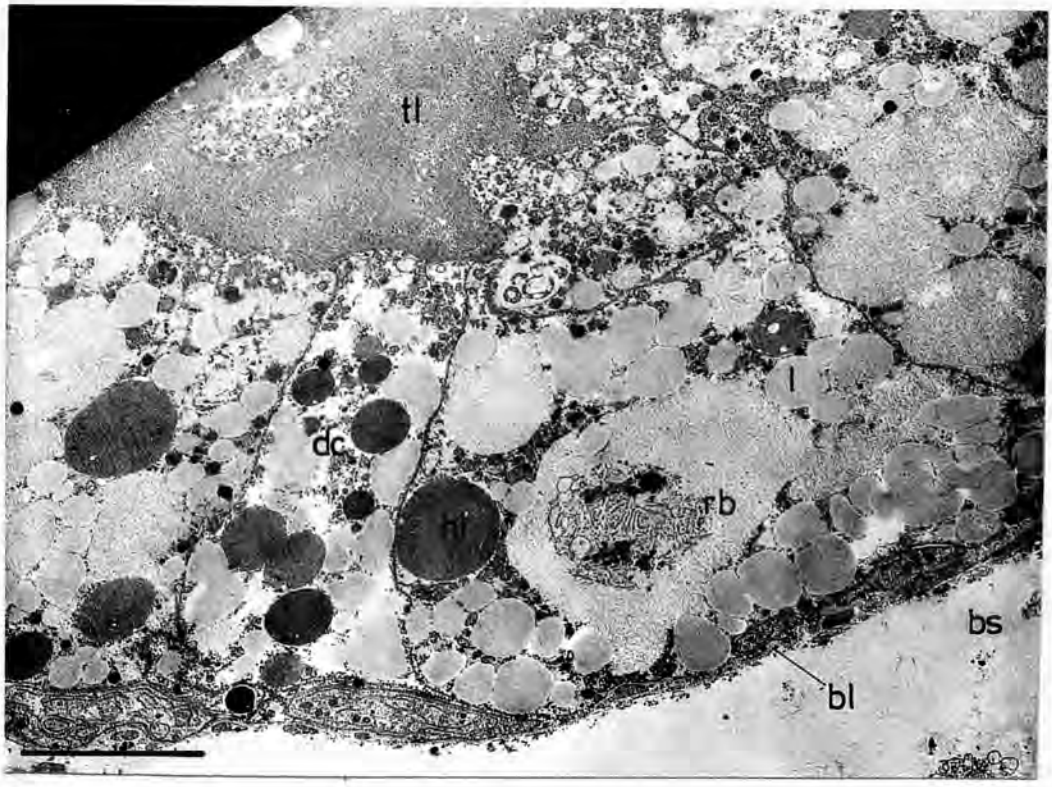


B

PLATE 55

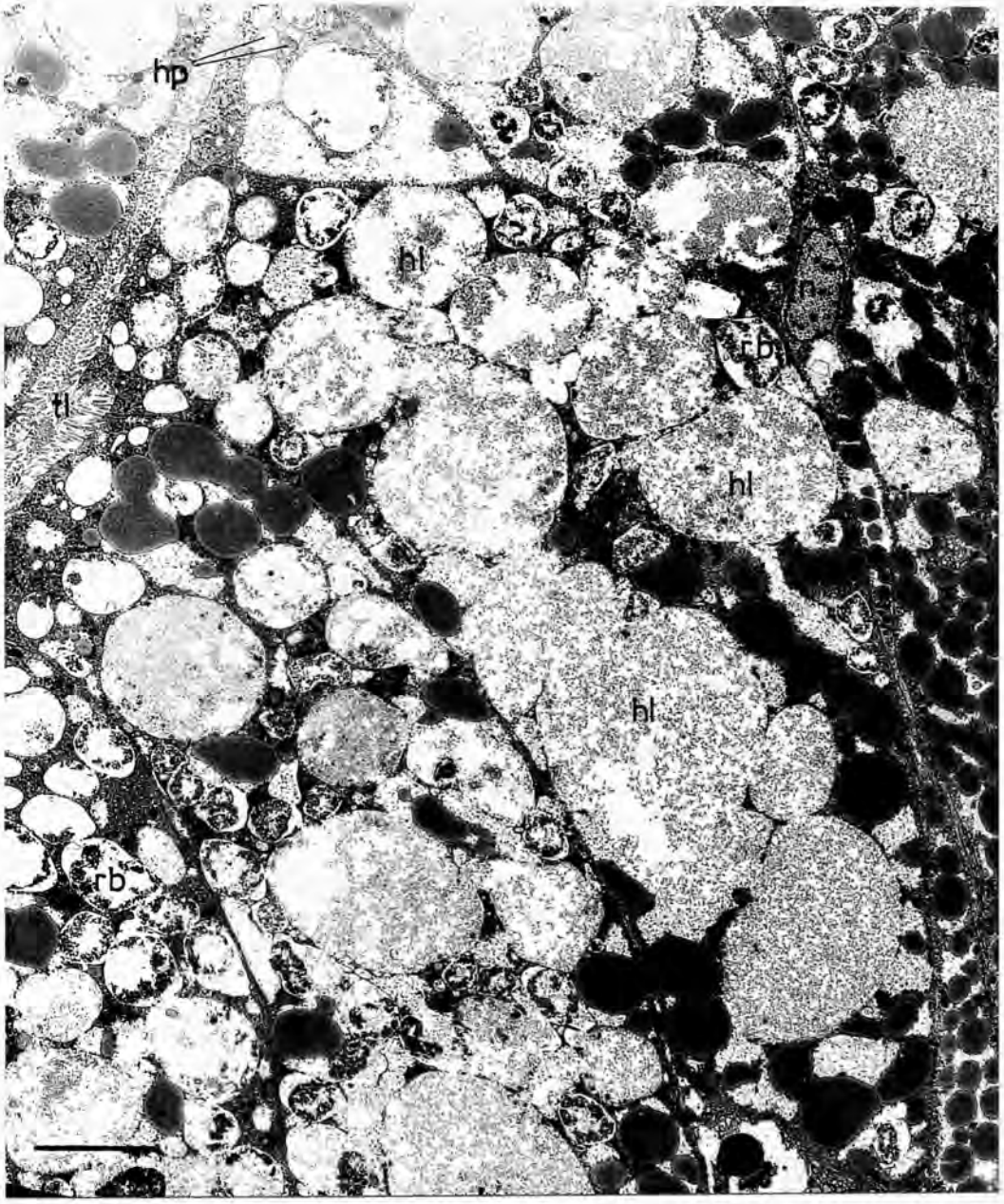


PLATE 56

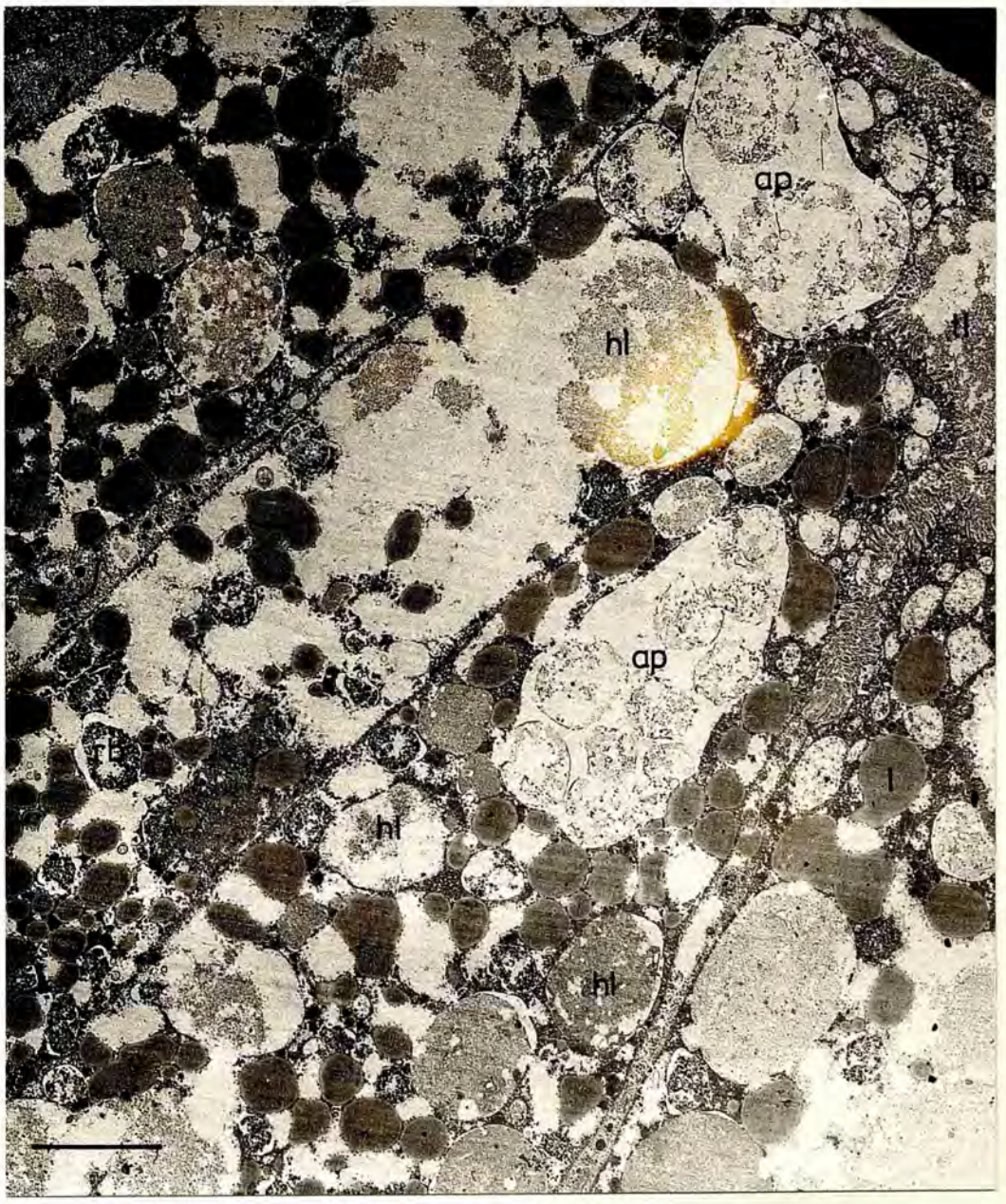
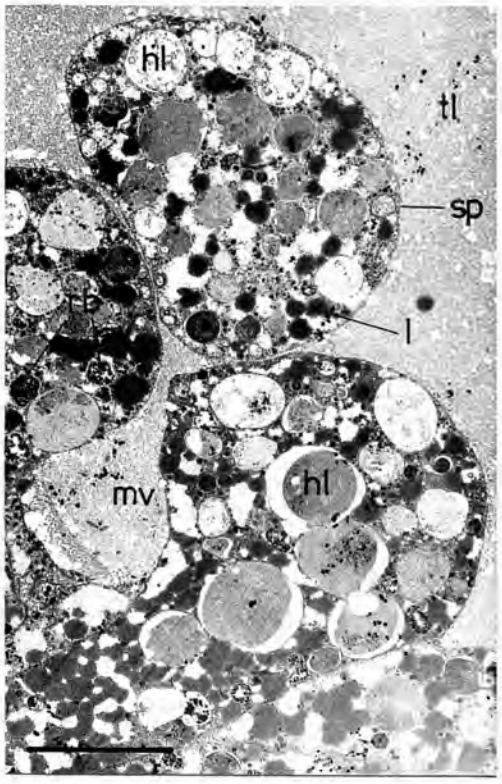


PLATE 57

A



B

C

RADIOTRACER EXPERIMENTS.

1-IN VIVO FEEDING EXPERIMENTS.

These pilot experiments were performed to determine if the gut can absorb D-glucose and if so which regions are involved. The first attempt (feeding limpets a 100µl meal with an activity of 5µCi ml^{-1} =10µMol L^{-1}) resulted in contamination of the control samples, poor counting efficiency due to colour quenching and detection of very little radioactivity, suggesting that the meal had been regurgitated. The results were considered invalid and the procedure repeated with modifications.

In the second experiment, limpets were fed a 10µl meal (206µCi ml^{-1} =412µMol L^{-1}) to prevent regurgitation caused by administering too large a meal. After feeding, each limpet was watched under a dissecting microscope to see if regurgitation occured and then placed in a closed bucket of aerated seawater (1L). Samples of this and contol seawater not exposed to radioactivity were taken and counted. Both samples gave higher counts than the backgrounds (control- 216 μl^{-1} =0.2µMol L^{-1}: experimental-432 μl^{-1} =0.4µMol $^{-1}$) suggesting that both were contaminated. The controls should not have been, but the experimental ones would be if the limpets were regurgitating. See appendix 1 for a discussion of the possibility of regurgitation and reasons for the unaccountability of much of the radioactivity.

The predicted outcome of these experiments is as follows. As the time after feeding increases, the gut fluid glucose level should fall if glucose is being absorbed, with a subsequent rise in the blood and tissue concentrations. Glucose should also appear in the epithelium of succesive regions of the gut in quantities reflecting their relative positions, if absorption occurs in preceding sections. The results of the second experiment are presented in table 18A and fig 25.

After 30 minutes and 1 hour, the oesophagus and intestine A

TABLE 18.

A). SUMMARISED RESULTS OF THE IN VIVO FEEDING EXPERIMENTS.

TIME AFTER FEEDING (hr)	SAMPLE	^{3}H-GLUCOSE CONTENT ($pMol\ mg^{-1}/\mu l^{-1}$)	^{3}H-GLUCOSE CONTENT OF ENTIRE SECTION (pMol)	TOTAL GLUCOSE CONTENT ($pMol\ mg^{-1}/\mu l^{-1}$)
0.5	BL	1.310	655.00	1.570
	GF	0.669	133.80	0.803
	OE	0.631	11.99	0.757
	IA	0.611	6.11	0.733
	IB	0.554	3.60	0.665
	IC	1.447	10.85	1.736
	ID	-	-	-
	IE	-	-	-
	DG	1.00	23.00	1.200
	TOTAL	6.222	844.35	7.466
1.0	BL	0.285	142.50	0.342
	GF	0.825	165.00	0.990
	OE	8.396	159.50	10.075
	IA	0.724	7.24	0.869
	IB	0.262	1.70	0.314
	IC	2.536	19.02	3.043
	ID	0.035	0.15	0.042
	IE	0.303	0.75	0.364
	DG	0.363	8.35	0.436
	TOTAL	13.729	504.21	16.475
2.0	BL	0.595	297.50	0.714
	GF	0.405	81.00	0.486
	OE	4.548	86.40	5.458
	IA	0.440	4.40	0.528
	IB	0.319	2.07	0.383
	IC	1.494	11.20	1.793
	ID	0.466	2.10	0.559
	IE	0.464	1.16	0.557
	DG	0.145	3.33	0.174
	TOTAL	8.876	489.16	10.651
4.0	BL	0.344	172.00	0.413
	GF	0.377	75.40	0.452
	OE	1.211	23.00	1.453
	IA	1.086	10.86	1.303
	IB	0.543	3.53	0.651
	IC	4.849	36.36	5.819
	ID	1.073	4.83	1.288
	IE	1.468	3.67	1.761
	DG	0.000	0.00	0.000
	TOTAL	10.951	329.65	13.141
8.0	BL	0.128	64.00	0.154
	GF	0.198	39.60	0.238
	OE	5.199	98.78	6.239
	IA	1.960	19.60	2.352
	IB	0.615	3.99	0.738
	IC	2.139	16.04	2.567
	ID	0.737	3.31	0.884
	IE	1.041	2.60	1.249
	DG	0.153	3.52	0.183
	TOTAL	12.17	251.44	14.604

CONTINUED....

TABLE 18 CONTINUED.

TIME AFTER FEEDING (hr)	SAMPLE	^{3}H-GLUCOSE CONTENT ($pMol\ mg^{-1}/\mu l^{-1}$)	^{3}H-GLUCOSE CONTENT OF ENTIRE SECTION (pMol)	TOTAL GLUCOSE CONTENT ($pMol\ mg^{-1}/\mu l^{-1}$)
16	BL	0.050	25.0	0.007
	GF	0.479	95.8	0.069
	OE	29.631	562.99	4.320
	IA	4.470	44.70	0.651
	IB	2.818	18.31	0.410
	IC	6.396	47.97	0.932
	ID	6.863	30.88	1.000
	IE	4.797	11.99	0.698
	DG	1.000	23.00	0.145
	TOTAL	56.50	860.64	8.228
32	BL	0.119	59.50	0.143
	GF	0.298	59.60	0.358
	OE	0.197	3.74	0.236
	IA	0.442	4.42	0.530
	IB	0.233	1.51	0.280
	IC	0.701	5.25	0.841
	ID	0.692	3.11	0.830
	IE	0.454	1.13	0.545
	DG	0.052	1.19	0.062
	TOTAL	3.188	139.45	3.825
CONTROL	BL	0.143	71.50	0.171
	GF	0.284	56.80	0.341
	OE	0.000	0.00	0.000
	IA	0.000	0.00	0.000
	IB	0.000	0.00	0.000
	IC	0.000	0.00	0.000
	ID	0.000	0.00	0.000
	IE	0.135	0.33	0.000
	DG	0.000	0.00	0.162
	TOTAL	0.562	128.63	0.000
				0.674
	EXPT SW	0.400	-	-
	CNTL SW	0.200	-	-

NOTATION:

BL...Blood, GF...Gut fluid, OE...Oesophagus, IA,B,C,D,E...Intestine sections, DG...Digestive gland, EXPT SW...Experimental sea water, CNTL SW...Control sea water.

The values in column 3 represent the tritiated glucose content of a mg or µl of the sample.
The values in column 4 represent the tritiated glucose content of the entire gut section, blood or gut fluid. They are obtained by multiplying the values in column 3 with the relevant weight or volume in table 18e.
The values in the last column reflect the total (tritiated and unlabelled) glucose.

B). TYPICAL WEIGHTS OF GUT SECTIONS AND APPROXIMATIONS OF THE BLOOD AND GUT VOLUMES FOR LIMPETS WITH SHELLS 35mm LONG.

Gut volume excluding the buccal cavity and digestive gland.....200µl.

Blood volume approximation.....500µl.

Oesophagus..... 19mg

Intestine A..... 10mg

" B..... 6.5mg

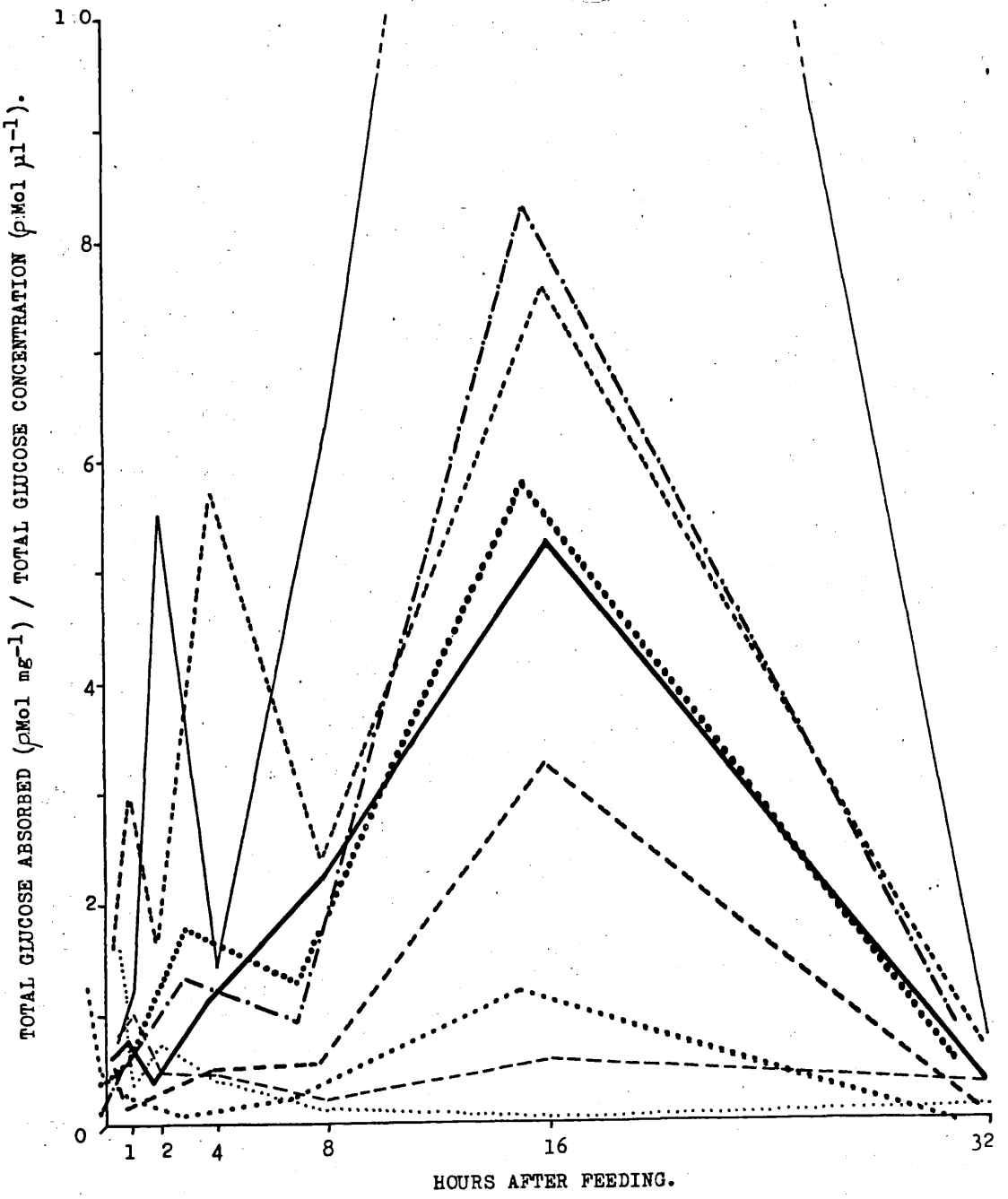
" C..... 7.5mg

" D..... 4.5mg

" E..... 2.5mg

Digestive gland..... 23mg

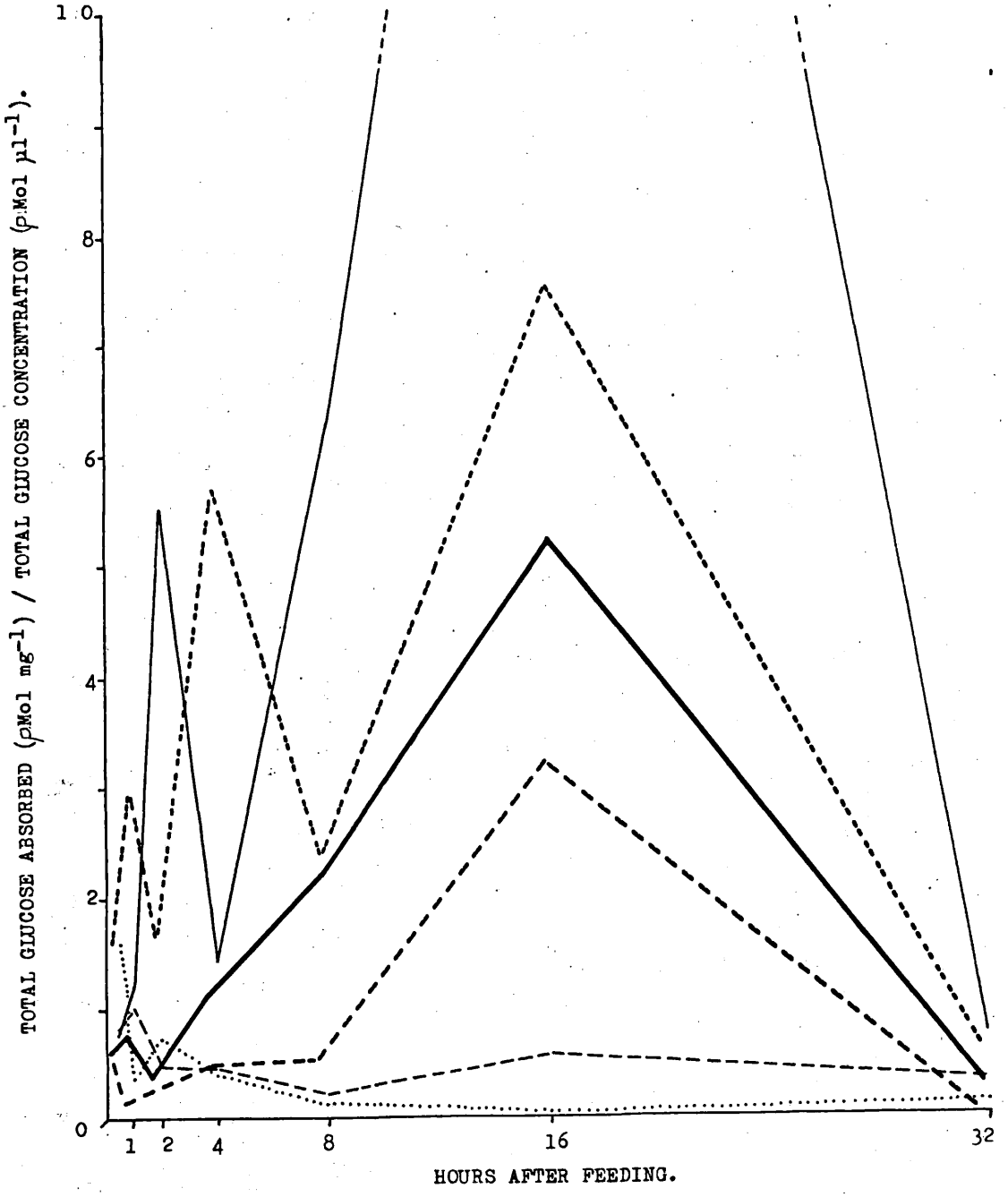
FIGURE 25 . IN VIVO FEEDING EXPERIMENTS:GLUCOSE UPTAKE BY THE GUT.



NOTATION:

- ..... BLOOD.
- - - - GUT FLUID.
- ——— OESOPHAGUS.
- ——— INTESTINE A.
- – – – " B.
- – – – – INTESTINE C.
- —·—· " D.
- ····· " E.
- ····· DIGESTIVE GLAND.

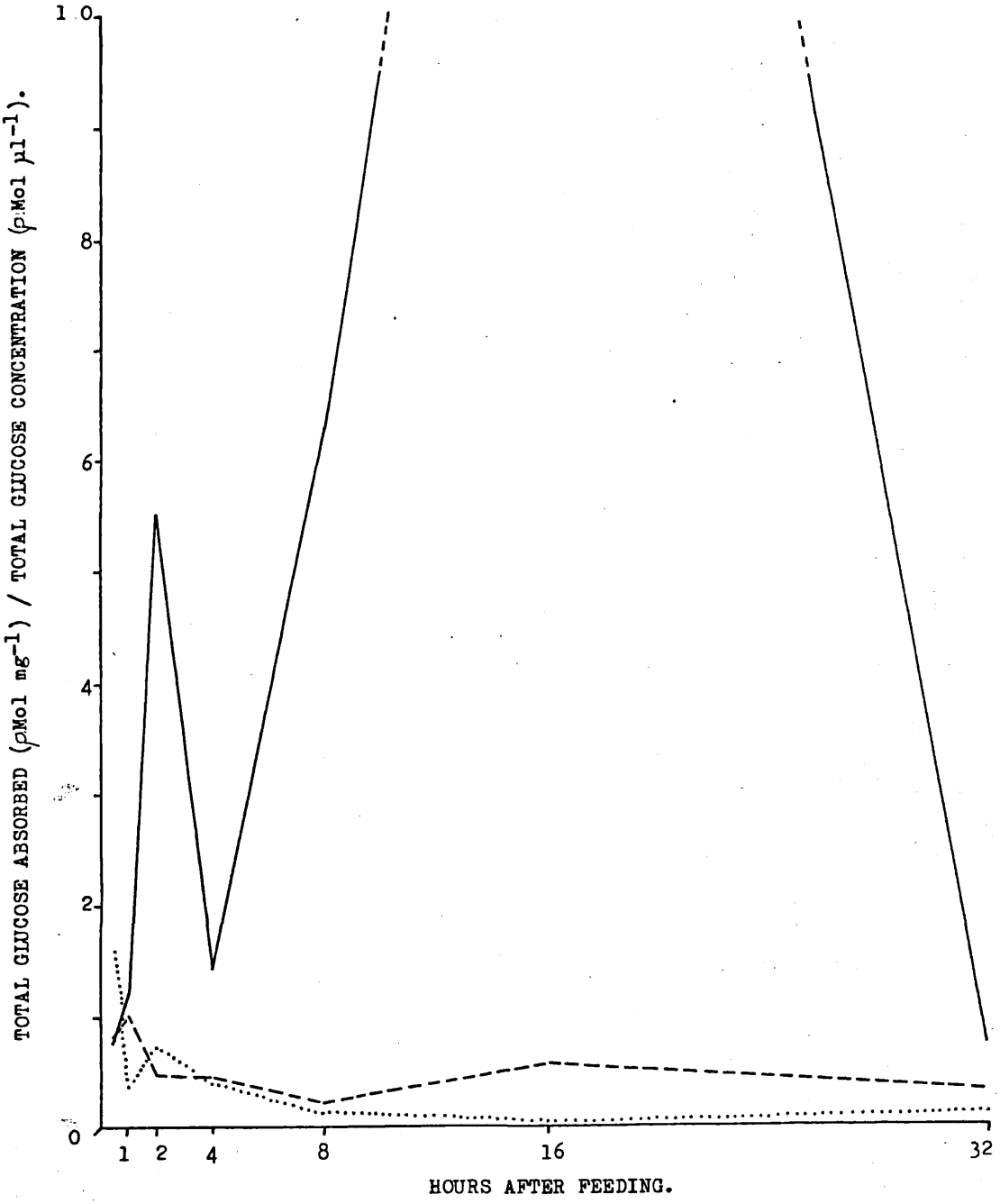
FIGURE 25 . IN VIVO FEEDING EXPERIMENTS:GLUCOSE UPTAKE BY THE GUT.



NOTATION:

..... BLOOD.
- - - - GUT FLUID.
——— OESOPHAGUS.
——— INTESTINE A.
- - - " B.
- - - - - INTESTINE C.
—·—· " D.
····· " E.
····· DIGESTIVE GLAND.

FIGURE 25 . IN VIVO FEEDING EXPERIMENTS:GLUCOSE UPTAKE BY THE GUT.



NOTATION:

..... BLOOD.
- - - - GUT FLUID.
——— OESOPHAGUS.
——— INTESTINE A.
- - - " B.
----- INTESTINE C.
—·—· " D.
•••••• " E.
····· DIGESTIVE GLAND.

contained quantities of glucose similar to those in the gut fluid sample. Oesophageal values peaked after 2 and 16 hours and then fell sharply. Glucose levels in sections A, B, D and E gradually peaked after 16 hours and then fell. Sections D and E were not sampled after 30 minutes because it was thought that the meal would not have reached these regions in this time, the low levels obtained in these sections after 1 and 2 hours support this view. Samples from section C showed greater radioactivity after 1, 4 and 16 hours, and digestive gland after 30 minutes and 16 hours, see appendix 1B.

The only sound conclusion that can be drawn from these results is that under in vivo conditions, small quantities of glucose are absorbed by all sections of the gut.

2-GLUCOSE-FLUX EXPERIMENTS. In these and the net water movement experiments, the luminal compartment represents the gut lumen lined by the intestinal epithelium and the external or haemocoelic compartment, faced by the subepithelial muscle layer, represents the blood space.

The statistical analyses (table 19) of the data presented in tables 20-23 and figs 26-29, indicate that there was not a net transport of glucose from the luminal to the haemocoelic compartment. The significance of the difference between the means of the two treatments (with or without phloridzin) was reduced because of the large variation within each treatment. In view of the statistical results, one can only conclude that the intestinal epithelium absorbed glucose under in vitro conditions. The results may have been significant if the phloridzin concentration had been greater or the variation within treatments less. See appendix 2.

3-GLUCOSE ABSORPTION EXPERIMENTS. The previous results indicate that all regions of the gut absorb glucose, the following experiments examined various physiological aspects of the mechanisms.

A).ABSORPTION. Pieces of oesophagus, anterior and posterior intestine, salivary and digestive gland accumulated glucose from the

TABLE 19.

SUMMARISED STATISTICAL ANALYSES OF THE GLUCOSE FLUX EXPERIMENTS.

ANTERIOR INTESTINE

TEST	TREATMENTS: WITHOUT PHLORIDZIN	TREATMENTS: WITH PHLORIDZIN	SIGNIFICANCE (P)
ANOVAR	LUMINAL [GLUCOSE]	LUMINAL [GLUCOSE]	> 0.05
t TEST			> 0.05
ANOVAR	HAEMOCOELIC [GLUCOSE]	HAEMOCOELIC [GLUCOSE]	> 0.05
t TEST			> 0.05
ANOVAR	TISSUE [GLUCOSE]	TISSUE [GLUCOSE]	> 0.05
t TEST			> 0.05

POSTERIOR INTESTINE

ANOVAR	LUMINAL [GLUCOSE]	LUMINAL [GLUCOSE]	> 0.05
t TEST			> 0.05
ANOVAR	HAEMOCOELIC [GLUCOSE]	HAEMOCOELIC [GLUCOSE]	> 0.05
t TEST			> 0.05
ANOVAR	TISSUE [GLUCOSE]	TISSUE [GLUCOSE]	> 0.05
t TEST			> 0.05

ANOVAR is an abbreviation for Analysis of Variance.

P values < 0.05 were taken as significant.

TABLE 20.

SUMMARISED GLUCOSE FLUX RESULTS: ANTERIOR INTESTINE WITHOUT PHLORIDZIN.

EXPT	SAMPLE	^{3}H-GLUCOSE CONTENT (pMol μl^{-1}/mg^{-1})	TOTAL GLUCOSE CONTENT (mMol L^{-1}) (nMol mg^{-1})	$\bar{x} \pm \frac{s}{\sqrt{n}}$
1	PrIn	0.435	0.870	0.907 ± 0.04
	PrIn	0.472	0.944	
	PoL	0.438	0.876	0.904 ± 0.03
	PoL	0.466	0.932	
	PoH	0.460	0.920	0.861 ± 0.06
	PoH	0.401	0.802	
2	PrIn	0.394	0.788	0.825 ± 0.04
	PrIn	0.431	0.862	
	PoL	0.231	0.462	0.615 ± 0.15
	PoL	0.384	0.768	
	PoH	0.350	0.700	0.689 ± 0.01
	PoH	0.339	0.678	
3	PrIn	0.445	0.890	0.875 ± 0.04
	PrIn	0.430	0.860	
	PoL	0.304	0.608	0.420 ± 0.18
	PoL	0.116	0.232	
	PoH	0.305	0.610	0.661 ± 0.05
	PoH	0.356	0.712	
4	PrIn	0.386	0.772	0.849 ± 0.08
	PrIn	0.463	0.926	
	PoL	0.350	0.700	0.700 -
	PoH	0.329	0.658	0.716 ± 0.06
	PoH	0.387	0.774	
5	PrIn	0.457	0.914	0.945 ± 0.03
	PrIn	0.488	0.976	
	PoL	0.397	0.794	0.800 ± 0.01
	PoL	0.403	0.806	
	PoH	0.436	0.872	0.852 ± 0.02
	PoH	0.416	0.832	
6	PrIn	0.451	0.902	0.941 ± 0.04
	PrIn	0.490	0.980	
	PoL	0.301	0.602	0.665 ± 0.06
	PoL	0.364	0.728	
	PoH	0.389	0.778	0.764 ± 0.01
	PoH	0.375	0.750	
7	PrIn	0.521	1.042	1.052 ± 0.01
	PrIn	0.531	1.062	
	PoL	0.284	0.568	0.633 ± 0.06
	PoL	0.349	0.698	
	PoH	0.416	0.832	0.834 ± 0.0
	PoH	0.418	0.836	
8	PrIn	0.507	1.014	1.046 ± 0.03
	PrIn	0.539	1.078	
	PoL	0.287	0.574	0.527 ± 0.05
	PoL	0.240	0.480	
	PoH	0.392	0.780	0.785 ± 0.00
	PoH	0.395	0.790	
1	TS	2.404 ± 0.10	4.808 ± 0.20	Same as for column 4.
2	TS	2.292 0.07	4.584 0.14	
3	TS	1.714 0.07	3.428 0.14	
4	TS	1.638 0.02	3.276 0.04	
5	TS	3.692 0.28	7.384 0.56	
6	TS	5.413 0.42	10.826 0.84	
7	TS	2.535 0.20	5.070 0.40	
8	TS	2.853 0.04	5.706 0.08	
Σ(1-8)	PrIn	-	-	0.930 ± 0.03
	PoL	-	-	0.658 0.05
	PoH	-	-	0.770 0.03
	TS	-	-	5.635 0.87

NOTATION:

PrIn.....Pre-Incubation.
PoL.....Post-Incubation Luminal Compartment.
PoH.....Post-Incubation Haemocoelic Compartment.
TS.....Residual in Tissue.

TABLE 21.

SUMMARISED GLUCOSE FLUX RESULTS: ANTERIOR INTESTINE WITH PHLORIDZIN.

EXPT	SAMPLE	^{3}H-GLUCOSE CONTENT (pMol $\mu l^{-1}/mg^{-1}$)	TOTAL GLUCOSE CONTENT (mMol L^{-1}) (nMol mg^{-1})	$\bar{x} \pm \frac{s}{\sqrt{n}}$
1	PrIn	0.599	1.198	
	PrIn	0.520	1.040	1.119 ± 0.08
	PoL	0.449	0.898	
	PoL	0.383	0.766	0.832 ± 0.07
	PoH	0.456	0.912	
	PoH	0.410	0.820	0.866 ± 0.05
2	PrIn	0.487	0.974	
	PrIn	0.543	1.086	1.030 ± 0.06
	PoL	0.323	0.646	
	PoL	0.336	0.672	0.659 ± 0.01
	PoH	0.484	0.968	
	PoH	0.459	0.918	0.943 ± 0.03
3	PrIn	0.522	1.044	
	PrIn	0.506	1.012	1.03 ± 0.02
	PoL	0.360	0.720	
	PoL	0.348	0.696	0.708 ± 0.01
	PoH	0.399	0.798	
	PoH	0.358	0.716	0.757 ± 0.04
4	PrIn	0.505	1.010	
	PrIn	0.534	1.068	1.039 ± 0.03
	PoL	0.371	0.742	
	PoL	0.341	0.682	0.712 ± 0.03
	PoH	0.385	0.770	
	PoH	0.366	0.732	0.751 ± 0.02
5	PrIn	0.519	1.038	
	PrIn	0.514	1.028	1.033 ± 0.01
	PoL	0.328	0.656	
	PoL	0.500	1.000	0.828 ± 0.17
	PoH	0.409	0.818	
	PoH	0.407	0.814	0.816 ± 0.00
6	PrIn	0.498	0.996	
	PrIn	0.526	1.052	1.024 ± 0.03
	PoL	0.316	0.632	
	PoL	0.324	0.648	0.640 ± 0.01
	PoH	0.373	0.746	
	PoH	0.543	1.086	0.916 ± 0.17
7	PrIn	0.501	1.002	
	PrIn	0.463	0.926	0.964 ± 0.04
	PoL	0.330	0.660	
	PoL	0.516	1.032	0.846 ± 0.18
	PoH	0.388	0.776	
	PoH	0.411	0.822	0.799 ± 0.02
8	PrIn	0.449	0.898	
	PrIn	0.531	1.062	0.980 ± 0.08
	PoL	0.383	0.766	
	PoL	0.413	0.826	0.796 ± 0.03
	PoH	0.393	0.786	
	PoH	0.377	0.754	0.770 ± 0.02
1	TS	2.220 ± 0.09	4.440 ± 0.18	
2	TS	2.990 0.05	5.980 0.10	Same as for column 4.
3	TS	2.650 0.05	5.300 0.10	
4	TS	3.650 0.29	7.300 0.58	
5	TS	2.880 0.13	5.760 0.26	
6	TS	3.210 0.16	6.420 0.32	
7	TS	0.611 0.01	1.222 0.02	
8	TS	0.691 0.01	1.382 0.02	
Σ(1-8)	PrIn	-	-	1.027 ± 0.02
	PoL	-	-	0.755 0.03
	PoH	-	-	0.827 0.03
	TS	-	-	4.725 0.80

TABLE 22.

SUMMARISED GLUCOSE FLUX RESULTS: POSTERIOR INTESTINE WITHOUT PHLORIDZIN.

EXPT	SAMPLE	^{3}H-GLUCOSE CONTENT (pMol $\mu l^{-1}/mg^{-1}$)	TOTAL GLUCOSE CONTENT (mMol L^{-1}) (nMol mg^{-1})	$\bar{x} \pm \frac{s}{\sqrt{n}}$
1	PrIn	0.650	1.300	1.3
	PoL	0.429	0.858	0.858
	PoH	0.518	1.036	
	PoH	0.505	1.010	1.023 ± 0.01
2	PrIn	0.597	1.194	
	PrIn	0.584	1.168	1.181 ± 0.01
	PoL	0.461	0.922	0.922
	PoH	0.427	0.854	
	PoH	0.476	0.952	0.903 ± 0.05
3	PrIn	0.568	1.136	
	PrIn	0.594	1.188	1.162 ± 0.03
	PoL	0.373	0.746	0.764
	PoH	0.445	0.890	
	PoH	0.370	0.740	0.815 ± 0.07
4	PrIn	0.568	1.136	
	PrIn	0.594	1.188	1.162 ± 0.03
	PoL	0.426	0.852	0.852
	PoH	0.467	0.934	
	PoH	0.264	0.528	0.731 ± 0.20
5	PrIn	0.419	0.838	
	PrIn	0.379	0.758	0.798 ± 0.04
	PoL	0.320	0.640	0.640
	PoH	0.402	0.804	
	PoH	0.353	0.706	0.755 ± 0.05
6	PrIn	0.497	0.994	
	PrIn	0.510	1.020	1.007 ± 0.01
	PoL	0.231	0.462	0.462
	PoH	0.381	0.762	
	PoH	0.349	0.698	0.730 ± 0.03
7	PrIn	0.514	1.028	
	PrIn	0.538	1.076	1.052 ± 0.02
	PoL	0.339	0.678	0.678
	PoH	0.375	0.750	
	PoH	0.409	0.818	0.784 ± 0.03
8	PrIn	0.534	1.068	
	PrIn	0.521	1.042	1.055 ± 0.01
	PoL	0.268	0.536	0.563
	PoH	0.371	0.741	
	PoH	0.381	0.762	0.751 ± 0.01
1	TS	5.495 ± 0.05	10.990 ± 0.10	Same as for oolumn 4.
2	TS	2.364 0.11	4.728 0.22	
3	TS	2.814 0.08	5.628 0.16	
4	TS	2.350 0.06	4.700 0.12	
5	TS	2.685 0.03	5.370 0.06	
6	TS	4.542 0.07	9.080 0.15	
7	TS	1.949 0.05	3.898 0.09	
8	TS	3.685 0.03	7.370 0.07	
Σ(1-8)	PrIn	-	-	1.089 ± 0.05
	PoL	-	-	0.711 0.05
	PoH	-	-	0.811 0.04
	TS	-	-	6.470 0.87

TABLE 23.

SUMMARISED GLUCOSE FLUX RESULTS: POSTERIOR INTESTINE WITH PHLORIDZIN.

EXPT	SAMPLE	^{3}H-GLUCOSE CONTENT (pMol $\mu l^{-1}/mg^{-1}$)	TOTAL GLUCOSE CONTENT (mMol L^{-1}) (nMol mg^{-1})	$\bar{x} \pm \frac{s}{\sqrt{n}}$
	PrIn	0.485	0.970	
	PrIn	0.535	1.070	1.020 ± 0.05
1	PoL	0.389	0.778	0.778
	PoH	0.365	0.730	
	PoH	0.444	0.888	0.809 ± 0.08
	PrIn	0.570	1.040	
	PrIn	0.478	0.956	0.998 ± 0.04
2	PoL	0.362	0.724	0.724
	PoH	0.411	0.822	
	PoH	0.419	0.838	0.830 ± 0.01
	PrIn	0.537	1.074	
	PrIn	0.501	1.002	1.038 ± 0.04
3	PoL	0.339	0.678	0.678
	PoH	0.437	0.874	
	PoH	0.389	0.778	0.826 ± 0.05
	PrIn	0.496	0.992	
	PrIn	0.477	0.954	0.973 ± 0.02
4	PoL	0.285	0.570	0.570
	PoH	0.468	0.936	
	PoH	0.427	0.854	0.895 ± 0.04
	PrIn	0.505	1.010	
	PrIn	0.543	1.086	1.048 ± 0.04
5	PoL	0.379	0.758	0.758
	PoH	0.426	0.852	
	PoH	0.448	0.896	0.874 ± 0.02
	PrIn	0.528	1.056	
	PrIn	0.509	1.018	1.037 ± 0.02
6	PoL	0.286	0.572	0.572
	PoH	0.429	0.858	
	PoH	0.376	0.752	0.805 ± 0.05
	PrIn	0.539	1.078	
	PrIn	0.538	1.076	1.077 ± 0.00
7	PoL	0.380	0.760	0.760
	PoH	0.395	0.790	
	PoH	0.419	0.838	0.814 ± 0.02
	PrIn	0.516	1.032	
	PrIn	0.557	1.114	1.073 ± 0.04
8	PoL	0.298	0.596	0.596
	PoH	0.346	0.692	
	PoH	0.354	0.708	0.700 ± 0.01
1	TS	4.509 ± 0.21	9.018 ± 0.42	
2	TS	2.411 0.10	4.822 0.20	Same as for
3	TS	3.741 0.05	7.482 0.10	column 4.
4	TS	2.181 0.09	4.362 0.18	
5	TS	1.937 0.05	3.874 0.10	
6	TS	3.744 0.17	7.488 0.33	
7	TS	2.040 0.06	4.080 0.12	
8	TS	4.139 0.08	8.278 0.16	
	PrIn	-	-	1.033 ± 0.01
(1-8)	PoL	-	-	0.679 0.03
	PoH	-	-	0.819 0.02
	TS	-	-	6.174 0.74

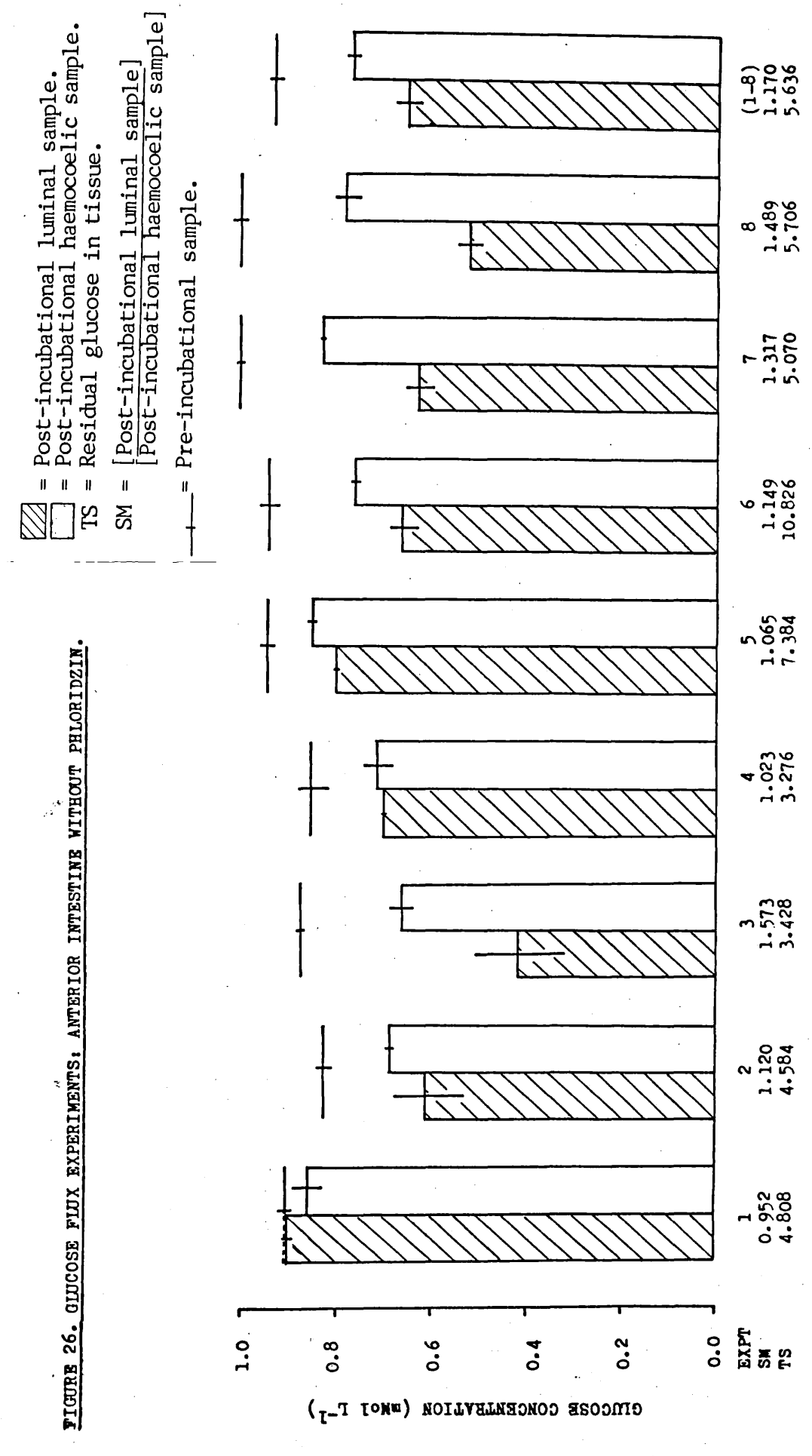


FIGURE 26. GLUCOSE FLUX EXPERIMENTS: ANTERIOR INTESTINE WITHOUT PHLORIDZIN.

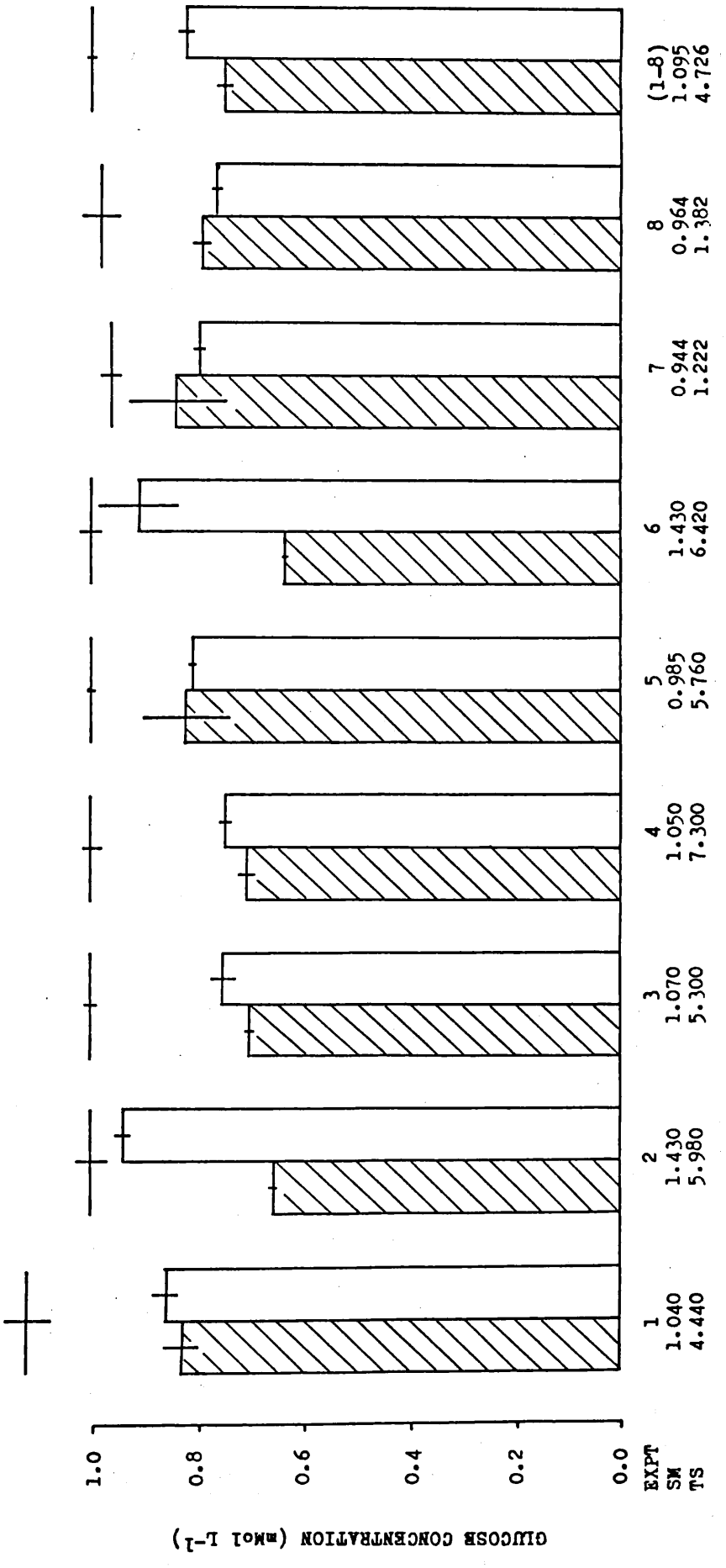


FIGURE 27. GLUCOSE FLUX EXPERIMENTS: ANTERIOR INTESTINE WITH PHLORIDZIN.

Notation as for figure 26.

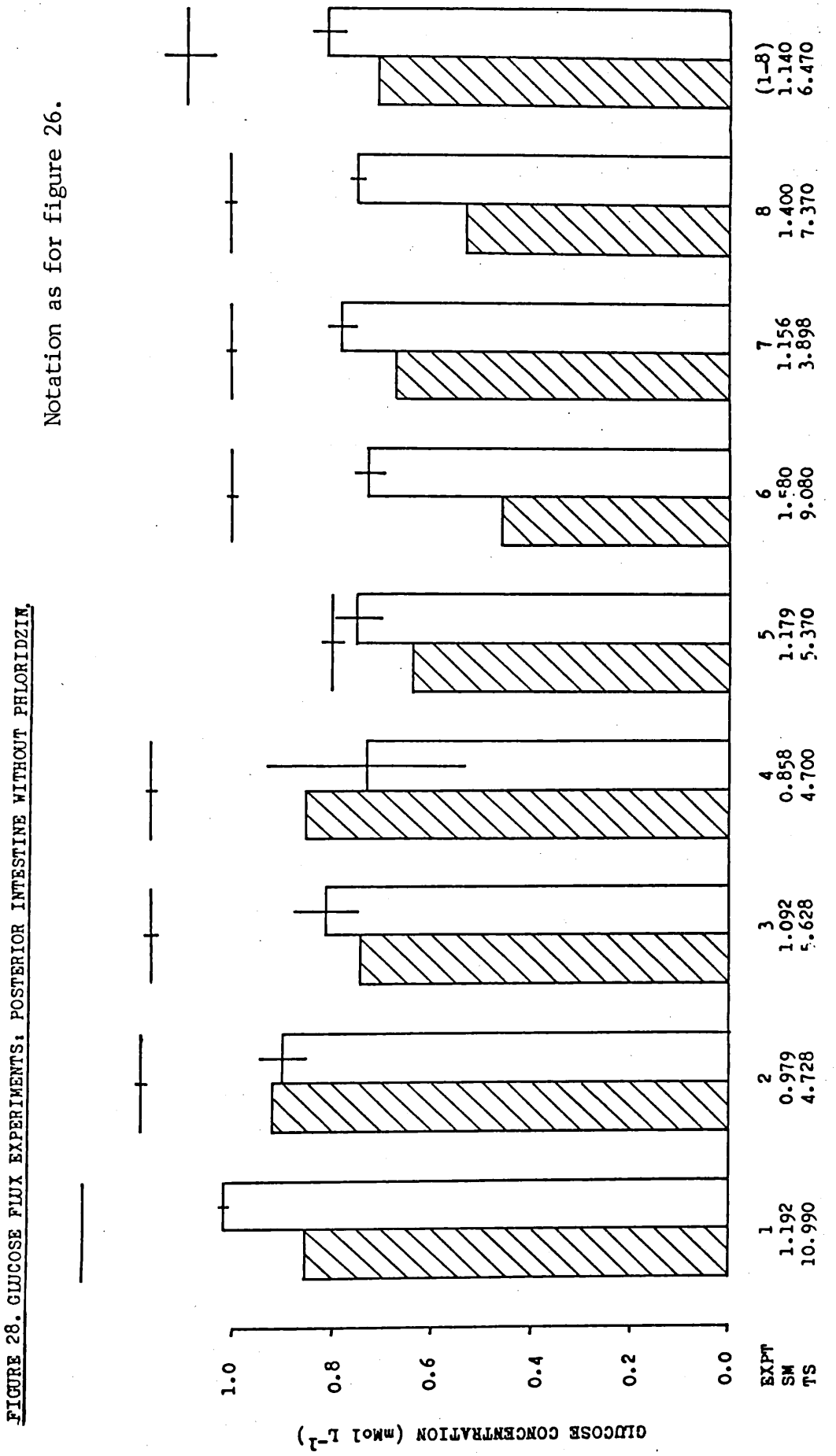


FIGURE 28. GLUCOSE FLUX EXPERIMENTS: POSTERIOR INTESTINE WITHOUT PHLORIDZIN.

Notation as for figure 26.

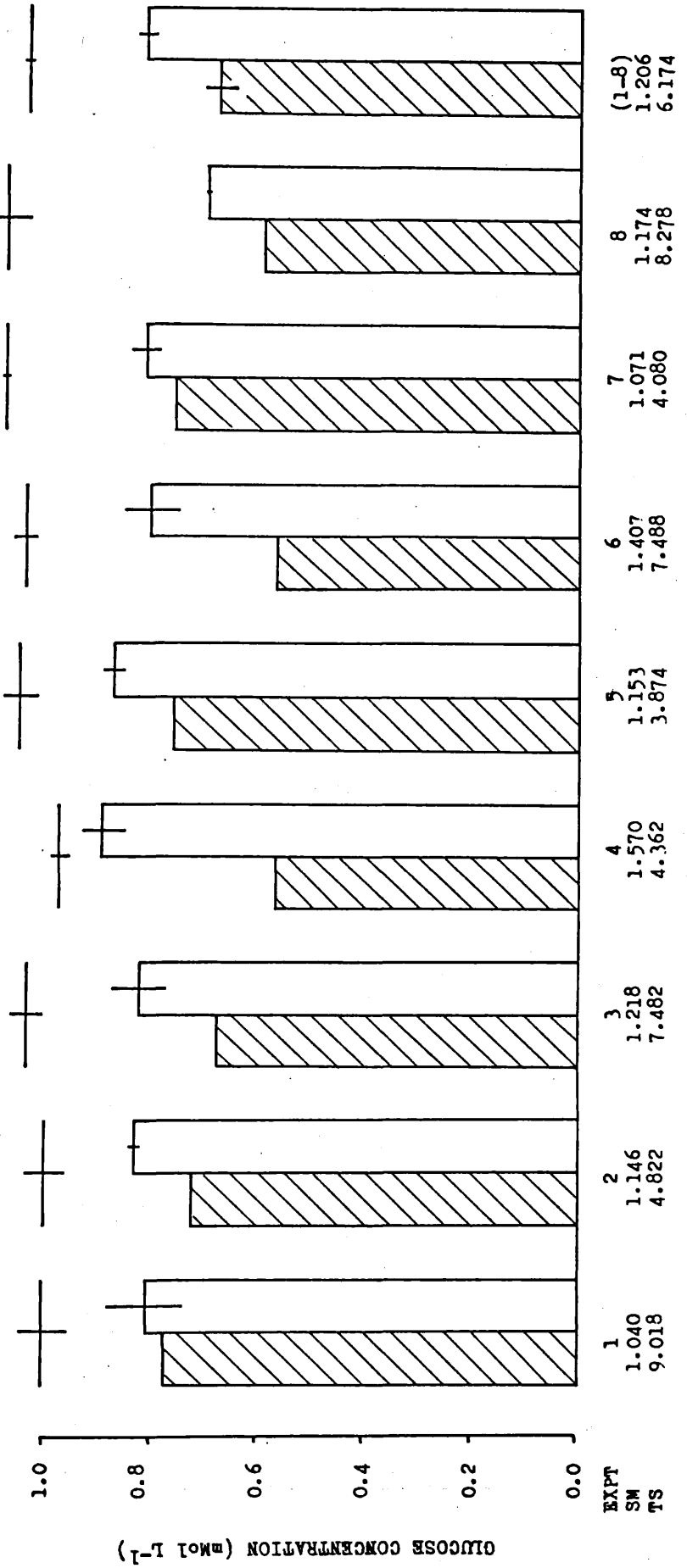


FIGURE 29. GLUCOSE FLUX EXPERIMENTS: POSTERIOR INTESTINE WITH PHLORIDZIN.

Notation as for figure 26.

incubation medium (fig 30A, table 24). Smaller quantities of glucose were absorbed by the two glandular tissues; this can be attributed to a reduced surface area:volume ratio than in the gut sections and also to the effects of unstirred layers in the digestive gland tubules, due to reduced ciliation. Glucose absorbed by the salivary gland represents uptake from the external compartment.

B).INHIBITORS. The effect of 2,4-DNP and phloridzin was significantly to reduce the glucose absorbed by all the tissues except salivary gland (figs 30B & C, tables 24 & 25A). 2,4-DNP reduced absorption by between 65-78% of the control values, suggesting that the absorption mechanism is linked, either directly or indirectly, to metabolism.

Phloridzin had inconsistent effects on absorption, the range of inhibition was between 37-86% of the control values. Inhibition had a highly significant effect on absorption by all tissues, except salivary gland, suggesting the involvement of a phloridzin-sensitive, carrier-mediated mechanism in the oesophagus, anterior and posterior intestine and digestive gland: with values of 86%, 50%, 64% and 37% respectively for inhibition. The inhibition of absorption by the glandular tissues is similar (38% & 37% respectively for salivary and digestive glands) and less than that for the other regions, suggesting that the phloridzin-sensitive mechanism is more important in the oesophagus and intestine.

Comparisons of 2,4-DNP and phloridzin treatments reveal that the differences of the means for oesophagus and intestine are not significant, but are highly significantly different for the two glandular tissues. This suggests that metabolic poisoning of the glandular tissues had a greater inhibitory effect than did the competitive inhibition. There are possibly two different mechanisms; a phloridzin-sensitive one accounting for 37-38% and one reduced by metabolic poisoning, accounting for 69% and 73% of the absorbed

FIGURE 30.

HISTOGRAMS SHOWING THE EFFECT OF INHIBITORS ON GLUCOSE ABSORPTION BY SECTIONS OF GUT.

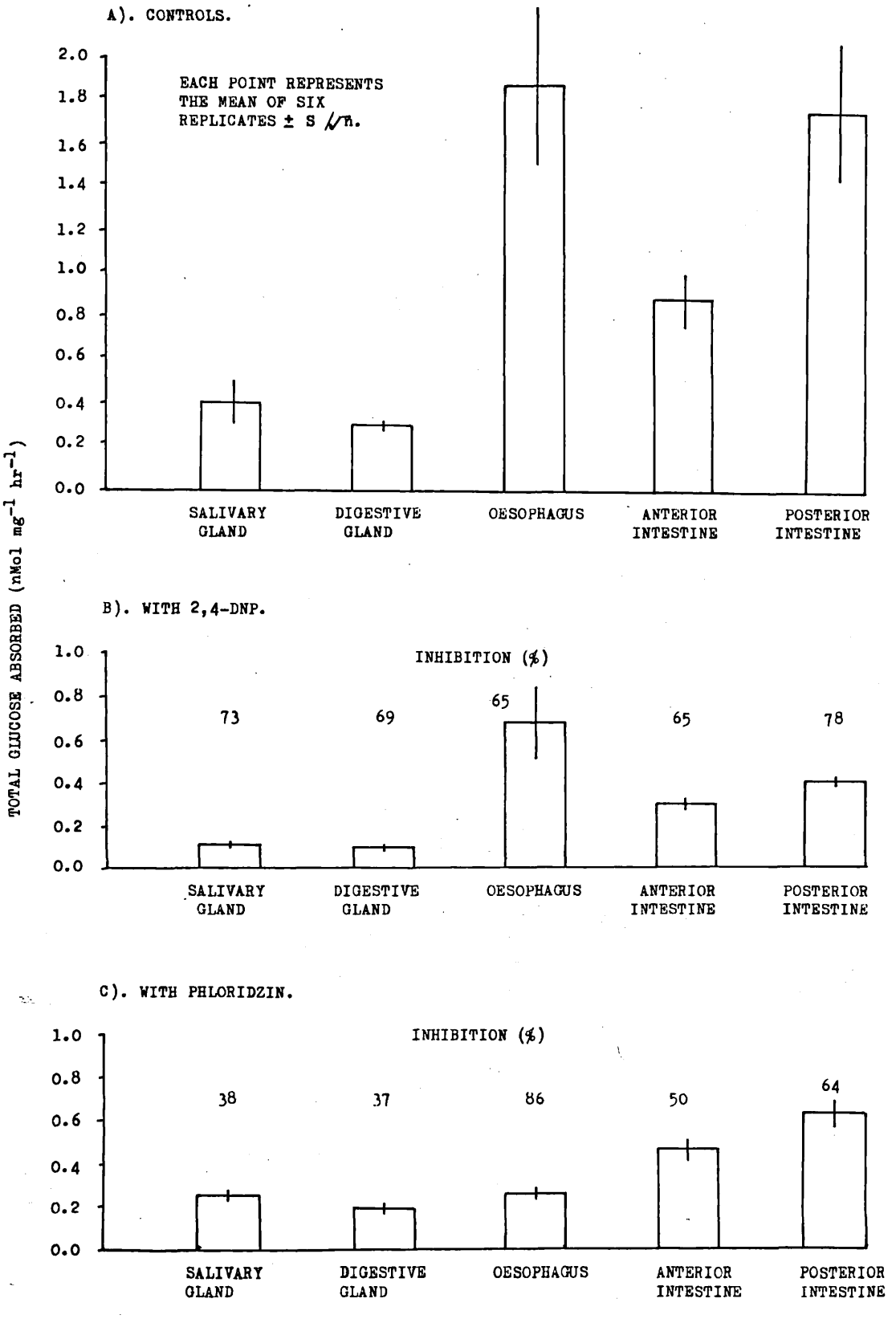


TABLE 24.

GLUCOSE ABSORPTION EXPERIMENTS: ABSORPTION RATES (nMol mg^{-1} hr^{-1}).

TREATMENT		OESOPHAGUS	ANTERIOR INTESTINE	POSTERIOR INTESTINE	DIGESTIVE GLAND	SALIVARY GLAND
CONTROL		1.875 ± 0.37	0.892 ± 0.13	1.750 ± 0.34	0.308 ± 0.02	0.409 ±0.12
2,4-DNP		0.656 ± 0.19	0.309 ± 0.03	0.388 ± 0.05	0.096 ± 0.01	0.109 ± 0.01
PHLORIDZIN		0.252 ± 0.03	0.447 ± 0.06	0.629 ± 0.06	0.194 ± 0.02	0.253 ± 0.03
$[Na^+]$ (%)	100	1.875 ± 0.37	0.892 ± 0.13	1.750 ± 0.34	0.308 ± 0.02	
	75	1.502 ± 0.24	0.717 ± 0.09	1.146 ± 0.29	0.230 ± 0.02	
	50	1.656 ± 0.14	0.921 ± 0.08	1.022 ± 0.09	0.203 ± 0.02	
	25	1.157 ± 0.06	0.705 ± 0.08	0.974 ± 0.09	0.239 ± 0.03	
	0	0.483 ± 0.06	0.596 ± 0.09	0.783 ± 0.09	0.277 ± 0.03	
pH	6.2	3.630 ± 0.19	2.185 ± 0.39	1.612 ± 0.15	0.812 ± 0.16	
	6.6	2.818 ± 0.44	1.462 ± 0.17	2.006 ± 0.35	0.436 ± 0.05	
	7.0	3.487 ± 0.62	0.666 ± 0.05	1.943 ± 0.42	0.369 ± 0.05	
	7.4	3.492 ± 0.61	1.095 ± 0.15	2.964 ± 0.76	0.511 ± 0.10	
	7.8	3.890 ± 0.78	1.184 ± 0.20	1.822 ± 0.29	0.485 ± 0.13	
	8.2	4.533 ± 0.59	0.755 ± 0.08	1.505 ± 0.13	0.402 ± 0.05	

TABLE 25.

SUMMARISED STATISTICAL ANALYSES OF THE GLUCOSE ABSORPTION EXPERIMENTS.

A). CONTROL-INHIBITOR EXPERIMENTS: STUDENT'S t TESTS.

GUT SECTION	TREATMENTS		
	CONTROL-2,4-DNP	CONTROL-PHLORIDZIN	2,4-DNP-PHLORIDZIN
OE	<0.002	<0.002	>0.05
AI	<0.002	<0.002	>0.05
PI	<0.001	<0.02	>0.05
DG	<0.01	<0.01	<0.001
SG	>0.1	>0.1	<0.002

B). Na^+ EXPERIMENTS.

i). ANOVAR TESTS.

All gut sections gave values for P >0.05

ii). STUDENT'S t TESTS.

GUT SECTION	% Na^+ TREATMENTS COMPARED	P
OE	100 vs 75,50,25	>0.05
	100 vs 0	<0.01
	50 vs 0	<0.001
AI	100 vs 0	>0.10
PI	100 vs 75,50	>0.05
	100 vs 25	<0.05
	100 vs 0	<0.002
DG	100 vs 0	>0.1

C). pH EXPERIMENTS.

i). ANOVAR TESTS.

All gut sections gave values for P >0.05

ii). STUDENT'S t TESTS.

GUT SECTION	pH TREATMENTS COMPARED	P
OE	6.2 vs 6.6,7.0,7.4,7.8	>0.05
	6.2 vs 8.2	<0.05
	7.2 vs 6.2,7.0,7.4,7.8,8.2	<0.05
AI	6.2 vs 6.6	>0.05
	6.2 vs 7.0,7.4,7.8,8.2	<0.05
	7.0 vs 7.8	<0.05
PI	6.2 vs 6.6,7.0,7.4,7.8,8.2	>0.05
	7.2 vs 7.4	>0.05
DG	6.2 vs 6.6	>0.05
	7.0 vs 7.2	>0.05
	6.2 vs 7.0	<0.05
	6.2 vs 7.2	<0.02

NOTATION: ANOVAR is an abbreviation for Analysis of Variance.
OE....Oesophagus AI....Anterior Intestine
PI....Posterior Intestine DG....Digestive Gland.
P values <0.05 were taken as significant.

glucose (fig 30).

The fact that the differences between the salivary gland 2,4-DNP-control and phloridzin-control values are not significant (table 25A) may be due to the large variation in the control values.

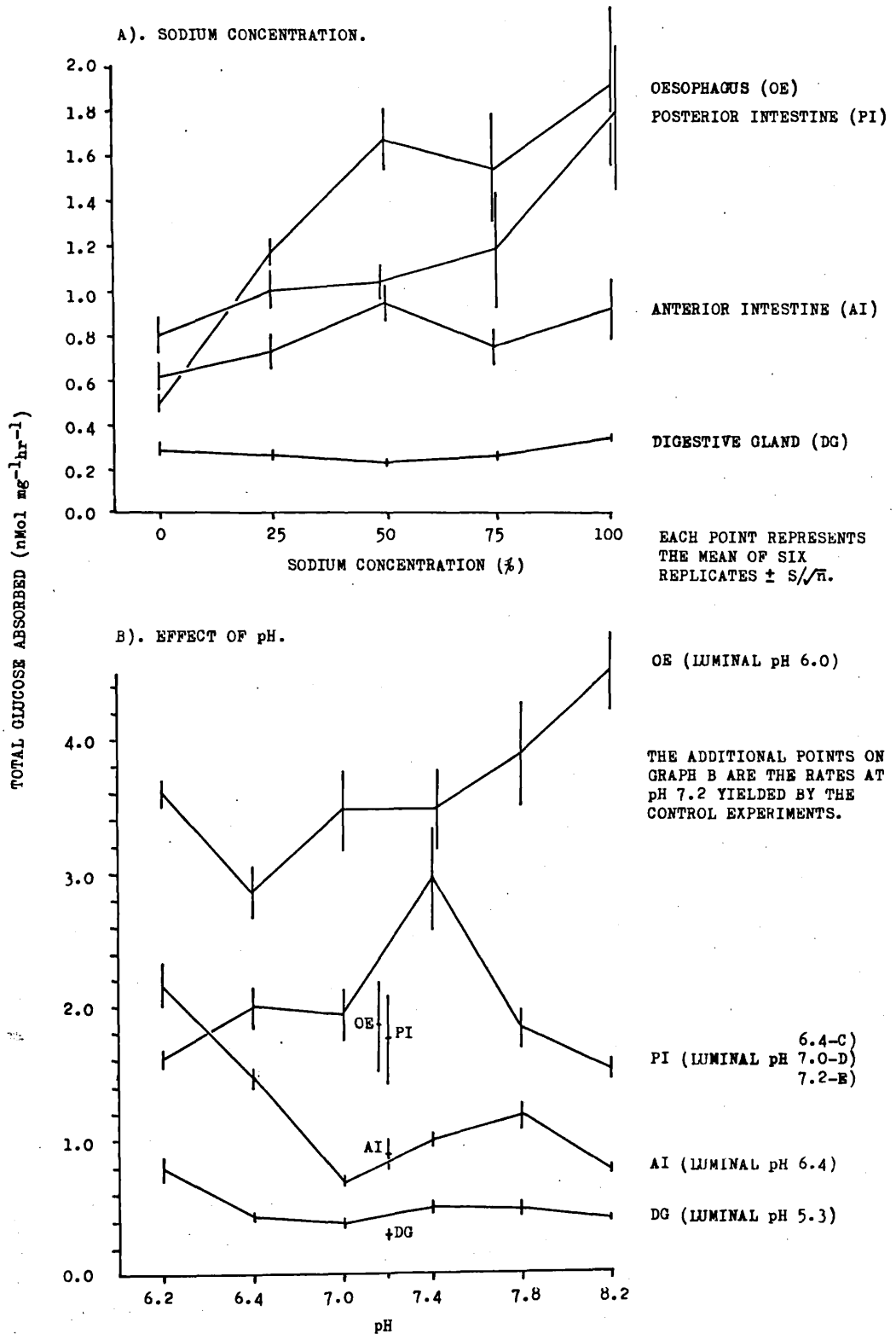
C).SODIUM CONCENTRATION. Reduction of the sodium ion concentration ($[Na^+]$), reduced absorption by the oesophagus and posterior intestine, but had little effect on the digestive gland and anterior intestine (fig 31A, table 24). Student's t test revealed that the differences in glucose absorption at the normal $[Na^+]$ in ASW (32‰ is taken to be 100%) and 0%, is significant for both oesophagus and posterior intestine; and that the differences between the 50% and 0% values for the oesophagus are very significant (table 25B). Glucose absorption by these regions is sodium-dependent. A one way analysis of variance performed on these results indicated that absorption was not affected by the sodium concentration.

Some glucose was absorbed at 0% $[Na^+]$ by the oesophagus and posterior intestine, this may be due to the small quantity of $NaHCO_3$ (which was not replaced with the equivalent lithium salt) being sufficient to operate the sodium-dependent mechanism. Alternatively, diffusion and a sodium-independent mechanism may be responsible.

D).EFFECT OF pH. A one way analysis of variance demonstrated that glucose absorption by all four tissues is not affected over the pH range 6.2-8.2 (fig 31B, table 24). However, Student's t tests revealed a significant difference between the digestive gland values at pH 6.2 and 7.0; the oesophageal values at 6.2 and 8.2 and between the anterior intestinal values at 6.2 and 7.0/7.4/7.8 and 8.2 and at pH 7.0 and 7.8 (table 25C). Significant differences were not found between any of the posterior intestinal values indicating that any correlation between pH and absorption was not shown. The results of the experiments on oesophagus and posterior intestine suggest that

FIGURE 31.

GRAPHS SHOWING THE EFFECTS OF SODIUM CONCENTRATION AND pH ON GLUCOSE ABSORPTION BY SECTIONS OF GUT.



significant differences may have been revealed in absorption at different pH values if variation between experiments had been less.

Maximal glucose absorption would be expected at the pH normally encountered in a particular section of the gut. The normal pH of the luminal contents of each section examined is displayed in fig 31B. The results from the digestive gland and anterior intestine experiments show a trend in increased glucose absorption towards a more acidic pH; their normal pH is 5.3 and 6.4 respectively. The oesophagus and posterior intestine do not show absorption trends related to their normal pH.

The absorption values displayed in fig 30A and table 24 are presented on fig 31B at the pH 7.2 mark, this was the pH of the incubation medium for that series of experiments. The reason for this is to determine how closely the absorption values at pH 7.2 lie to the experimental pH plots, this will indicate if the buffer system is affecting absorption.

The digestive gland and anterior intestine values at pH 7.2 lie very close to their respective plots and are in fact not significantly different from them, indicating a close correlation between the two series of experiments for these tissues and that the buffer does not affect absorption. The posterior intestinal value at pH 7.2 is not significantly different from any of the other values on the line, so the pH 7.4 peak is possibly anomalous. The oesophageal value at pH 7.2 is significantly smaller than the values on the plot suggesting that in this instance the buffer stimulated absorption.

E).KINETICS EXPERIMENTS.

The inhibition of glucose absorption by phloridzin demonstrated that the absorption mechanism is carrier-mediated and so a membrane-bound protein that complexes with glucose and translocates it across the membrane into the cell, is probably involved. The complexing reaction between carrier and substrate can be explained in terms of

the Michaelis-Menten equation;

$$V=Vmax.[S] / Km + [S]$$

where V=the velocity of absorption, Vmax=the maximal velocity, [S]=the substrate concentration and Km=the Michaelis-Menten constant. The kinetics experiments were performed to determine Vmax and Km.

The results are presented graphically in two different forms. In figs 32 and 33, V is plotted against [S], if the absorption mechanism obeys the Michaelis-Menten equation the plot will be a rectangular hyperbola. Linear transformations of the data, effected by means of an Hofstee plot (Neame and Richards, 1972), are shown in figs 34 and 35; V is plotted against V/[S] and a straight line is obtained if the equation is obeyed.

The curves in figs 32 and 33 do not correspond exactly to rectangular hyperbolae, those of the digestive gland and posterior intestine are almost straight lines, although the other two are more reminiscent of a hyperbola. The straight lines indicate that the velocity of glucose absorption is directly related to its concentration, suggesting the involvement of diffusion. Diffusion effects were expected to be more noticable at the higher glucose concentrations but less so at the lower ones. This would give a hyperbolic curve at first which would tail off as a diagonal straight line.

Each of these figures display "corrected" and "uncorrected" curves. The latter represents the total glucose absorbed by the tissue in each experiment. It is known that a certain percentage of this total is affected by phloridzin and it is this component of the total that must be examined in the kinetic experiments. The "corrected" curve takes this inhibition into account and is derived as shown below for the oesophagus experiment at [S]=500µMol.

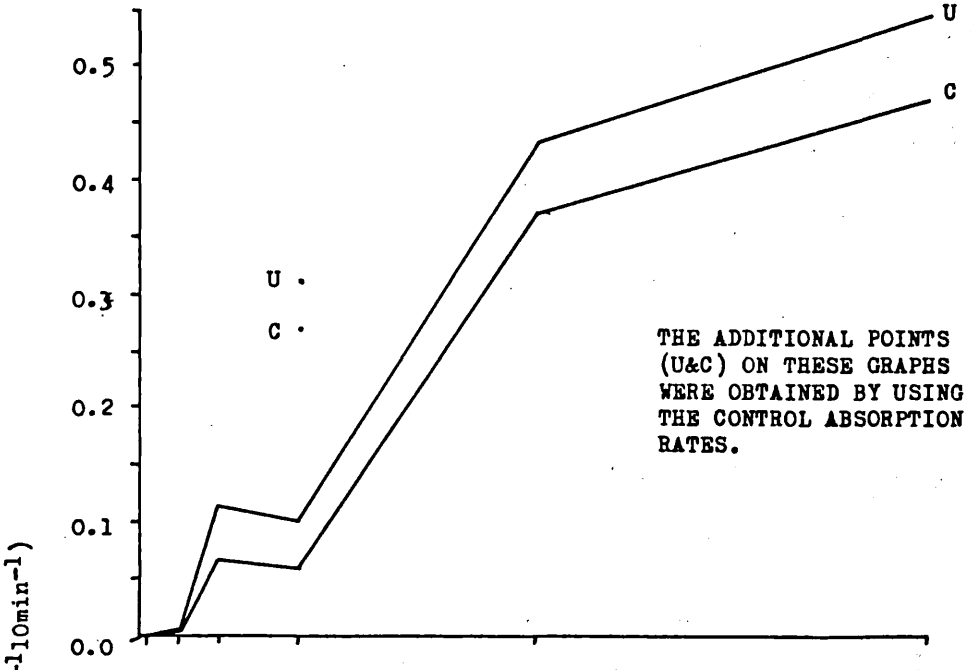
total glucose absorbed= 0.55nMol mg^{-1} 10 $mins^{-1}$ (=V)

phloridzin-inhibition of oesophageal absorption= 86%

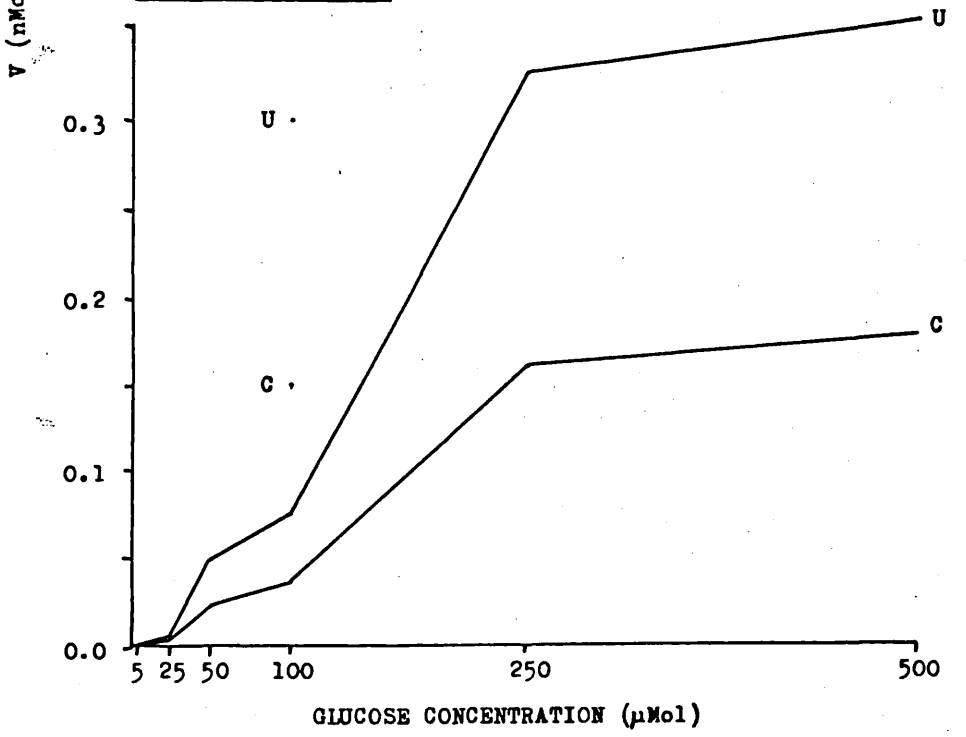
FIGURE 32.

KINETICS EXPERIMENTS: V/S PLOTS.

A) OESOPHAGUS.



B). ANTERIOR INTESTINE

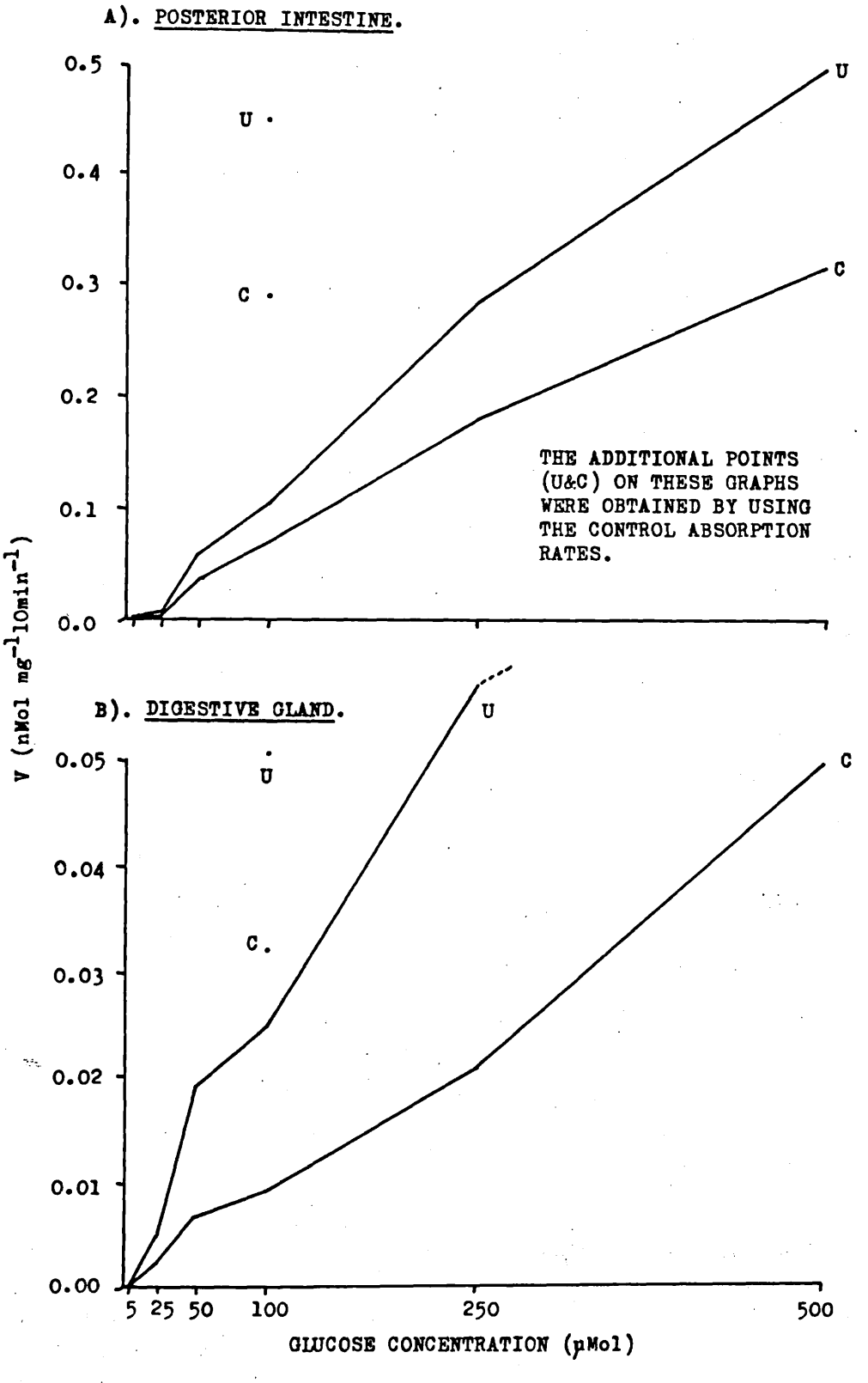


V (nMol $mg^{-1}10min^{-1}$)

NOTATION: U...Uncorrected plot.
C...Corrected plot.

FIGURE 33.

KINETICS EXPERIMENTS: V/S PLOTS.



NOTATION: U...Uncorrected plot.
C...Corrected plot

FIGURE 34.

KINETICS EXPERIMENTS: HOFSTEE PLOTS OF "CORRECTED" RESULTS.

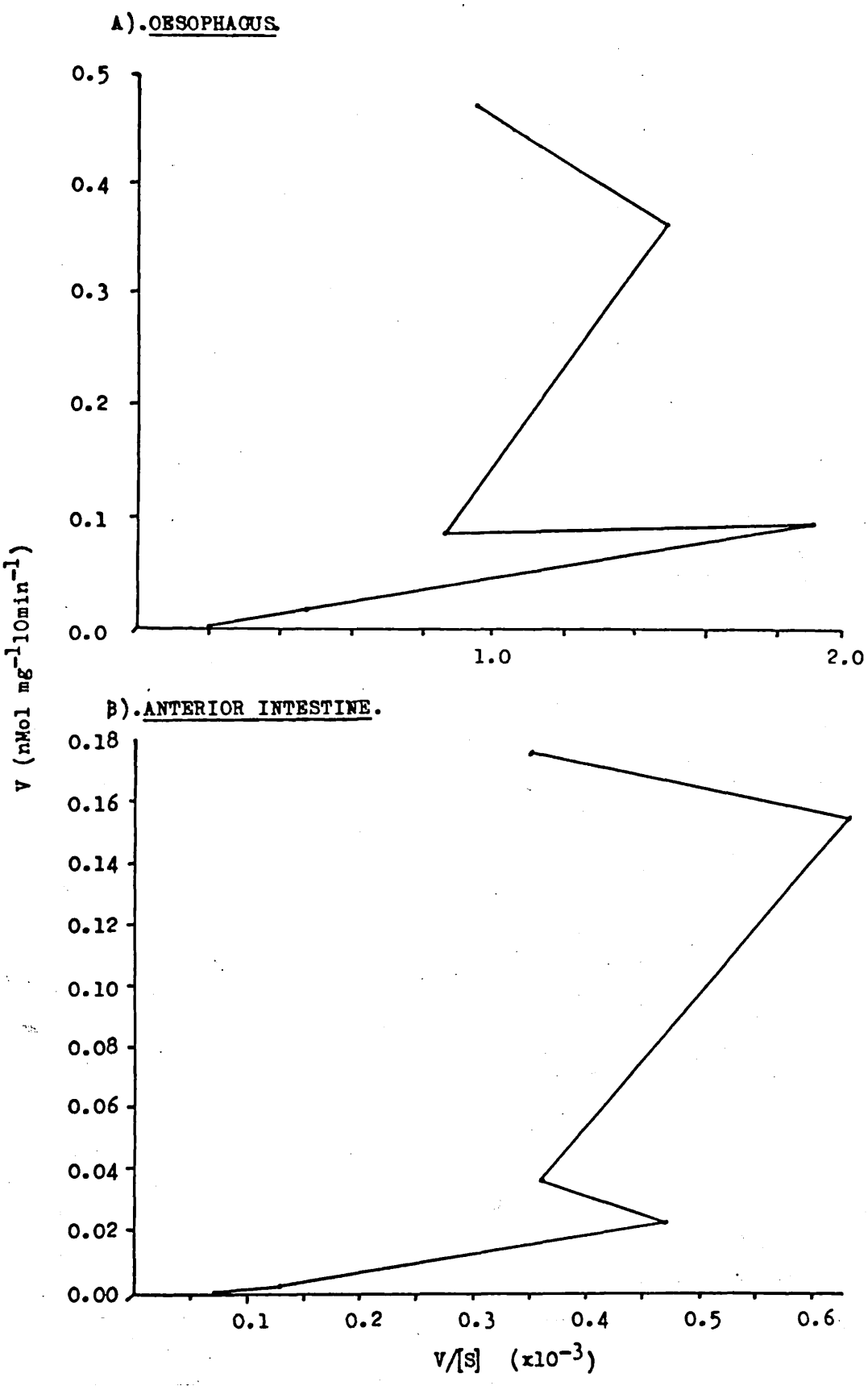
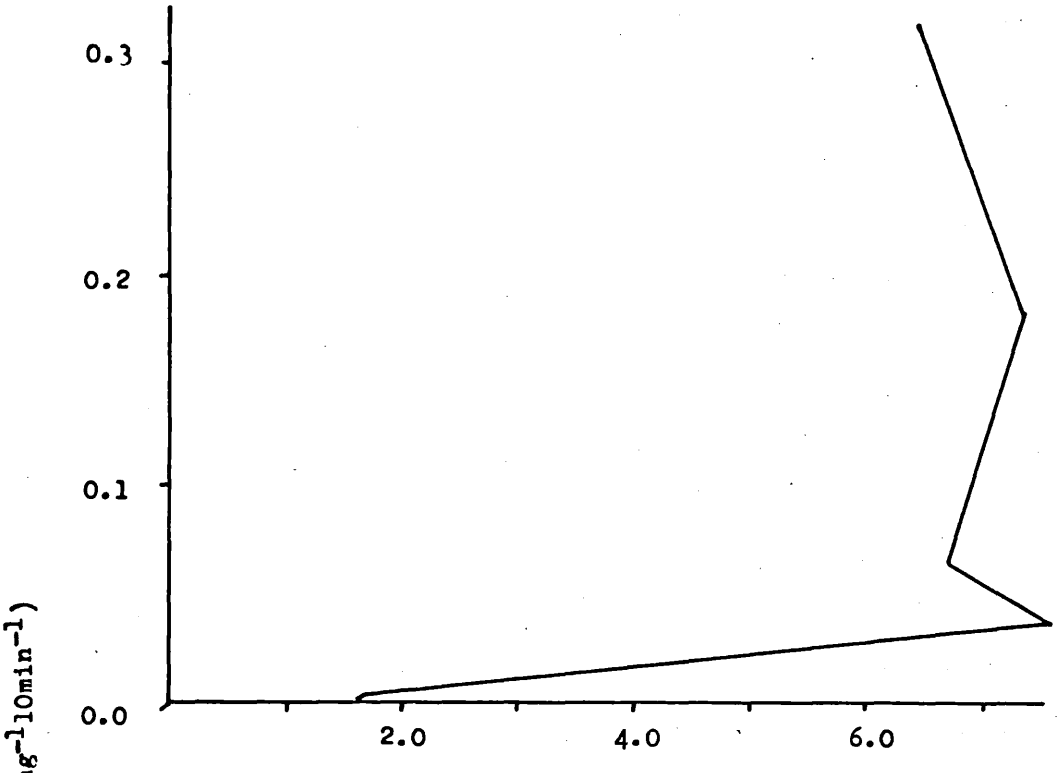


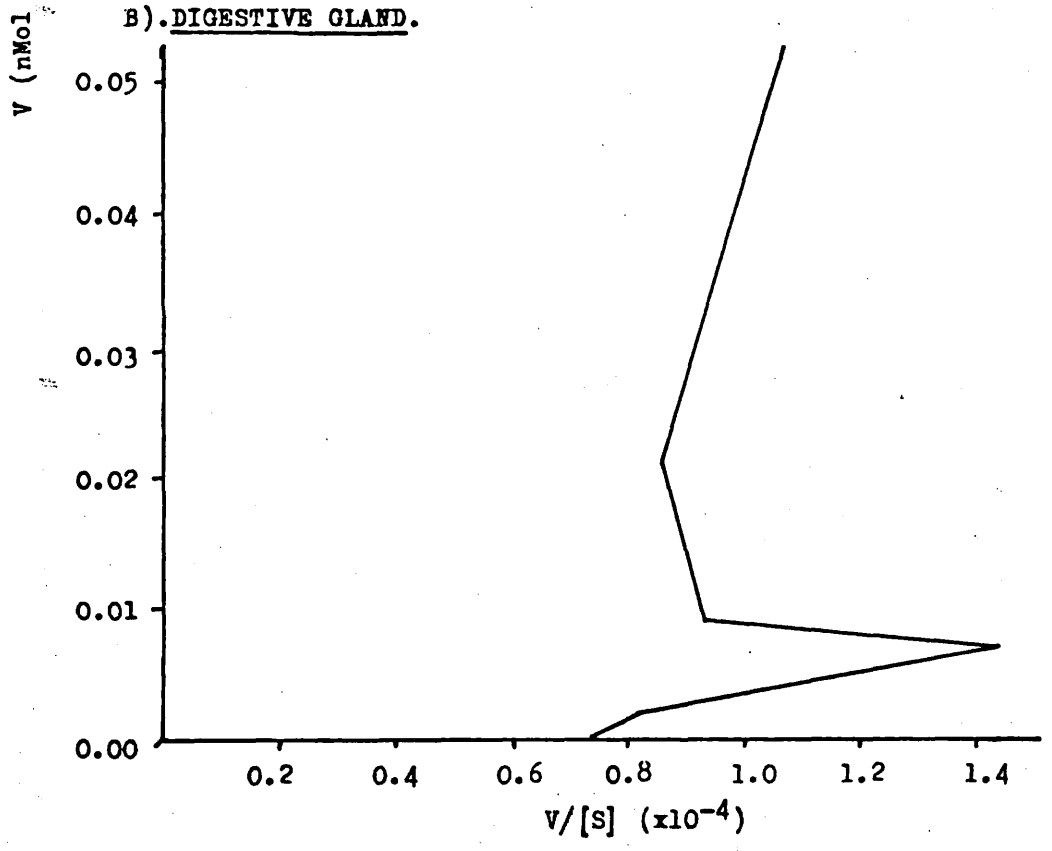
FIGURE 35.

KINETICS EXPERIMENTS: HOFSTEE PLOTS OF "CORRECTED" RESULTS.

A).POSTERIOR INTESTINE.



B).DIGESTIVE GLAND.



"Corrected" value= 86.V/100 =0.473nMol mg^{-1} 10 $mins^{-1}$.
This was done for each tissue at each [S] experiment (table 26) and the values plotted. Although this does not alter the shape of the curve, it is a necessary step to bring the values into perspective.

The absorption values displayed in fig 30A and table 24 are presented on figs 32 and 33 at the 100μMol mark as this was the glucose concentration used in that series of experiments. Each of these values was converted from glucose absorbed per 30 mins to 10 mins (the duration of the kinetic experiments) and similarly corrected for phloridzin-inhibition. The usefulness of this is only justified if the absorption rates over 10 and 30 minute intervals are similar. These values fit a hyperbolic curve better than the actual 100μMol kinetic experimental values.

Definite conclusions cannot be drawn from the V against [S] plots because of the subjectivity involved in defining a rectangular hyperbola, a straight line from an Hofstee plot is easier to interpret. The resemblance of the oesophageal and anterior intestinal curves to a hyperbola would suggest that their Hofstee plots should conform more closely to a straight line than the plots of the other two tissues. This was not so, none of the Hofstee plots correspond to a straight line that intersects the ordinate (V) to give Vmax and crosses the abscissa to give Vmax/Km (figs 34 & 35).

These irregular lines reflect the V against [S] curves and suggest that the absorption mechanism acting in all four sections of gut, does not obey the Michaelis-Menten equation. This implies that a carrier protein, liable to saturation at high glucose concentrations, is not responsible for glucose absorption which conflicts with the results of the phloridzin experiments.

4-CHROMATOGRAPHIC EXPERIMENTS.

The results are displayed in figs 36 and 37. The chromatographs of the ^{3}H-glucose stock and 100μMol glucose medium (figs 36A & B),

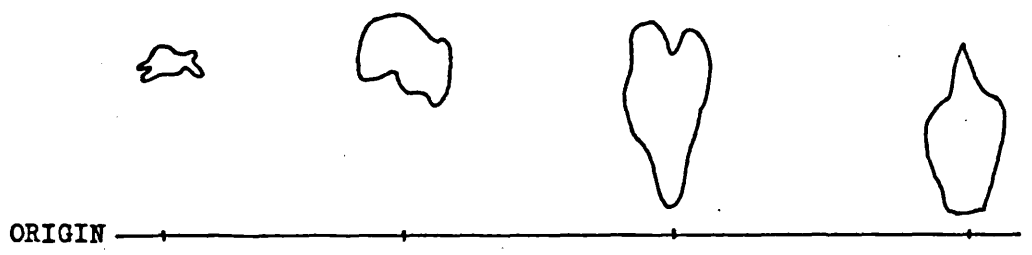
TABLE 26. COMPILATION TABLE OF "UNCORRECTED" (U) AND "CORRECTED" (C) V AND V/[S] VALUES.

[S] (μMol L^{-1})		GUT SECTION: OESOPHAGUS U	OESOPHAGUS C	ANTERIOR INTESTINE U	ANTERIOR INTESTINE C	POSTERIOR INTESTINE U	POSTERIOR INTESTINE C	DIGESTIVE GLAND U	DIGESTIVE GLAND C
500	V	0.55	0.47	0.35	0.17	0.49	0.32	0.14	0.05
	V/[S]	1.10×10^{-3}	9.46×10^{-4}	7.06×10^{-4}	3.53×10^{-4}	9.98×10^{-4}	6.38×10^{-4}	2.88×10^{-4}	1.06×10^{-4}
250	V	0.43	0.37	0.31	0.16	0.28	0.18	0.06	0.02
	V/[S]	1.72×10^{-3}	1.48×10^{-3}	1.26×10^{-3}	6.30×10^{-4}	1.14×10^{-3}	7.28×10^{-4}	2.28×10^{-4}	8.40×10^{-5}
100	V	0.10	0.096	0.072	0.036	0.104	0.067	0.025	9.25×10^{-3}
	V/[S]	1×10^{-3}	8.60×10^{-4}	7.20×10^{-4}	3.60×10^{-4}	1.04×10^{-3}	6.70×10^{-4}	2.50×10^{-4}	9.25×10^{-5}
50	V	0.11	0.095	0.05	0.023	0.059	0.037	0.019	7.18×10^{-3}
	V/[S]	2.20×10^{-3}	1.89×10^{-3}	9.40×10^{-4}	4.7×10^{-4}	1.18×10^{-3}	7.54×10^{-4}	3.88×10^{-4}	1.43×10^{-4}
25	V	6.35×10^{-3}	5.46×10^{-3}	5.66×10^{-3}	2.83×10^{-3}	6.40×10^{-3}	4.09×10^{-3}	5.50×10^{-3}	2.04×10^{-3}
	V/[S]	2.54×10^{-4}	2.18×10^{-4}	2.26×10^{-4}	1.13×10^{-4}	2.56×10^{-4}	1.64×10^{-4}	2.20×10^{-4}	8.14×10^{-5}
5	V	1.19×10^{-3}	1.03×10^{-3}	0.78×10^{-3}	0.39×10^{-3}	1.23×10^{-3}	0.79×10^{-3}	9.92×10^{-4}	3.67×10^{-4}
	V/[S]	2.39×10^{-4}	2.05×10^{-4}	1.55×10^{-4}	7.78×10^{-5}	2.47×10^{-4}	1.58×10^{-4}	1.98×10^{-4}	7.34×10^{-5}

FIGURE 36.

^{3}H-GLUCOSE CHROMATOGRAPHIC EXPERIMENTS.

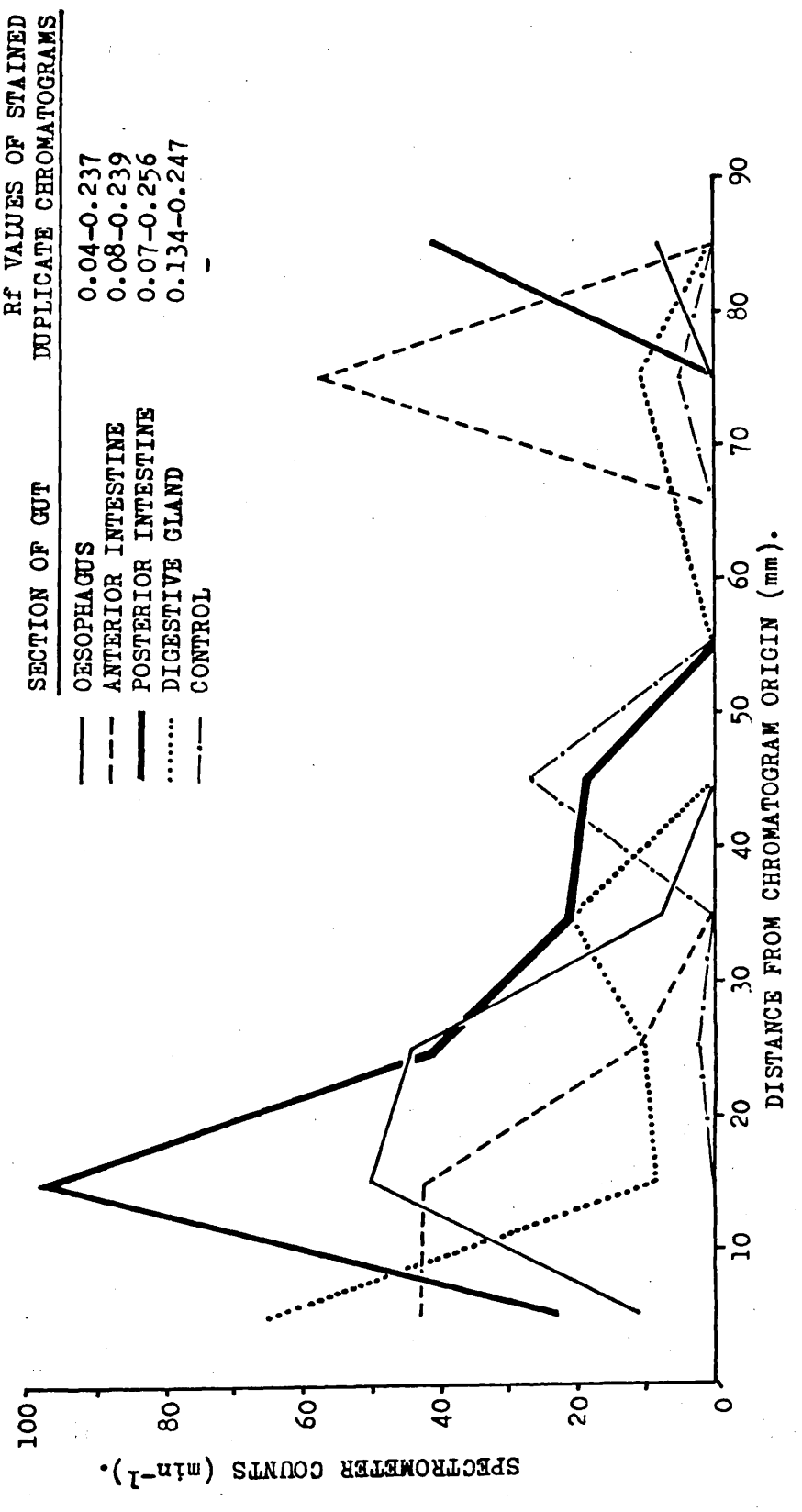
	A). D-GLUCOSE	B). ^{3}H-GLUCOSE	C). HOMOGENATE ON ^{3}H-GLUCOSE	D). HOMOGENATE CONTROL
Rf VALUE	0.26	0.262	0.132-0.306	0.202-0.298



TISSUE INCUBATED IN ^{3}H-GLUCOSE MEDIUM.

	E). OESOPHAGUS	F). ANTERIOR INTESTINE	G). POSTERIOR INTESTINE	H). DIGESTIVE GLAND
Rf VALUE	0.04-0.237	0.083-0.239	0.07-0.256	0.134-0.247

FIGURE 37. CHROMATOGRAPHIC EXPERIMENTS:RADIOACTIVITY OF CHROMATOGRAMS.



show that both were pure; the small spots indicate that only one sugar was present, the Rf values of 0.26 corresponds with that quoted for glucose (Smith, 1969). The control appeared blank after silver staining, demonstrating that the paper-solvent interaction does not produce artefactual spots.

Chromatographs of homogenised tissue previously incubated in the labelled medium (figs 36E, F, G & H), suggest the presence of a range of sugars and that their Rf values are less than that for glucose, indicating that the sugars have a greater molecular weight than glucose. A similar range of values was obtained for the oesophagus and both intestinal sections (Rf=0.04-0.237; 0.083-0.239 and 0.07-0.256 respectively). Digestive gland gave a range of 0.134-0.247 indicating that the sugars (di-, oligo- or polysaccharides) with Rf values corresponding to 0.04-0.083 found in the other tissues were absent. These sugars may be en route to the synthesis of glycogen, demonstrated cytochemically in the ciliated and unciliated columnar epithelial cells.

The effect of tissue homogenate on the Rf value of ^{3}H-glucose can be seen in fig 36C, where a sample of homogenate was spotted onto a dried glucose spot. The values range from 0.132-0.306, compared to 0.202-0.298 for the homogenate controls (fig 36D) and 0.26 for pure glucose. This suggests that the sugars with Rf values <0.132, demonstrated in the chromatographs of the tissues incubated in the tritiated medium, were not present in the homogenate controls or produced by the interaction of the homogenate and dried glucose spots. These sugars must therefore be a product of the metabolism of ^{3}H-glucose by live, intact cells; as their Rf values are less than that for glucose they must be of greater molecular weight and a product of glycogenesis.

Evidence to support this came from the duplicate chromatographs cut into strips and counted; the measure of radioactivity from the strips

is displayed in fig 37. Comparisons of the count peaks and the Rf values of the stained duplicates gives a close correlation. The majority of the radioactivity was confined to the first two strips (0-20mm corresponding to Rf values 0-0.2) indicating that the ^{3}H-atoms remained associated with a sugar molecule that had increased in molecular weight. Both intestinal chromatographs display peaks around the 70-90mm mark, suggesting that some ^{3}H-atoms exist in a highly mobile form or that these particular strips were contaminated. The greatest peak from the digestive gland was in the first strip (0-10mm, Rf= 0-0.10) suggesting that the ^{3}H-atoms were associated with a less mobile sugar molecule.

5-NET WATER MOVEMENT EXPERIMENTS.

The results are expressed graphically in figs 38 and 39 and the statistical analyses presented in table 27. Differences between the means of the pre- and post-incubational luminal samples for both intestinal sections are highly significant. The radioactivity of the luminal medium increased during the incubation period; this could only occur if there was a net flow of water out of the intestinal lumen either into the epithelial cells or through the epithelium into the haemocoelic compartment.

A significant difference was not found between the means of the pre-incubational samples of the two intestinal sections (table 27), this was expected because all the samples were taken from the same source (the microsyringe used to fill the gut). However, the difference between the means of the post-incubational samples of the two intestinal sections was significant, confirming that the net flow of water was significantly greater across the epithelium of the anterior intestine with respect to the posterior intestine. The hydrostatic pressure within the anterior intestine, which is responsible for the gut fluid spurting through any deliberately inflicted punctures, may facilitate water flow across the epithelium.

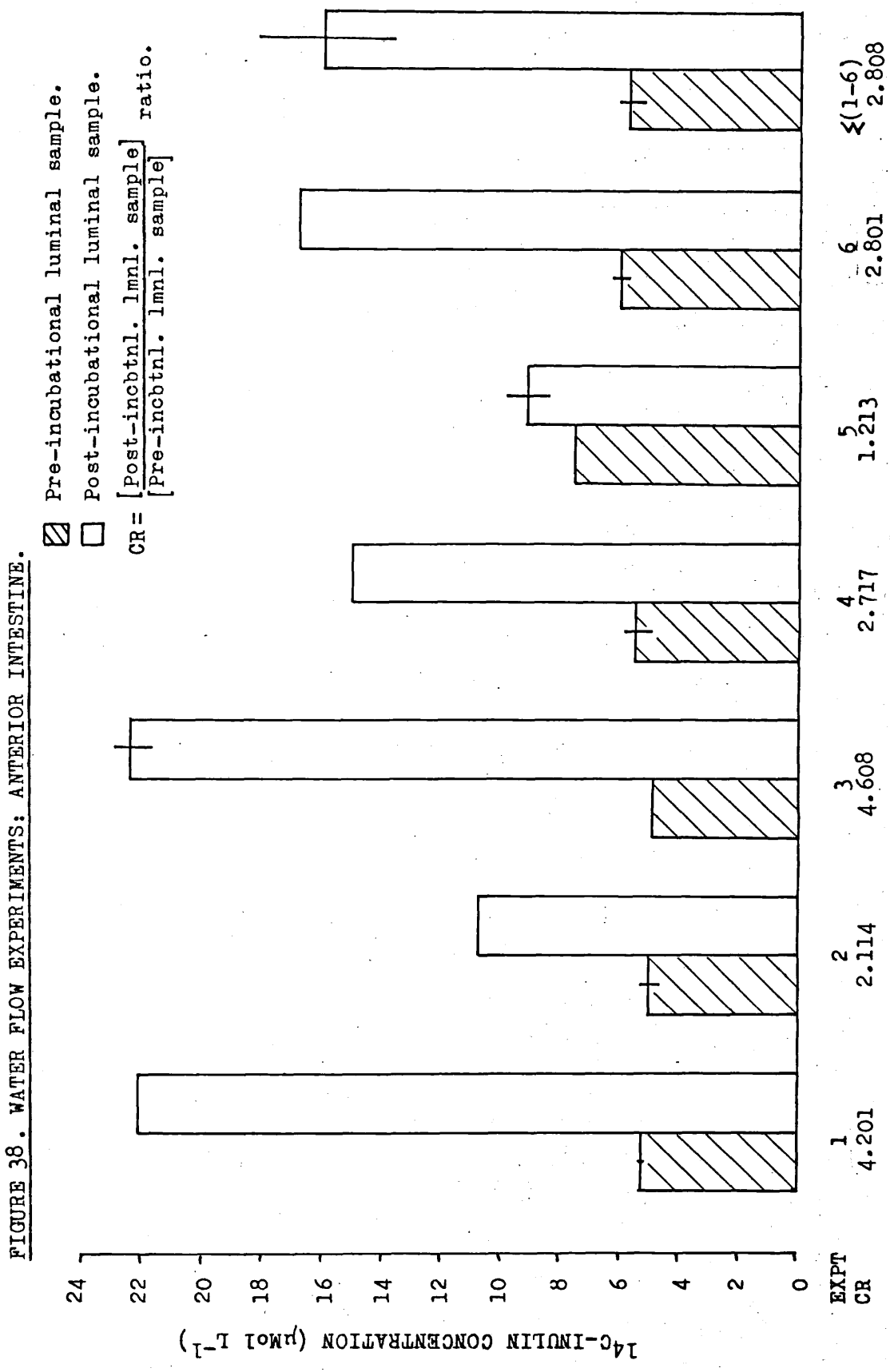


FIGURE 38. WATER FLOW EXPERIMENTS: ANTERIOR INTESTINE.

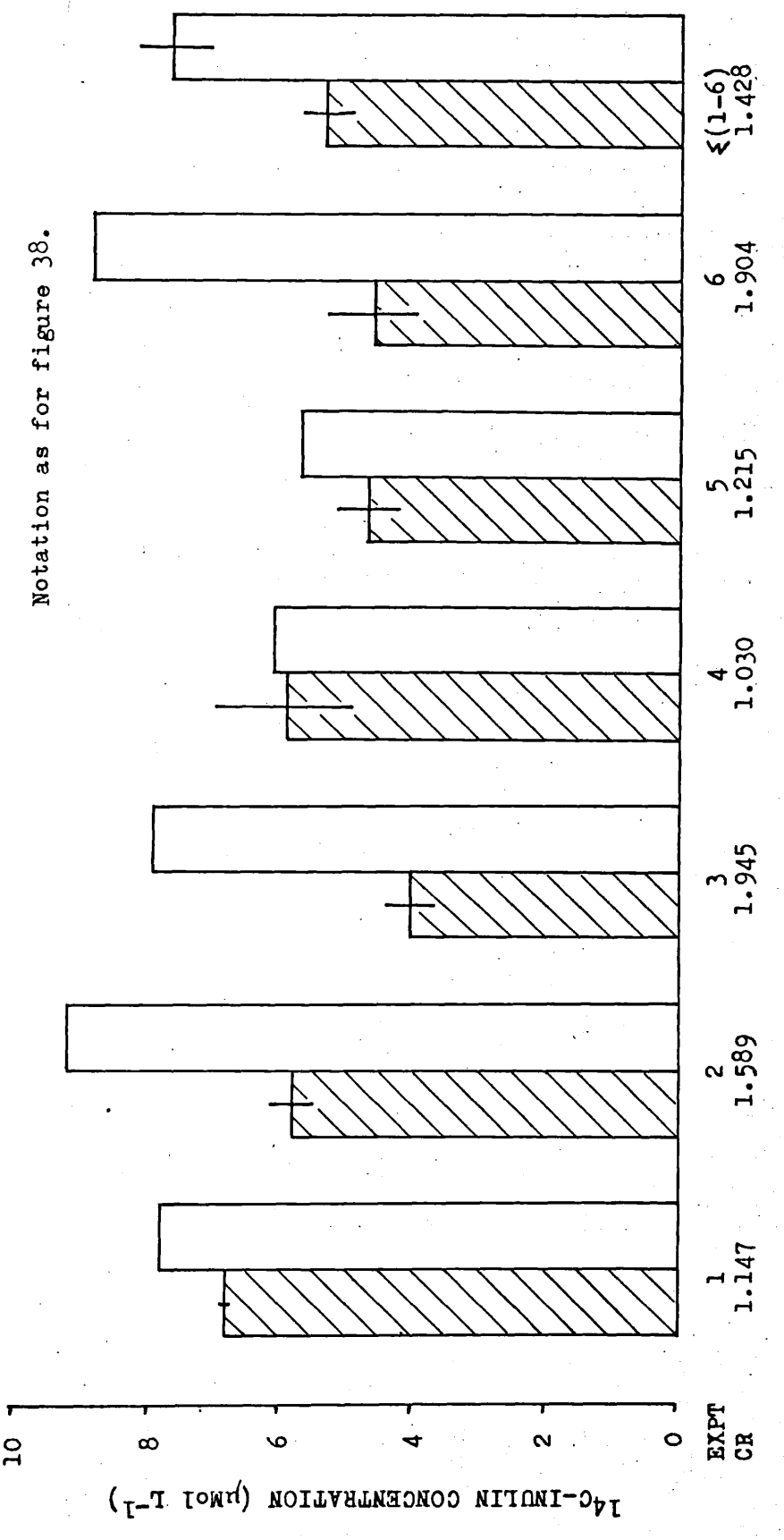


FIGURE 39. WATER FLOW EXPERIMENTS: POSTERIOR INTESTINE.

TABLE 27 .

WATER FLOW EXPERIMENTS:SUMMARISED STATISTICAL ANALYSES.

STUDENT'S t TESTS.

A).TO DETERMINE IF THE DIFFERENCE BETWEEN THE LUMINAL PRE- AND POST-INCUBATIONAL SAMPLES OF THE TWO INTESTINAL SECTIONS IS SIGNIFICANT.

INTESTINAL SECTION	P
ANTERIOR	0.02
POSTERIOR	0.01

B). TO DETERMINE IF THE DIFFERENCE BETWEEN THE ANTERIOR AND POSTERIOR INTESTINAL LUMINAL PRE- AND POSTINCUBATIONAL SAMPLES IS SIGNIFICANT.

SAMPLE	P
PREINCUBATIONAL	0.05
POSTINCUBATIONAL	0.01

P values < 0.05 were taken as significant.

AUTORADIOGRAPHY.

Attempts to demonstrate which cell types of the oesophagus, digestive gland and intestine absorb glucose, by autoradiography failed to yield positive results. Black deposits occurred over the sections and emulsions of experimental and control slides, (see appendix 3).

The histological preservation of the epithelium lining the oesophageal gland was very poor, the lateral folds could just be distinguished, but the two cell types could not be identified. Better histological detail was obtained with the digestive gland; ducts were obvious and the basophilic cells distinct from the digestive cells. The anterior intestinal epithelium was also fixed well, the brush border and apical cytoplasm staining red and the nuclei blue. However, the posterior portion was less distinct, but similarly stained. Had the experiments worked it would have been possible to correlate the silver grain distribution with a particular cell type of the digestive gland and anterior intestine only.

DISCUSSION.

Most of Graham's observations on the functional anatomy and histology of the limpet gut have been confirmed by this ultrastructural study. Only limited comparisons of the ultrastucture of the gut of *Patella* with that of other molluscs is possible since there are few similar investigations, consequently most comparisons will be made with insect and mammalian species where ultrastructural knowledge is greater. The observations reported above together with those of Graham (1932), indicate that the various regions of the gut contribute to digestion as summarised below.

The radular action ruptures algal cells releasing their cytoplasm and assimilatory products. Small molecules that do not require digestion can be absorbed directly by the oesophagus. Cellular debris and other detritus rasped up is passed to the roof of the buccal cavity as the radula ends its stroke and receives saliva to lubricate and entrap material rasped from the substratum. Passing along the oesophageal food channel, the food is mixed with mucus from the subepithelial mucous cells and the amylase from the oesophageal gland. Carbohydrate digestion starts in the mid-oesophagus and the sugars produced are absorbed by a sodium-dependent, carrier-mediated mechanism situated on the apical membrane of the epithelial cells. Oesophageal contractions may force fluids out of the food channel into the lateral pouches, where sugar absorption continues and suspended proteinaceous particles are pinocytosed by the amylase-secreting cells.

Squeezing of the mucous string by the posterior oesophagus releases soluble nutrients into the stomach fluids. It is carried through the stomach into the style sac by the folds, F1 and F3, here and in the anterior intestine blebs of cytoplasm are added to it. Blebs circulating the lumen, collide with suspended particles and cause their precipitation on the ventral wall, where the ciliary currents

add them to the developing faecal rod. The pH of the style sac, anterior intestine and oesophagus are similar, so amylase activity may continue.

Occlusion of the style sac by the muscular valve, enables stomach fluids and fine suspended particles to be forced into the digestive gland by increased hydrostatic pressure, caused by oesophageal contractions. Large particles are rejected by the ciliary currents of the duct directed towards the stomach, these particles are taken into the intestine along the intestinal groove. At some stage the duct cells absorb fatty acids and glycerides.

Pinocytosis of particulate material and absorption of small molecules is a function of the digestive cell which exhibits phasic activity unrelated to the tidal cycle. Throughout the absorptive phase pinocytosis is rife, as food accumulates in the digestive vesicles digestion begins to produce soluble and insoluble by-products. The former diffuse out of the vesicles and pass across the basal membrane into the blood, whilst the insoluble material is sequestered in residual bodies that are excreted within spherules abstricted from the cells during the fragmentation phase. Spherules in the tubule lumen are mixed with the secretion of the basophilic cells and cemented into a liver string; it emerges from the duct aperture and is carried by the typhlosoles into the style sac.

Intracellular digestion and autolysis may continue in the spherules until they disintegrate (releasing lysosomal enzymes), allowing reclamation of some cell components by absorption along the intestine. The oesophageal amylase and lysosomal enzymes may be adsorbed onto the glycocalyx of the intestinal cells, this is highly speculative and the terminal digestion of molecules prior to transport into cells would only occur if the enzymes are active at the intestinal pH.

Glucose absorption by a carrier-mediated mechanism occurs in the intestine, it is sodium-dependent in the posterior section, but not

the anterior. Water is moved out of the lumen aiding consolidation of the faecal rod, which is compacted by muscular contractions; more importantly it may serve an osmoregulatory function, compensating for the atrophy of the left kidney.

The function of the basal gland cell is unknown. When the faecal rod reaches intestine section D, the secretion of the clavate gland cells is applied, forming a "cement" layer around it. Its passage through the rectum is lubricated by sparse mucous cells, pellets are chopped off by the contraction of the circular muscle fibres and they fall into the mantle cavity, where they remain unchanged. Expulsion from the cavity occurs once the limpet is submerged.

Research on the physiology of the molluscan gut is concerned exclusively with organic solute and ion transport. Webber (1969) and Forester (1977) are the only authors to report water resorption from the molluscan gut lumen. Specimens of Acmaea scutum (Rathke) placed upside down in sea water double their blood volume in 6-12 hours, the blood ionic composition remains unchanged, so both ions and water are absorbed (Webber, 1969). When methylene blue was added to the sea water, the intestine stained blue, prompting Webber to conclude that this was the site of absorption. Forester suggested that as the intestine of H.pomatia did not absorb organic solutes, it is principally involved in water resorption and that this is an adaptative feature of a terrestrial mollusc.

Fretter and Graham (1976) state that P.vulgata survives a 60% water loss and salinities down to 3‰, but that they do not normally occur in salinities lower than 25‰, (British coastal waters= 32‰). The left kidney, responsible for resorption of solutes and possibly water in archaeogastropods (Andrews, 1985) is markedly reduced in Patella, so could not resorb large quantities of water. The long intestine of Patella may have an osmoregulatory role, partly compensating for the reduction in size of the left kidney. The left

kidney may be most important in solute resorption, whilst the right one acts as a water resevoir.

Numerous authors have found a correlation between tolerance of desiccation and zonation patterns (Davies, 1969; Wolcott, 1973; Branch, 1975). Branch (1975) found that size had a strong influence on the tolerance of Patella granularis L., the larger, more tolerant individuals occur higher up the shore. A similar trend was found in P.vulgata (Das and Seshappa, 1947; Lewis, 1954). If body proportions are held constant, then an increase in size results in a proportional decrease in surface area relative to volume. Water content is dependent on body volume, whilst water loss is related to surface area, large bodies are thus more resistant to desiccation.

Shotwell (1950) found that the volume of water stored in the extravisceral space (that between body and shell) was greater in small limpets and concluded that this store helped offset the desiccating effects due to their small size. Wolcott (1973) believed the total volume of body water is the determining factor in resistance to desiccation. He suggested that deaths due to desiccation are actually caused by osmotic stress, a result of water loss concentrating the tissue ions. Immersion of limpets in an hyperosmotic solution also caused death by ionic concentration, but without removing as much water. Chaisermartin (1971; in Branch, 1981) produced results indicating that hypermagnesia reduced muscular activity, thus reducing the force of attachment to the rock and allowing limpets to be washed off.

The water in the gut and large right kidney of P.vulgata might act as a useful reservoir, which could be tapped to maintain a constant blood osmolarity during times of desiccation.

Wigglesworth (1932) first realised that the rectal pads of Calliphora are responsible for absorbing ions and water from the rectal lumen. The lateral intercellular spaces of this epithelium

are elaborated into extensive channels, with which are associated mitochondria and an ATPase (Berridge and Gupta, 1968). Sodium is actively pumped out of cells into the channels and water follows by osmosis, the hydrostatic pressure in the channels increases, forcing the isosmotic absorbate into the blood (Berridge and Gupta, 1967). This is the basic "standing osmotic gradient" model described in the introduction and it applies also to cockroach mid-gut cells (O'Riordan, 1969) and vertebrate gallbladder and intestine (Berridge and Oschman, 1972).

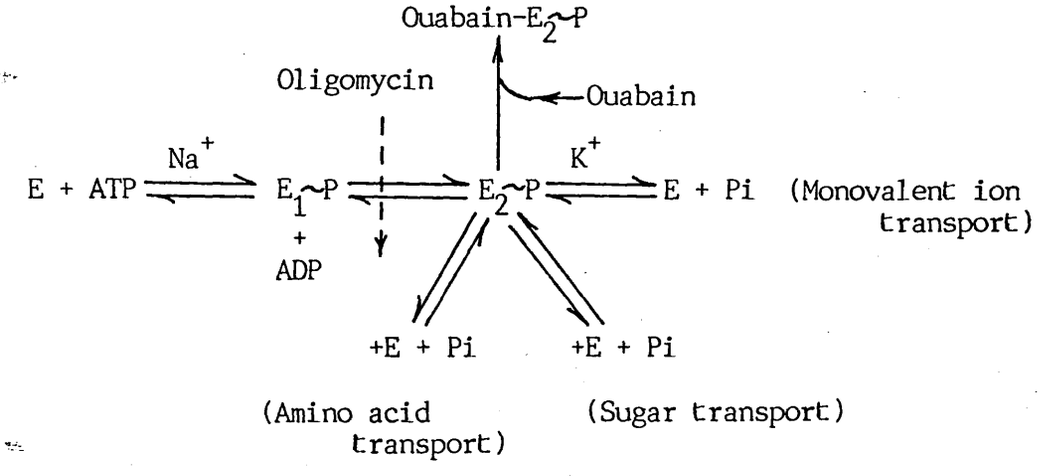
The transcellular transport of glucose by the vertebrate intestine is due to an asymmetric distribution of transport proteins in the cell membrane. Glucose and sodium are pumped across the apical cell membrane (developed into a brush border) into the cells by a sodium-dependent glucose carrier protein. Transport of sodium into cells (down its electrochemical gradient), produces a local osmotic gradient across the apical membrane, causing a passive flow of water into the cells through hydrophilic pores in the membrane. A solvent drag effect is established that sweeps solutes towards the microvilli from the lumen, and further into the cells on the cytoplasmic side of the membrane. Microvilli act as "backward channels" (Berridge and Oschman, 1972).

The anionic sugars of the glycocalyx create a favourable electrical force attracting sodium and other cations to the membrane. In the microvilli of the mammalian intestine is a core of actin filaments, enabling individual microvilli to move and disrupt the unstirred layer in the brush border (Alberts, Bray, Lewis, Raff, Roberts and Watson, 1983)

The sodium gradient driving the sodium-dependent glucose carrier is maintained by a Na^+-K^+ATPase in the basal and lateral membranes which keeps the cellular sodium concentration low. Absorbed glucose passes across the basal regions of the cell (down its

concentration gradient) by facilitated diffusion, mediated by a sodium -independent glucose carrier protein in the lateral and basal membranes. These solute movements produce another osmotic gradient favouring a passive efflux of water from the cells into the lateral intercellular spaces acting as "forward channels". Another solvent drag effect is established which carries solutes to the basal membrane where they are concentrated, and sweeps them towards the blood capillaries on the other side of the membrane (Berridge and Oschman, 1972).

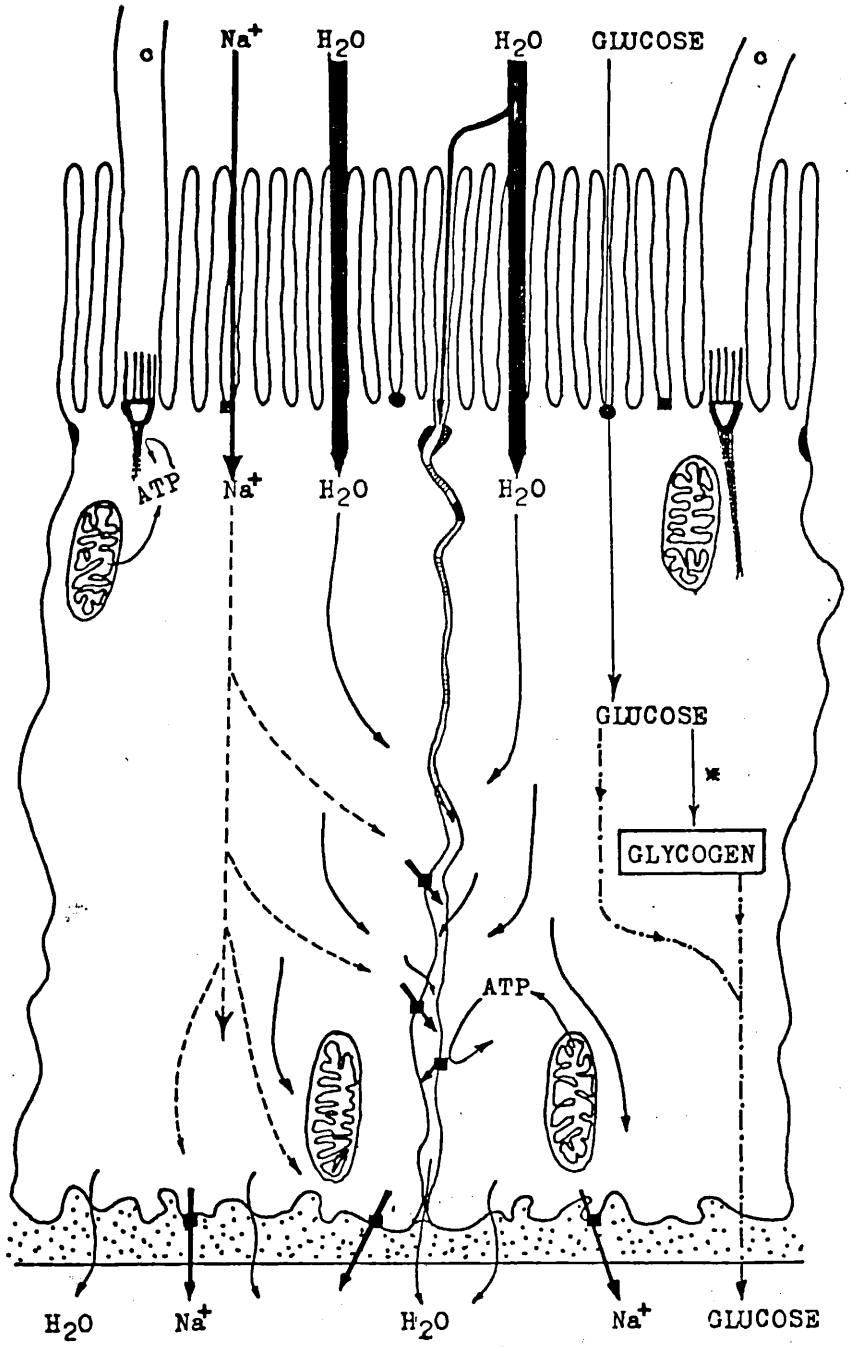
Komnich (1970) proposed that intestinal transport of sugars and amino acids in mammals is energised directly by metabolism rather than by a sodium gradient. He envisaged a membrane-bound ATPase that requires sodium for the energised intermediate (E_2-P). Potassium, phloridzin, oligomycin and ouabain all inhibit this mechanism;



where E is the ATPase, (after Komnich, 1970). Potassium dissipates energy in monovalent ion transport, oligomycin affects the binding of the ATPase with the lipid bilayer and prevents the formation of the intermediate, ouabain prevents the utilisation of it and phloridzin competes with the sugars for their binding site.

The proposed models of glucose and water transport in the oesophagus and intestine of Patella are similar to those described above and are shown in figures 40 and 41.

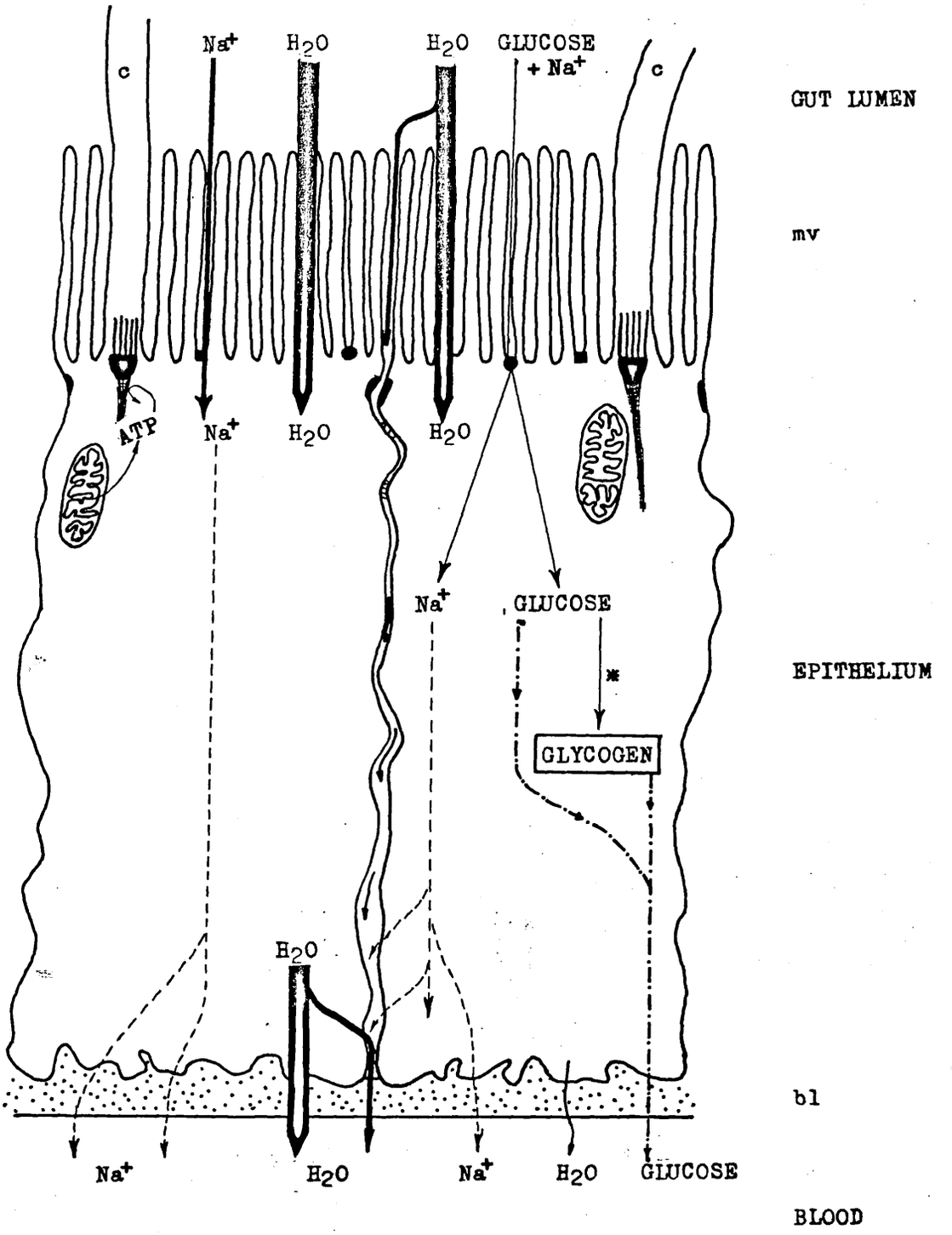
FIGURE 40 . ANTERIOR INTESTINE.



NOTATION FOR FIGURES 40 AND 41.

■.....Sodium transport protein.
——►.....Transport of sodium down electrochemical gradient.
- - - - ►.....Passive diffusion of ions (Na^+) aided by solvent drag.
●.....Glucose carrier protein.
-·-·-·►.....Possible routes.
▬► ~►..Water flow.
* .....Glycogenesis.

FIGURE 41. POSTERIOR INTESTINE AND OESOPHAGUS.



In Patella glucose is absorbed by the oesophagus and posterior intestine by facilitated diffusion, mediated by a carrier powered by the sodium gradient across the apical membrane; this mechanism is thus affected by competitive inhibition with phloridzin and the external sodium concentration. The mechanism operating in the anterior intestine is not powered by a sodium gradient, but is otherwise similar.

A large ciliated surface area is presented by the oesophagus and intestine for absorption. It is necessary to distinguish between the glucose absorbed from the gut lumen and that from the blood, but since the glucose flux experiments were of doubtful validity, one must rely on the ultrastructural features of particular cells to determine the direction of absorption. Cells with large basal surfaces (mucous, basophilic and salivary cells) probably absorb glucose from the blood and those with increased apical surfaces (columnar, amylase-secreting and digestive cells) from the lumen. Autoradiographs failed to identify the cells involved.

In the oesophagus and posterior intestine of P.vulgata solvent drag effects established as described above, concentrate glucose and sodium (as well as other solutes) at the luminal surface of the cells. Both ligands are transported into the cells by a sodium-dependent, phloridzin-sensitive carrier, but in the anterior intestinal cells this mechanism is sodium-independent. In the former, glucose and sodium complex with the carrier at the luminal receptor site, bringing about either an increased mobility of the carrier in the lipid bilayer so that it traverses the bilayer or an allosteric change causing the ligand-binding site to cross the bilayer. The result is that the complex dissociates on the cytoplasmic side releasing both ligands.

The dissociation is due to an increased tendency for the sodium to leave the carrier because of the lower concentration of this ion inside the cell. Both solutes are swept into the cell (by solvent

drag) maintaining the favourable gradient, conversion of the cytoplasmic glucose pool into glycogen and the loss of sodium across the basal and lateral membranes facilitate this.

The absence of basal mitochondria in the oesophageal and posterior intestinal cells, suggests that active extrusion of sodium does not occur. Perhaps the basal membrane is more permeable to sodium than the apical membrane and that the increased cellular hydrostatic pressure, due to the apical water influx, flushes sodium out across the basal membrane.

Metabolic poisoning with 2,4-DNP would effect this model by uncoupling the oxidative phosphorylation of ADP at the inner mitochondrial membrane, directly reducing all ATP-dependent steps; ciliary activity and the ATPases. Consequently the solvent drag effect and reduction of unstirred layers are disrupted and absorption reduced.

Uptake by the digestive cell of Patella may be by pinocytosis or a carrier-mediated system, the former would be inhibited by 2,4-DNP and the latter by phloridzin. The fact that 69% of glucose absorption was inhibited by 2,4-DNP, whilst phloridzin reduced it by 37%, suggests that most absorption occurs by pinocytosis. The phloridzin-sensitive mechanism may be operating in the basophilic cell.

The fate of the absorbed glucose appears to be conversion to glycogen in the columnar cells of the gut and transfer to the blood from the digestive gland in Patella. This suggestion is based on the fact that large glycogen deposits occur in the columnar cells but not in the digestive cells. Absorption by the salivary gland from the blood represents the glucose utilised in the synthesis of the saliva. The mechanism is probably carrier-mediated.

The models presented here can be tested by further experimentation by locating the sites of ATPase activity histochemically, using

ouabain to inhibit the ATPases, determining the direction of net sodium fluxes by short circuit techniques (Gerenscer, 1982) and attempting more autoradiographic experiments to determine which cells absorb glucose. Experiments using labelled amino acids and fatty acids would also be worthwhile.

The functional anatomy of the gut of Patella needs reappraisal since the work of Graham and also in the light of this more advanced work.

The buccal cavity being the first section of the gut, is lined by ectoderm of stomodaeal origin. In Patella, it is a relatively simple region involved with the collection of food, that is lined by an epithelium containing mucous cells (Graham, 1932). Their mucous secretion aids the transfer of food from the radula to the roof of the buccal cavity. Projecting forward from the floor is the buccal mass bearing the docoglossan radula; Graham (1964) describes its functional anatomy.

The radular formula of Patella has recently been revised by Jones et al. (1984), who showed using SEM that the central rachidian tooth, reported by Fretter and Graham (1962), is absent. They suggested that a refraction artefact or shadow seen in light microscope preparations was mistaken for this tooth, which is common in other prosobranchs. Both accounts agree that the dominant pluricuspid teeth are formed by the fusion of other teeth and the latter suggest that the docoglossan radula is derived from a rhipidoglossate form by reduction in the numbers of teeth. Branch (1981) proposed that this has resulted in few teeth, with broader stronger attachments to the radular membrane.

The teeth of Patella are composed of tanned protein, chitin, silica and geothite ($Fe_2O_3.H_2O$ -unique to acmaeid and patellid limpets), which has a hardness of 5 on the Mohs scale and so is harder than some rocks (Jones, McCance and Shackleton, 1935;

Lowenstam, 1962). As the posterior edge of a tooth has a lower (26%) iron content than the anterior (47%), it is softer, tends to wear faster and ensures that each tooth is self-sharpening (Branch, 1981).

A peculiarity of the limpet radula is that the teeth are immobile and do not erect as they move over the radular bending plane. There is no general agreement concerning the mechanism by which new teeth migrate out of the radular sac, but Fretter and Graham (1962) suggested that the "licker" (peculiar to limpets and topshells), is an accumulation of radular membrane that has progressed forward.

After rasping against the rock, the radula is retracted and pressed against the roof of the buccal cavity, transfering any particles which it has picked up, to the dorsal food channel. In this position the radula lies below the apertures of the salivary ducts which pour saliva over it. Most authorities agree that the saliva of most prosobranchs lacks digestive enzymes and acts purely as a lubricant (Fretter and Graham, 1962; Purchon, 1978), although in "advanced herbivores" and carnivores the salivary gland secretes enzymes.

The regions of gut behind the buccal cavity are derived from embryonic endoderm, except the rectum which is a terminal length of proctodaeal ectoderm. The oesophagus of Patella, as is typical of lower prosobranchs, consists of a central, dorsal food channel either side of which, the lateral walls of the mid-section are elaborated into sac-like extensions. In the Patellacea, Littorinacea, Cypraeceae, Lamellariacea and Doliacea they are divided into separate pouches by lamellate lateral folds. In the Pleurotomariacea and Trochacea numerous villi project from the lateral walls. Both lamellae and villi are composed of a glandular epithelium regarded by Fretter and Graham (1962) as a primitive feature; their presence being "an index of extracellular digestion taking place in more posterior parts of the alimentary canal" (Graham, 1939).

Prosobranchs with a crystalline style (Rissoacea, Strombacea,

Tiaridae, Cerithiacea and Calyptraeacea), possess vestiges of this glandular epithelium and thus bear reduced pouches or none at all. Graham (1939) correlated this situation with the presence of the crystalline style, on which is adsorbed an amylase, so an extracellular oesophageal amylase would be redundant. The secretion of a protease is incompatible with the presence of a style, since the protein of the style itself would be digested. In those species where digestive enzymes are absent from the saliva, these oesophageal gland cells produce the first extracellular enzyme (Graham, 1932; Owen, 1958; Fretter and Graham, 1962; Campbell, 1965; Ward, 1966; Balaparameswara Rao, 1975). Fretter (1937) reported an amylase in the "sugar gland" of chitons, which is homologous to the oesophageal gland of prosobranchs and so might function similarly to that of Patella. It would be worthwhile to determine if the sugar gland is ultrastructurally similar to the limpet oesophageal gland.

In comparison with a generalised prosobranch stomach (Graham, 1949), that of Patella is highly modified by reduction in size, simplification and by the change in the topographical arrangements of the apertures of the oesophagus, style sac and duct of the digestive gland. The oesophageal aperture has migrated anteriorly (an evolutionary trend in advanced prosobranchs) so that it opens into the stomach from the left, instead of the right. The style sac leaves the stomach posteriorly and to the left, instead of anteriorly and the digestive duct opens on the left side away from the oesophageal aperture, instead of the right close to it.

The features lost during this remarkable transformation are most of the globular posterior portion of the stomach, the gastric shield, spiral caecum and posterior sorting area. Graham (1949) reported that the latter occurs in Patella, this cannot be confirmed by the present study. Unlike typical typhlosoles, those of Patella originate from within the aperture of the digestive gland duct.

Consequently the limpet stomach is structurally simple and in such respects parallels the stomach of higher monotocardians, opisthobranchs and pulmonates which are macrophagous feeders with extracellular digestive processes. But this does not explain the simplification in Patella, a microphagous herbivore. If one examines the functions of the features that have been lost and relates them to the present idea of stomach function, a reasonable explanation becomes clear.

In Monodonta lineata (da Costa) and lower monotocardians, the spiral caecum ensures a thorough mixture of particles and mucus to form a stomach string, which is squeezed by the muscular stomach walls (releasing nutrients and products of extracellular digestion) protected from sharp particles by the gastric shield; loose particles are carried into the intestinal groove by the ciliated posterior sorting area (Graham, 1949). In Patella, the highly developed oesophagus produces a mucus string that passes into the stomach and then into the style sac without being squeezed by the stomach; the posterior oesophagus squeezes the string and this must have the same effect. This makes a caecum and gastric shield redundant, both could become reduced and finally lost. The function of the sorting area has been replaced by the stomach wall in general, collecting and compacting any free particles with the white secretion from the columnar cells.

Graham's suggestion (1932, 1949) that the muscular valve directs the stomach string into the duct of the digestive gland, cannot be confirmed by the present study; although it is possible, it would be quite unusual. In prosobranchs (Fretter and Graham, 1962) including Haliotis cracherodii (Leach) (Campbell, 1965), Pomacea canaliculata(D'Orbigny) (Andrews, 1965), bivalves and also the scaphopod Dentalium entalis L. (Purchon, 1978), it is the soluble products of digestion and suspended particles that are injected into

the duct of the digestive gland by stomach contractions. The stomach string passes into the style sac. Graham's proposal that the valve can be closed down on either side of the duct aperture is accepted. The significance of this is to prevent stomach fluid being transfered into the style sac instead of the digestive gland.

Stomach contractions were not observed in Patella, so possibly the stomach contents are forced into the duct system by oesophageal contractions or by the bulk fluid flow created by the cilia in the oesophagus and the stomach. In either case the valve would be closed down on the intestinal side of the dilated aperture of the duct. The fact that the duct cilia normally beat towards the stomach suggests that large particles are excluded (as in Nucula; Owen, 1973), however iron saccharate particles entered the tubules (Graham, 1932), so ciliary activity may be altered when stomach fluids enter the duct.

A stoppage or reversal of the duct cilia, together with an expansion of the digestive gland would facilitate the entry of fluids containing soluble nutrients and suspended particles into the gland. This was proposed for Haliotis (Campbell, 1965) and Jorunna tomentosa (Cuvier) (Millot, 1937).

Elimination of faecal material (as a liver string) from the digestive gland into the style sac will occur whether or not the valve is closed down on the oesophageal side of the aperture, because all ciliary currents are directed towards the intestine. The currents generated by the duct walls, typhlosoles and F3 carry the string into the style sac, where it is spiralled into the intestine as the white secretion of its walls build it into a faecal rod. This is not a protostyle (Morton, 1953), because it does not act as a capstan drawing food strings into the stomach and neither does it sweep loose particles across ciliated sorting areas.

Free particles not entangled in the liver string are carried along the intestinal groove and any escaping this route are carried in

suspension into the style sac. Here blebs of cytoplasm thrown into the lumen from the secretory tracts, collide with the particles and remove them from suspension by settling on the walls where they are added to the faecal rod.

Typically the prosobranch style sac is clearly defined from the globular stomach, but because of the great modification of the limpet stomach, such a clear anatomical distinction is impossible (Graham, 1949). The stomach merges imperceptibly into the style sac and similarly this merges into the intestine. The limits of these sections can only be determined on the basis of their characteristic features.

A typical prosobranch stomach is globular and the style sac leaves it anteriorly, on the ventral wall the intestinal groove extends to the intestine, followed by T1, and bordered on its left by T2, which does not pass into the globular part of the stomach (Graham, 1949). Thus the limpet stomach can be defined as that region which does not receive T2, the section between the oesophageal and digestive duct apertures. The style sac starts on the intestinal side of the digestive gland duct. Its distal end is less easy to identify.

The fold, F3, ends at a point where the style sac lies on the right side of the animal (fig 2). The loss of this fold coincides with the loss of the secretory tracts either side of the typhlosoles, which continue into the intestine. The primary function of the style sac is the formation of the faecal rod (Fretter and Graham, 1962), so any specialisations facilitating this would be peculiar to the style sac. The loss of the tracts and F3 must mark the distal end of the style sac.

Apart from its indistinct limits, the limpet style sac functions like that of a typical prosobranch; consolidating the stomach and liver strings into a faecal rod. Unlike typical typhlosoles, those of Patella lack mucous cells, though the unciliated cells function

similarly.

Intestinal length can be correlated with the diet and sanitation mechanism in the mantle cavity, herbivorous prosobranchs have longer intestines than carnivorous species. That of Diodora apertura (Montagu), a carnivore, forms just one loop around the visceral mass, whilst that of Emarginula (Fretter and Graham, 1962), Fissurella barbadensis Gmelin (Ward, 1966), Haliotis (Campbell, 1965) and Pomacea (Andrews, 1965) forms two or more loops. All except Pomacea eject their faecal pellets into water currents that leave the mantle cavity dorsally via a slit or hole.

In Patella the intestine forms six loops, with a total length eight times the shell length. During periods of emersion the pellets are retained in the mantle cavity and would foul and clog the gills if they disintegrated. They are therefore compacted and packaged in the intestine, the posterior portion of which bears two types of gland cell. In contrast, the intestine of aeolids lacks gland cells, the faeces are ejected in a particulate form because there is no mantle cavity to foul (Graham, 1938).

The white secretion of the style sac and anterior intestine increases the diameter of the faecal rod until it fills the lumen of section B, here it is compressed by the muscular walls aided by the reduced diameter of this section. The contents of the intestinal groove are applied to the faecal rod where both typhlosoles merge with a transverse fold in this section. The blebs released from the fold, F4 and the secretory gutter, stick this component to the surface of the rod. The transport of water from the intestinal lumen by the epithelium also aids compaction of the faeces.

The diameter of the posterior intestine is smaller than that of the preceding sections and muscular compaction continues here. The reappearance of completely ciliated typhlosoles, signifies that they no longer serve a secretory role, but help transport the faecal rod.

In Lepidochitona, ciliated cells are attached to the haemocoelic surface of the intestine and it is suggested that they supplement the action of the heart by facilitating the flow of blood through the voluminous visceral blood space (Brimble, 1982; Malacological Conference poster abstract). In Patella this space is less capacious so the effect of a large "dead space" is reduced, and it is suggested that the action of the heart is sufficient to circulate the blood.

Patella lacks an anal gland, which in the muricacean stenoglossans (Fretter and Graham, 1962) and Pomacea (Andrews, 1965) has an excretory function, but in trochids (Fretter and Graham, 1962) and Marisa (Lufty and Demian, 1967) it plays a lubricatory role.

The ultrastructure and function of the cell types in the oesophageal, digestive and salivary glands of Patella needs reviewing considering the recent interest in the last two glands in other molluscs.

The salivary ducts of the Patellacea and most mesogastropods are lined by a ciliated epithelium lacking gland cells (Fretter and Graham, 1962). In P.vulgata this is not so, a secretory epithelium lines the ducts too, suplementing that of the tubules; this coupled with the migration of the enlarged salivary glands into the visceral mass indicates the importance of the saliva. Secretory cells also occur in the salivary ducts of Lymnaea stagnalis apressa Say (Carriker and Bilstad, 1946), L.stagnalis L. (Boer et al., 1967) and Marisa (Lufty and Demian, 1967).

The epithelium lining the salivary ducts of Limax (Beltz and Gelperin, 1979), Agriolimax (Walker, 1970b), mammals (Augustus, 1976) and insects (Kendall, 1969; Oschman and Berridge, 1970) has features typical of an absorbtive epithelium and so may modify the secretion as it travels along the duct. The water and ion content of the saliva may be adjusted and this in slugs would be an adaptation to the terrestrial environment.

The secretory epithelium of the salivary gland of Patella is composed of a single secretory cell type, displaying a secretory cycle (as suggested by Pugh, 1963), interspersed with ciliated cells. The glands as a whole do not show phasic activity. However, recent investigations on L.stagnalis L., Agriolimax, and Limax (Boer, et al., 1967; Walker, 1970b; Beltz and Gelperin, 1979 respectively) suggest that the secretory cells in these species do not undergo a secretory cycle and imply that there are several cell types, chemically and histologically distinct adding a separate component to the saliva.

Two types of secretory cell occur in Achatina fulica Fer. (Ghose, 1963), Aplysia punctata Cuvier (Howells, 1942), Haliotis (Campbell, 1965), Marisa (Lufty and Demian, 1967) and Thais (Bhanu, Shyamasundari and Hanumantha Rao, 1981a). But in Agriolimax (Walker, 1970b) there are ten, in Limax (Beltz and Gelperin, 1979) there are four and in L.stagnalis L. (Boer et al., 1967) there are nine. Carriker and Bilstad (1946) believed that only one secretory cell type occurs in L.stagnalis Say, and that it exhibits different phases. In all these species, except Haliotis and Marisa, the saliva consists of mucus and enzymes.

The secretory vesicles of Patella salivary cells resemble those of the cell type I (mucocyte) and cell type II (serous) of Limax (Beltz and Gelperin, 1979), which correspond to the mucocyte I and grain cell of Agriolimax (Walker, 1970b). The type 1 vesicles of Patella are smaller than the granular vesicles in the slugs.

In the various species investigated, mucous cells typically have a well developed golgi complex but few RER cisternae, whilst the opposite is true of the serous cells. The fact that both of these organelle systems are well developed in limpet cells suggests, together with the histochemical results, that a mucoprotein is synthesised. The presence of proteins in the secretion may increase

its viscosity by cross-bridging and may therefore be responsible for its viscous nature: "..thick, whitish, slightly ropy.." (Graham, 1932).

Very little is known of the gland cells of the oesopageal gland, common histological features are the presence of secretory droplets in the vacuolated cytoplasm and a lack of cilia from the bulging apical portions of the cells which may be pinched off (Fretter and Graham, 1962). Ward (1966) suggested that mucous cells synthesise the amylase demonstrated in the oesophageal gland of Fissurella. Considering the proteinaceous nature of the secretory granules in these cells of Patella, Haliotis (Campbell, 1965) and Thais (Bhanu et al., 1981b) and that they are the only gland cells in the gland, it is reasonable to assume that they are the source of the enzyme.

The presence of a morphologically and histologically distinct mid-oesophagus that secretes an amylase is related to the primitive intermittent, microphagous feeding habit (Graham, 1939; Purchon, 1978). Limpets situated on the littoral zone have a cyclical pattern of emersion and submersion imposed upon them. Because of this they cannot feed continuously and according to Graham (1939), this was the major factor that led to the development of the oesophageal gland. A crystalline style has evolved in species which are continuous feeders.

Lasaea is a discontinuous feeder and according to Morton (1956) its crystalline style shows a pattern of secretion and dissolution correlated with the tidal cycle. Dreissena polymorpha Pall shows style breakdown and secretion, but Morton (1969) related this to a circadian rhythm of adductor activity. Another intermittent feeder that possesses a crystalline style is Rissoa, the storage capacity of the stomach ensures that the style can be involved with digestion when the animal is not feeding, Rissoa can thus achieve continuous digestion.

Amylase secretion by the oesophageal gland, would be under nervous

or hormonal control (Graham, 1939), nerves have been demonstrated in the glandular epithelium and the cells exhibit phasic activity in Patella, which is consistent with this view.

During periods when feeding is possible (submergence) in Patella, the amylase-secreting cells contain secretory granules released in blebs of cytoplasm, that are carried into the food channel by the cilia of the pouches and three longitudinal folds. The method of enzyme release is unknown, but it is possible that autolysis begins once the blebs are shed. The amylase initiates carbohydrate digestion in the mass of food and mucus passing along the food channel. The pH optimum of this amylase is 6.2 at 30°C (Graham, 1932) and this is similar to the pH of the oesophageal, stomach, style sac and anterior intestinal contents (6.0 and 6.4 respectively), so the enzyme may be active in these regions too.

As an extracellular cellulase is absent from Patella, digestion of plant cell walls cannot occur, so the amylase must act on food reserves released from cells ruptured by the radula. All algal phyla, except the Phaeophyta, have unicellular representatives and each has peculiar final products of photosynthesis and metabolism; these two processes determine the nature of the food store. The Chlorophyta contains true starch, Chrysophyta - oil and leucosin, Cyanophyta - proteinaceous cyanophysin and cyanophycean starch, Rhodophyta - floridean starch, galactan sulphate and floridioside (Kumar and Singh, 1979). Diatoms (Bacillariophyta) contain leucosin, lipids and sterols (Miller, 1962), all of which may be assimilated by Patella. Leucosin is a polymer of glucose, containing at least eight glucose molecules, in some diatoms this may account for 15-30% of the cell mass. It is converted to glucose by Cryptochiton digestive enzymes (Meeuse, 1962).

The amylase-secreting cells of Patella are not simple gland cells, they show ultrastructural similarities with the digestive cell;

the presence of pinosomes, an endocytotic canal system, a lysosomal system consisting of numerous vesicles and the abstriction of the cell apices. The pinocytosis of ferritin by clathrin-coated pits suggests the uptake of proteins by these cells. Other non-proteinaceous organic solutes may be taken up by the uncoated vesicles and canal system. As proteases are not produced by salivary and oesophageal glands, the proteins must be derived from the algal cells or dissolved organic material in the sea water. Mono- and disaccharides from the digestion of polysaccharide storage products are also absorbed at this stage by the ciliated cells of the oesophagus.

Because the ciliary currents oppose the movement of fluid into the oesophageal pouches, the dissolved solutes must be squeezed into them by contractions of the muscles that ensheath the oesophagus, together with the radial fibres of the lateral folds which, on contraction, reduce the depth of the pouches.

The thin mucous lamellae that occur between the longitudinal folds of the posterior oesophagus have a high surface:volume ratio, facilitating the diffusion of nutrient molecules from the food-mucus mixture into solution. The molecules can then be absorbed here and in subsequent sections of the gut.

The discovery that the amylase-secreting cells can pinocytose material from the lumen and that as a whole the oesophagus can absorb D-glucose, sheds new light on the function of the prosobranch oesophagus. It has only been shown to be a site of absorption in H.rufescens (McLean, 1970) and A.reticulatus (Walker, 1972). Together with its primary function of mixing the amylase with the food entangled in the saliva and mucus from the subepithelial mucous cells of the food channel, the oesophagus also assimilates the by-products of carbohydrate digestion (which it initiates) and dissolved organic solutes. The large surface area of the pouches and the thin basal lamina of its epithelium are condusive to this. The food absorbed by

the amylase-secreting cells, including amino acids, peptides and proteins may be used in the synthesis of the amylase enzyme during the secretory phase.

The digestive gland of Patella is histologically similar to that of other prosobranchs in that the ducts are lined by an epithelium resembling the stomach, whilst the tubules are composed of distinct digestive and basophilic cells. Graham (1932) and Pugh (1963) did not find unciliated cells in the duct epithelium of Patella, nor did they identify the numerous lipid droplets reported here. Unciliated cells have also been found in the ducts of other molluscs (Owen, 1958 & 1972a; Campbell, 1965) and in each case they produce a clear secretion in the form of apical blebs and absorb lipids.

Pugh (1963) demonstrated glycogen and proteins in the apical cytoplasm of the duct cells in Patella, confirming the absorptive role indicated by their ultrastructural features. The duct epithelium of H.aspersa, Nucula and Ostrea is also absorptive (Sumner, 1965a; Owen, 1972a; Mathers, 1972 respectively). This region supplements the endocytotic activity of the digestive cell, which most authors agree is the main site of nutrient absorption in the molluscan gut (Yonge, 1926a; Fretter and Graham, 1962; Owen, 1972a; Purchon, 1978).

The observations reported here on the digestive cell of Patella support the observations of Graham (1932) and confirm the absorptive function. Exogenous material is endocytosed into an elaborate heterophagic lysosomal system where intracellular digestion occurs. A number of questions remain as to the functioning of this system, concerning the way material is endocytosed and then transfered to the digestive vesicles and the interrelationship of the different vesicles.

In Patella, the uptake of ferritin in clathrin-coated pinosomes (as in the amylase-secreting cell) is similar to the endocytotic

process in Lasaea (McQuiston, 1969b), Cardium (Owen, 1970), Mytilus (Owen, 1972a), Mya (Pal, 1972) and Rissoa (Wigham, 1976). These authors also reported an endocytotic canal system and all except Pal suggested that it is involved in the transfer of food to the heterophagosomes, by direct connections similar to those found in Patella. A canal system has also been reported in the pericardial gland cells and the cells lining the proximal limb of the kidney in Scrobicularia plana (Da Costa) (Jennings, 1984) and the left and right kidneys of archaeogastropods (Andrews, 1985).

Connections of the canal system with the apical membrane have not been observed, so Owen (1972a) suggested that pinosomes empty their contents into the system, thereby filling the heterophagosomes indirectly. McQuiston (1969b) suggested and Wigham (1976) agreed, that the canals lead from the lumen to the heterophagosomes, although they did not propose a mechanism by which food enters the system. It is possible that the connection to the lumen is transient, lasting long enough to allow the diffusion of food into the canals as separate pulses, thus establishing a concentration gradient along the canal-heterophagosome complex.

Merdsoy and Farley (1973) and Boghen and Farley (1974) adopted the view that the torus-shaped pinosomes found in L.littorea and L.saxatilis, are formed by the invagination of the apical membrane around a rigid microvillus and do not represent sections of a canal system. Similarly shaped pinosomes occur in Patella, but are believed to be sections of the canal system. Phagocytosis has been reported in H.aspersa (Sumner, 1965a), Anodonta anatina (L.) (Sumner, 1966b & c), Cardium (Owen, 1970), Ostrea (Mathers, 1972) and Arctica islandica (L.) (Palmer, 1979), but was not seen in Patella.

The presence of a glycocalyx coat in a pinosome reduces its permeability. Marshall and Nachmias (1965) investigating phagocytosis

by amoebae, found that when the glycocalyx lining a phagocytotic vacuole was digested away, the bounding membrane became 100 times more permeable to water. The significance of this in endocytosis is that water movement out of the vesicle will concentrate the solutes present and establish a gradient across the membrane favouring their diffusion into the cytoplasm.

Food accumulates in heterophagosomes (= "apical phagosomes", McQuiston, 1969b: "type 1 macrovesicles", Owen, 1970; Boghen and Farley, 1974: "secondary vesicles", Merdsoy and Farley, 1973) which lack enzymes (Owen, 1972a). McQuiston (1969b) proposed the idea that they are continuously formed by the fusion of pinosomes and Owen (1970 & 1972a), Merdsoy and Farley (1973) and Wigham (1976) support this view, although Owen suggested the possibility that once formed they are permanent structures. The evidence obtained from Patella agrees more closely with McQuiston's concept; the increase in heterophagosome size as they migrate deeper into the cell, suggests that they are pushed away by the formation of new, smaller vesicles due to pinosome fusion. The canal system makes new connections with the recently formed vesicles.

Digestion of food occurs in the heterolysosomes (= "phagolysosomes", McQuiston, 1969b: "type 2 macrovesicles" Owen, 1970; Boghen and Farley, 1974: "digestive vacuoles", Merdsoy and Farley, 1973) which are the enzyme-containing digestive vesicles of the cell. McQuiston (1969b) suggested that they form as the heterophagosomes migrate into the cell and fuse with lysosomes. If the heterophagosomes are permanent as Owen proposed, food must be passed from heterophagosome to heterolysosome by small vesicles budding from the former and fusing with the latter, the origin of which he does not explain.

Unfortunately, the work on Patella has not resolved the problem of how heterolysosomes are formed and how they receive food and

enzymes. There was no evidence of fusion between heterophagosomes or with lysosomes to form heterolysosomes. The Ω-shaped evaginations of their bounding membrane can be interpreted in different ways. They could represent pinosomes fusing with the heterolysosomes instead of the heterophagosomes, that they are lysosomes transfering enzymes, that they are small vesicles nipped-off from the heterophagosomes and fusing with the heterolysosomes or that they are evaginated vesicles containing "breakdown products of digestion", which pass their contents across the basal membrane of the cell by exocytosis as proposed by McQuiston (1969b).

The last two suggestions can be ruled out for Patella, because the heterolysosomes are not permanent and any final products of digestion would be in the form of diffusable molecules, rendering their exocytosis improbable. The possibility that they are pinosomes rather than lysosomes is more likely because of their ultrastructure and lack of acid phosphatase. In Lasaea (McQuiston, 1969a), Cardium and other bivalves (Owen, 1970) and L.saxatilis (Boghen and Farley, 1974), the swollen margins of the golgi cisternae and the primary lysosomes derived from them, contain "membranous elements" consisting of disc-shaped vesicles (Owen, 1972a) or particles 30nm in diameter (McQuiston, 1969a). Such a feature was not seen in any of the vesicles or golgi cisternae of Patella.

Acid phosphatase was demonstrated in the heterolysosomes of Patella only at the histological level, so it is difficult to speculate how and where the lysosomal enzymes are derived. The amylase and protease demonstrated by the substrate-film methods probably occur in the lysosomal system of the digestive cell. A lipase may be absent from the cells, so the lipid droplets in the duct and digestive cells may be composed of fatty acids and glycerides from the algal cells, which could be absorbed without prior lipolytic digestion.

Lysosomes can be derived from the ER or golgi (Novikoff, Essner and

Quintana, 1964). In Lasaea, lysosomes derived from SER cisternae fuse with heterophagosomes to form heterolysosomes which migrate to the base of the digestive cell and fuse with other lysosomes from the golgi complex (McQuiston, 1969a). The characteristic primary lysosomes containing "membranous elements" were thought to contain acid phosphatase, but the variable results of enzyme incubations have only confused matters (Owen, 1970). The golgi of Patella digestive cells was so rarely encountered that nothing definite can be said about its role in the cell. Peroxisomes (Owen, 1972b) were not seen in the digestive cells of Patella.

As intracellular digestion proceeds and undigested waste residues accumulate in the heterolysosomes they transform into residual bodies (Owen, 1972a & 1973; Wigham, 1976) which in Cardium are shed in spherules abstricted from the cell (Owen, 1955). McQuiston (1969b) proposed that in Lasaea waste material is lost by cellular disintegration, whilst in Mytilus (Owen, 1972a), L.littorea (Merdsoy and Farley, 1973) and Rissoa (Wigham, 1976) residual bodies are exocytosed across the apical membrane. In L.saxatilis (Boghen and Farley, 1974) the type 4 vesicles rupture and the released wastes are shed by exocytosis or diffuse out of the cell.

The apocrine excretion of wastes shown by Patella resembles that of Cardium, but the transformation from heterolysosome to residual body may not be as simple as Owen suggests it is in bivalves. For instance, fusion between heterolysosomes forms "apical granules", and residual bodies may also connect to heterolysosomes, as if sequestering material from their lumen. Residual bodies may form by budding from heterolysosomes or "apical granules".

The darkly staining cell type has been given many names (basophilic cell- Owen, 1970, 1972a & 1973; Pal, 1971; Wigham, 1976; Palmer, 1979: crypt cell- Campbell, 1965; Mathers, 1972; Nelson and Morton, 1979: excretory cell- Owen, 1958; Andrews, 1965; Merdsoy and Farley, 1973:

secretory cell- Graham, 1932; Howells, 1936 & 1942; McQuiston, 1969b; Boghen and Farley, 1974: young cell- Yonge, 1926b; Owen, 1955) and attributed different functions (the secretion of enzymes- Howells, 1936 & 1942; McQuiston, 1969b; Sumner, 1969; Owen, 1970; Pal, 1971; Mathers, 1973; Boghen and Farley, 1974; Wigham, 1976; Nelson and Morton, 1979: secretion of a "cement"- Graham, 1932: excretion- Owen, 1958; Andrews, 1965; Campbell, 1965; Lufty and Demian, 1967; Merdsoy and Farley, 1973: regeneration of tubules- Yonge, 1926b; Palmer, 1979).

It has been established here that the basophilic cell of Patella is a protein-secreting cell, ultrastructurally resembling the homologous cells in Anodonta (Sumner, 1966c), Lasaea (McQuiston, 1969a), Cardium (Owen, 1970), Mya (Pal, 1971), Nucula (Owen, 1973), L.saxatilis (Boghen and Farley, 1974), Rissoa (Wigham, 1976) and also the exocrine cells of the mammalian pancreas and salivary gland (Threadgold, 1976; Leeson, 1967). It has all the ultrastructural features associated with protein secretion. Hypertrophied mitochondria have also been reported in rabbit submandibular gland cells and are suggestive of intense activity; the large nucleolus is indicative of ribosome synthesis (Threadgold, 1976).

The fenestrations in the golgi cisternae are reminiscent of the GERL region in various mammalian exocrine cells (Hand and Oliver, 1977). In these cells the GERL may be derived from the trans golgi cisternae , it is joined to condensing vesicles by multiple tubular connections both of which are intensely reactive to acid phosphatase tests. This is the first time such a golgi has been reported in the basophilic cell of the molluscan digestive gland. The GERL region is involved with the formation of secretory vesicles in the exocrine cells and the basophilic cell in Patella.

The function of the basophilic cells in Patella is still in

doubt. Graham (1932) proposed that they produce a mucus-like secretion that cements pieces of plant cell walls and diatoms (and in the light of the present work, the fragmentation spherules also) into a liver string.

The secretory granules contain carbohydrates and proteins, a condition typical of mucus, one would expect an enzyme to be only protein. However, Nelson and Morton (1979) reported a similar glycoprotein in the crypt cells of Maoricrypta to be enzymatic. If extracellular enzymes were released into the tubule lumen, the duct cilia would sweep them into the stomach and Graham (1932) would have detected them. Furthermore, enzymes should be released when food is present in the tubule; the only occasion that release of secretion was noticed coincided with the fragmentation phase of the digestive cell, such timing indicates that the secretion is a "cement". As the food entering the tubules of the digestive gland consists of dissolved molecules and suspended particles, extracellular digestion is unlikely.

In at least some species two or three basophilic cell types exist. Sumner (1966c) described immature and mature cell types, McQuiston (1969a) suggested that stem cells at the blind endings of the tubules replace the epithelium after its disintegration. Owen (1970) reported a columnar, flagellated, immature basophilic cell possessing numerous free ribosomes instead of an extensive RER and suggested that they replace either or both of the other two cell types. Fankboner (in Owen, 1972a) suggested that stem cells give rise to flagellated and pyramidal basophilic cells in Tridacna and that the latter are a transitory stage in the formation of mature digestive cells. Nelson and Morton (1979) proposed that in Maoricrypta the crypt cells repopulate the tubules after epithelial breakdown, either by groups of them budding from old tubules to form new ones or by remaining in the old tubules and simply replicating. Mathers, Smith and Colins (1979)

also proposed a budding method. Sumner (1983) found that 6 hours to 30 days after injecting H.aspersa with ^{3}H-thymidine, the main site of labelling was in the ducts, few digestive cells were labelled but on one occasion they were near a duct.

Since immature or stem cells have not been identified in Patella the mechanism of cell renewal is unknown. The possession of a flagellum by the basophilic cell does not imply that they are immature because the well developed RER, golgi complex, nucleus (and nucleolus) and the secretory granules are all indicative of a mature secretory cell. The function of the flagellum is to circulate the luminal contents, so that during the absorption phase unstirred layers are reduced and during the fragmentation phase, the excretory spherules are mixed with the "cement". The activity of the flagellum may also aid the entry and exit of materials from the tubules.

The presence of profiles of an endocytotic canal system in the apical cytoplasm suggests that the cells are capable of absorbing food from the lumen, although neither ferritin or iron saccharate (Graham, 1932) were taken up by it. Wigham (1976) reported that the homologous cells in Rissoa endocytose material from the lumen by a canal system.

The digestive cells of Patella clearly exhibit different phases that give the epithelium of the tubules various appearances, the digestive and fragmentation state being the most frequently encountered. Phasic activity could not be related to the tidal cycle and in this respect Patella resembles L.littorea (Merdsoy and Farley, 1973) and Maoricrypta (Nelson and Morton, 1979). Cyclical activity may be related to an animal's feeding habits and distribution governed by the tide, those experiencing long periods of immersion during which feeding may be continuous, do not show tide-related rhythms. Conversely, long periods of emersion and intermittent feeding, imposes a tide-related cycle upon the digestive activity of

some molluscs (Lasaea, McQuiston, 1969b; L.saxatilis, Boghen and Farley, 1974; Rissoa, Wigham, 1976; Chlamys and Venerupis, Mathers, Smith and Colins, 1979).

The length of time for the completion of digestion dictates whether the activity cycle is mono- or diphasic. Both Venerupis and Rissoa exhibit a monophasic cycle, in which all the tubules are at the same phase and digestion is completed within 12 hours; all the tubules are ready to receive food every 12 hours. When digestion is slower, two tubule conditions co-exist, half of the tubules exibit one phase (usually that of digestion) whilst the other half are regenerating or in a holding phase. Both Chlamys and Lasaea show a diphasic cycle.

A more detailed study of Patella from different levels on the shore, might reveal tide-related digestive rhythms with increasing length of emersion.

The marked change in the appearance of the gland cells from the oesophagus and digestive glands of starved limpets, indicates reduced activity related to the insignificant volume of food consumed. The ultrastructural features of the amylase-secreting and digestive cells were predicted. Amylase synthesis was stopped since there was no longer a need for it and pinocytosis was reduced in both cell types, because the low concentration of food was insufficient to stimulate extensive activity. However, since the endocytotic canal system and pinosomes were not totally absent, both cell types were probably absorbing some nutrients. Although the limpets were deprived of visible quantities of food, dissolved organic material was present in the common sea water supply of the aquarium. These nutrients could be absorbed by the mechanisms discussed earlier and this may explain the unaltered condition of the ciliated epithelium.

Shrinkage of the digestive gland and gonad may represent resorption of tissues as a physiological solution to starvation; the fact that

the lipid droplets of the digestive cells were not utilised as a food source is surprising.

Purchon (1978) proposed that the primitive molluscan gut was composed of a "multipurpose digestive cell" capable of phagocytosis, intracellular digestion, absorption, secretion and excretion, that in some cases these cells have been retained and in others have been superseded by specialised gland cells. On the ultrastructural evidence, the ciliated and unciliated columnar cells of Patella have features suggesting absorptive and pinocytotic activity, as well as ciliary and secretory functions. This, together with the lack of specialised gland cells from the stomach, style sac, anterior intestine and digestive gland ducts suggests that these cells are primitive according to Purchon's definition. A feature of the epithelium lining these regions in Patella is its ability to produce a white secretion.

Gibson (1887) reported that the limpet gut can secrete a "whitish rod" and Mackintosh (1925) mistook this for a crystalline style. It was Graham (1932) who suggested that the ciliated cells of the anterior intestine were producing "some kind of secretion" and Pugh (1963) reported that the cells lining the digestive gland duct are secretory, noting that the secretion is elaborated by the apical cytoplasm.

Columnar cells from the gut of other molluscs produce a similar secretion. The pigmented columnar cells of the oesophagus, stomach, style sac, intestine and digestive gland ducts of H.cracherodii release a clear secretion. As in Scutus breviculus (Blainville), the unciliated cells lining the ducts of the digestive gland of Nucula produce "vesicular outpushings" that are nipped-off from the cells (Owen, 1958). Carriker and Bilstad (1946) reported "secreting ciliated cells" in the gut of L.stagnalis Say and Lufty and Demian (1967) described an acidophilic secretion from the ciliated cells of

the gut of Marisa.

In all these species the secretion performs a lubricatory and cementing role and in Patella, abundant blebs are formed in regions where specialised gland cells are absent. Furthermore these regions are where the faecal rod is formed and compacted, the secretory tracts and ciliary currents in the style sac and anterior intestine are specialisations for this. Similar cytoplasmic blebs in other species carry secretory vesicles into the gut lumen.

The intestinal ciliated cells of Mytilus galloprovincialis Lamarck, secrete a proteinaceous substance of unknown significance in the form of individual vesicles or within blebs of cytoplasm (Giusti, 1970). Judd (1979) described two types of ciliated style sac cell (B and D) responsible for secreting the crystalline style in Amphidesma australe (Gmelin), Macomona liliana (Iredale) and Pholadidae spathulata (Sowerby). These cells and their blebs contain PA-TSC-SP-reactive droplets.

The blebs formed by the style sac gutter cells of Lasaea contain cytoplasmic ground substance, ribosomes and small vesicles containing the pre-cursors of the style matrix. In Dentalium, Taib (1976) reported that ciliated cells in the stomach produce a secretion rich in β-glucoronidase and galactosidases. In the opisthobranch Jorunna, the ciliated cells of the caecum and mid-gut are secretory (Millot, 1937), but those of Cymbulia peronii Blainville and Aplysia (Howells, 1936 & 1942 respectively), aeolids (Graham, 1938) and tectibranchs (Fretter, 1939) are not.

The blebs of Patella lack secretory vesicles and were unreactive to all the histochemical tests employed. They do not form a "mucin" as Pugh (1963) proposed. The absence of specialisations typical of secretory cells (extensive RER, large golgi complex and secretory vesicles) from the columnar cells, together with the mechanism of bleb formation indicates that they contain only cytoplasmic ground matrix

derived from the microvilli, when they dilate explosively into the lumen. Contraction of the apical cytoskeleton which in mammalian intestinal cells contains actin and myosin filaments (Mooseker and Tilney, 1975), could cause the constriction of the bleb to form a neck, which disappears as the opposing membranes eventually meet.

The microvilli may be regenerated by the polymerisation of actin filaments pushing the apical membrane outwards into finger-like evaginations. Biochemical tests on sea urchin eggs, indicate that extension of microvilli is accompanied by large scale polymerisation of actin (Alberts et al., 1983). A similar mechanism of bleb formation and microvillus regeneration probably occurs in the amylase-secreting and digestive cells of Patella.

Bleb production may be under nervous control. Nerve fibres intrude into the epithelium and contact the basal membrane of the columnar cells in those regions where bleb release is common. Connections between nerve and epithelial cells or release of a neurosecretion were not seen, so the stimulus may be related to the membrane potential of the neurilemma.

With the advent of electron microscopy came the discovery of the cytoskeleton which is implicated in amoeboid movement, organelle transport, maintenance of shape, structural support and cell division. Recent high-voltage TEM has revealed a lattice of microtrabeculae pervading the cytoplasm of animal cells (Porter and Tucker, 1981). The degree of development of the cytoskeleton in the columnar cells of Patella is related to their height, and is significant in the compaction of the faecal rod and resisting the hydrostatic pressure in the lateral intercellular spaces during water flow across the epithelium.

Mechanical support is afforded by the cytoskeleton and in the intestine also by the finger-like extensions of the basal lamina. Cellular support is essential to prevent damage during the squeezing

of the faecal rod by the muscular intestine. In their description of a typical prosobranch style sac cell, Fretter and Graham (1962) reported intracellular fibrillae converging to the side of the nucleus or the basal surface from the basal granules of the cilia. These fibrillae correspond to the cytoskeleton, which in Patella is not associated with the ciliary rootlets.

An ATPase is associated with the cross-bands of certain rootlets (De Robertis and De Robertis, 1980), which suggests that the apposition of the mitochondrial cristae and rootlet bands in Patella, ascidians and rotifers (Threadgold, 1976), is to reduce the distance that ATP molecules travel between the organelles.

Long cilia can develop a larger arc of swing and provide a greater surface area which acts on the fluid in the gut lumen, than short cilia. This condition would be suitable for transporting viscous secretions as in the salivary ducts and the oesophageal food channel, for generating strong currents in the intestinal groove or "painting" secretions onto the faecal rod as in the posterior intestine. The smaller arc of short cilia produces a more "rigid beat", suitable for moving the bulk of the faecal rod as is the case of the general surface of the stomach, style sac, intestine and the rectum.

The cilia of the oesophageal gland need only generate weak currents to carry the amylase-containing blebs into the food channel. The slightly longer cilia of the cells lining the digestive gland ducts transport loose particles, aid formation of the liver string and transport it towards the stomach, so the cilia are of intermediate length.

The junctional complex of the limpet gut cells resemble those of Sepia officinalis L., both include a belt desmosome encircling the cell and a pleated-sheet type of septate desmosome common to annelids, arthropods (in tissues of ecto- and mesodermal origin) and other molluscs (Boucaud Camou, 1980). Tight junctions (zonula occludens)

typical of the vertebrate intestine are absent, but the septate junctions of invertebrate cells function similarly. The junctional proteins themselves form a seal without bringing the opposed membranes together (Alberts et al., 1983), although they remain permeable to water. The belt, septate and hemidesmosomes strengthen the epithelium by locking the cells and basal lamina together, so distributing mechanical forces to the epithelium and connective tissue as a whole (Staehelin and Hull, 1978) and minimising any damage during compaction of the faecal rod.

The ciliated columnar epithelium has several ultrastructural features indicative of absorption (Berridge and Oschman, 1972). The apical membrane is elaborated into a well developed brush border of microvilli (except in the salivary gland and its ducts) covered by an anionic glycocalyx. This and the folded basal membrane increases the surface area of the cell across which solutes can be transported. Mitochondria in the apical and basal regions of cells suggests the presence of ATP-dependent processes there. PA-TSC-SP-reactive glycogen deposits are more abundant in those cells with a PAS-positive brush border, indicating that this carbohydrate moiety of the glycocalyx plays a part in glucose absorption.

The highly anionic acid mucopolysaccharides (hyaluronic acid and chondroitin sulphate) in the glycocalyx of the columnar cells resemble those found in vertebrate glycocalyces (Threadgold, 1976). Cationic solutes in the gut lumen will be attracted towards and concentrated at the apical membrane, the significance of which was discussed earlier.

The negative charge of the glycocalyx may vary with pH. Assuming that the carboxyl and sulphate groups in the sugars exist in the following equillibria:

$COOH \rightleftharpoons COO^- + H^+$ and $OSO_3H \rightleftharpoons OSO_3^- + H^+$

then the equillibria will be shifted to the left in acid conditions and to the right in alkaline ones. These groups will be dissociated to

a greater extent (have a greater negative charge) in the posterior intestine where the pH is higher than the rest of the gut. Glucose absorption in this region is sodium-dependent and this cation will be attracted to the glycocalyx with greater force at the higher pH than elsewhere.

Parsons (1975) suggested that in the vertebrate small intestine, digestive enzymes are adsorbed onto the glycocalyx of the epithelial cells. It is therefore possible that the extracellular amylase and the lysosomal enzymes released from the spherules of the digestive cells may be adsorbed onto that of the columnar cells. Moog (1981) reported that in the mammalian small intestine, alkaline phosphatase, disaccharidases and aminopeptidases are actually glycoproteins embedded in the microvillar membrane, the carbohydrate tail forming the glycocalyx that traps those enzymes secreted by the pancreas.

Acid phosphatase occurs on the brush borders of the columnar cells lining the digestive gland ducts and section A of the intestine in Patella. Acid and alkaline phosphatases have been demonstrated at the brush borders of other animal cells; the excretory cells of A.hortensis digestive tubules (Bowen and Davies, 1971), the digestive gland ducts of Nucula (Owen, 1973), Artica (Palmer, 1979) and Dentalium and mouse duodenal cells (Hugon and Borgers, 1966). Alkaline phosphatase has been implicated in active transport of extracellular molecules against concentration gradients (Rothstein, Meier and Scharff, 1953; Monin and Rangneker, 1974).

Pugh (1963) reported β-glucoronidase in the apical regions of the digestive, basophilic and duct cells of Patella and β-glucosamidase in the digestive cell. The former enzyme hydrolyses glucoroids in plant celluloses and its activity is greater in herbivorous molluscs (Corner, Leon and Bulbrook, 1960).

An increased surface area of membrane is a significant absorptive feature because a higher density of protein carrier molecules and

enzymes can be situated on the extra membrane, so increasing the transporting potential of the cell.

That the columnar cells of Patella are pinocytotic is suggested by the presence of "uncoated" depressions in the apical membrane and an endocytotic canal system, although the formation of vesicles and the uptake of ferritin was not seen. The PTA reactivity of the glycocalyx and the canal system suggests that they are continuous. The connection of the canal system with the apical electron-opaque bodies and the depressions, indicates that the former may be "uncoated" pinocytotic vesicles or secretory vesicles, that release their content into the canal system instead of at the base of the microvilli.

Opaque bodies are common in the cells lining the stomach, style sac, intestine and ducts of the digestive gland of Patella, which are involved in absorption. However, the derivation of similar bodies from the margins of the golgi cisternae suggests that they are either secretory or lysosomal in nature. Acid and alkaline phosphatases are absent from the opaque bodies suggesting that they are not lysosomes, although other enzymes could be present. On no occasion was the release of the bodies observed, either by exocytosis or within the blebs of cytoplasm. However, if their contents are released into the canal system, there could be a gradual loss of secretion which would be less conspicuous than that by normal exocytosis.

The opaque bodies in the anterior intestinal cells stain for carbohydrates (PTA), this could be interpreted as a secretion or a carbohydrate-rich material pinocytosed from the lumen, but neither the golgi or the lumen in those sections contained a similar material. They could be storage vesicles; those derived from the golgi containing a PTA-positive matrix that binds certain molecules sequestered by the canal system. If this was so one would expect them to be depleted in the starved animals, but they were not. Clearly from the existing evidence their exact nature can not be determined.

Further feeding experiments using tracers such as colloidal gold or horseradish peroxidase and more enzyme incubations may establish their role in the cell.

The presence of acid phosphatase in the golgi, primary and secondary lysosomes confirms their lysosomal nature. Endogenous material was only encountered in the multivesicular bodies which, because of their lack of phosphatase enzymes and derivation from pre-existing cytomembranes of two or three layers, suggests that they are autophagosomes (Threadgold, 1976).

Multivesicular bodies have been noted in numerous cell types of different species, yet their function remains enigmatic. The internal vesicles may arise when SER membranes envelop golgi vesicles and in this case may show acid phosphatase activity (Novikoff, Essner and Quintana, 1964). Farquhar and Palade (1962) found them in cells of the renal glomerulus concerned with protein uptake. Bowen (1970) found them in the crop and intestinal epithelial cells of A.ater. McQuiston (1969a) found them in the style sac and basophilic cells of the digestive gland of Lasaea, and Pal (1972) described them in Mya digestive and basophilic cells; all are secretory cells. However, Robbins, Marcus and Gonotas (1964) suggested that they are involved in endocytosis and Lane (1968) has demonstrated their lysosomal nature.

The secondary lysosomes do not contain cellular debris so are probably involved in the digestion of exogenous material obtained by endocytosis and are therefore heterolysosomes. Their enzymes are derived from the primary lysosomes by fusion. Autolysosomes typically contain endogenous material (such as fragments of organelles or membrane structures derived from them) together with enzymes, these were not seen in the columnar cells.

The residual bodies are typical of those described by Threadgold (1976), their myelin figures, granular deposits, lack, or low

activity, of phosphatase enzymes, deposits of lipid and lipofuscin pigment are indicative of the terminal lysosomal phase. Newbigin (1898) found that the gut pigment of Patella is a metabolic derivative of chlorophyll in the diet, suggesting the absorption of this component by the columnar cells.

The golgi complex is involved with the formation of the electron-opaque bodies, multivesicular bodies and primary lysosomes, the latter supplying the heterolysosomes with acid phosphatase and possibly other enzymes for intracellular digestion. The absence in the golgi of any reactions to cytochemical tests for carbohydrates, implies that the cells do not actively synthesise a secretion; this is supported by the scarcity of RER in the cytoplasm. Synthesis of the cytoplasmic ground matrix to replace that lost in the blebs must take place at the RER and the few ribosomes free in the cytoplasm. The golgi must also be responsible for the production and packaging of the glycocalyx on the apical membrane which is periodically lost when blebs of cytoplasm are released.

The SER has been implicated in glycogenolysis (depolymerisation) in mouse and rat liver cells and is believed to initiate glycogen synthesis, but not to be involved in the actual addition of glucose to existing glycogen (Threadgold, 1976). The enzymes responsible for this actually form a coat around the glycogen granule (Alberts et al., 1983). In skeletal muscle the polymerisation of glycogen is controlled by the ubiquitous protein, calmodulin, which is activated by an allosteric change brought about by binding calcium (Cheung, 1982).

In muscle, calmodulin is one of the four sub-units forming the phosphorylase kinase molecule, the gamma sub-unit is the catalytic region and the alpha and beta units are the sites where the cyclic-AMP-dependent protein kinase acts. This enzyme phosphorylates both units (beta more rapidly than alpha), so that once the beta is phosphorylated, the phosphorylase kinase complex is activated and it

activates glycogen phosphorylase, which converts glycogen to glucose-1-phosphate. Inactivation of the phosphorylase kinase occurs when the alpha sub-unit is phosphorylated, this time delayed switch stops depolymerisation. When cyclic-AMP levels are low, all of the cyclic-AMP phosphorylations are reversed by a phosphoprotein phosphatase, thus inactivating the enzymes involved in glycogen breakdown and activating glycogen synthase, the enzyme involved in polymerisation (Alberts et al., 1983). Perhaps a similar mechanism is responsible for glycogen metabolism in the columnar cells of Patella.

Previous reports of glycogen deposits in ciliated cells from the gut of molluscs have been cited by Heidermanns (1924, in Carriker and Bilstad, 1946), Judd (1979), McQuiston (1969a), Owen (1973) and Taib (1976). In Patella, the columnar cells lining the oesophageal food channel, stomach, style sac and anterior intestine store carbohydrates in the form of glycogen. The glycogen augments the food reserves in the digestive gland (Pugh, 1963). Barry and Munday (1959) found that the glycogen content of the visceral mass of Patella fluctuates throughout the year, being highest between July and November and lowest during the period November to March. The peak spawning period is between October and January (Fretter and Graham, 1976), which coincides with the period when glycogen stores diminish.

The abundance of lipid droplets in the columnar cells lining the ducts of the digestive gland, suggests that they are involved in lipid absorption from the lumen of the duct. Graham (1932) reported "fatty droplets" in the ciliated cells lining intestine section B only. Heidermanns (1924, in Carriker and Bilstad, 1946) showed experimentally that the ciliated cells of L.stagnalis can absorb fats and carbohydrates, whilst Sumner (1965a) found lipid absorption occurring in the intestine, rectum and the ducts of the digestive gland of H.aspersa. Lipid spheres occur in the cells lining the

ducts of the digestive gland of Nucula (Owen, 1973) and in the apical halves of the cells lining the gut of Dentalium (Taib, 1976).

Absorption of lipids by the epithelial cells of the mammalian intestine is believed to be by pinocytosis of droplets and partly by uptake of the soluble products of hydrolysis (Napolitano and Kleinerman, 1964; Novikoff and Holtzman, 1970). In Patella, pinocytosis of lipid droplets was not seen. The absence of a lipase sensitive to those methods employed suggests that lipid digestion does not occur in the gut lumen. This does not preclude the absorption of fatty acids, glycerol, and mono- and diglycerides released from the lysosomal system of the free digestive cell spherules or, those naturally occuring in the algal cells consumed by the limpet. Algal lipids consist almost entirely of glycerides of straight chained fatty acids and 80% of the lipid content of Phaeodactylum sp (=Nitzschia, a diatom) is fatty acid (Clarke and Mazur, 1941).

Lipoproteins are pinocytosed by a process of receptor-mediated endocytosis; hen oocytes take up lipoprotein particles in clathrin-coated vesicles. Cholesterol is packaged into low density lipoprotein (LDL) particles by mammalian liver cells and secreted into the blood, the LDL particles are then pinocytosed by cells and hydrolysed by the lysosomal system releasing cholesterol, fatty acids and protein (Dautry-Varsat and Lodish, 1984).

Damage to the basal lamina of the columnar epithelium was never seen, suggesting that amoebocytes do not invade the gut lumen or epithelium to absorb nutrients as they do in some molluscs (most filter-feeding bivalves, Purchon, 1978; chitons, Fretter, 1937; opisthobranchs, Fretter, 1939, Millot, 1937; Dentalium, Taib, 1976; H.cracherodii, Campbell, 1965 and pilids, Andrews, 1965). The lack of phagocytotic amoebocytes in prosobranchs contrasts with that in lamellibranchs and opisthobranchs, where they play an important role in the digestion and assimilation of particles in the gut lumen

(Fretter and Graham, 1962).

The large surface area of the oesophageal lateral pouches provides a site for the absorption of solutes, but it is equally a good site for the invasion of parasites and bacteria. As the amoebocytes in the sub-epithelial space lack glycogen and lipid stores, they may play a defensive role rather than an absorptive one. The cells reported as intestinal amoebocytes could be regeneratve cells that divide and grow to replace dead cells; there is no evidence to support this view (except for the fact that damaged cells must be replaced somehow), but there is none to suggest they are amoebocytes.

The small nerve fibres that intrude into the stomach, style sac, intestinal and rectal epithelia correspond to the "intra-epithelial canals" described by Graham (1932) in Patella, and by Mackintosh (1925), Yonge (1926a) and Campbell (1965) in the style sac epithelia of Crepidula fornicata (L.), Ostrea and Haliotis respectively. In Patella the nerves supplying the oesophageal dorsal folds originate from the buccal ganglion, those innervating the salivary glands come from the right oesophageal ganglion and the remainder of the gut is supplied by fibres from the visceral ganglion (Fretter and Graham, 1962).

The intra-epithelial nerve network is believed to control the activities of the columnar and basal gland cells. Bleb release may be under neural control but there is only circumstartial evidence to support this; the nerves only penetrate the epithelia of the gut where bleb release and basal gland cells occur. The contol mechanism may be via a calcium-calmodulin system; a depolarisation of the membrane may cause an influx of calcium ions into the cells, thereby activating calmodulin which somehow elicits a response. Control of the basal gland cells is discussed later.

One of the most unusual histological features of the gut of Patella is the limited distribution of mucous gland cells, they are

restricted to the epithelium lining the buccal cavity, dorsal food channel and ventral channel of the rectum where they are rare.

The ultrastructure of the mucous cells resembles that of the goblet cell of the mammalian small intestine (except in shape) and because of this, the secretory pathway (Neutra and Leblond, 1966 & 1969) is believed to be similar. The extensive RER indicates that there is a proteinaceous component in the secretion, although this was not demonstrated histochemically in the cells of Patella. Presumably the concentrations in these secretions are too low to detect in this way.

A survey of the literature reveals the abundance of mucous cells in the gut of other gastropods, however, the chemical nature of the secretion is not always specified. Glandular cells are common in the gut of pilids; Andrews (1965) reported cells secreting neutral and acid mucopolysaccharides and mucoproteins in P.canaliculata and Demian and Michelson (1971) described six histologically distinct gland cells in Marisa. Campbell (1965) found mucous cells, "secretion cells" and two types of "secretory cell" in the gut of Haliotis, whilst Ward (1966) reported PAS-positive goblet cells in most regions, and uncharacterised gland cells in the oesophagus and digestive gland ducts.

Secretory cells are equally common in pulmonate gastropods. Ghose (1963) found four types in the gut of Achatina and Kulkarni (1972) described five in Laevicaulis alte (Fer.). There are twelve histologically distinct gland cells in L.stagnalis Say which are distributed such that each section of the gut receives a mucoid secretion (Carriker and Bilstad, 1946). Howells (1936 & 1942) examined the opisthibranchs Cymbulia and Aplysia respectively, in both species mucous cells are common along the gut, as they are in Jorunna (Millot, 1937).

The limited distribution of mucous cells in Patella is correlated with the production of cytoplasmic blebs by the columnar cells.

The secretory function of the "primitve" columnar cells has not been superseded by specialised mucous cells in the stomach, style sac, anterior intestine and digestive gland ducts, but has in the buccal cavity, oesophageal food channel and rectum and also by the clavate gland cells in section D of the intestine.

Hunt (1973) reported that in the hypobranchial gland of Buccinum undatum L., acid mucopolysaccharides have a high viscosity, whilst neutral ones are more fluid. Presumably the combination of both groups in the saliva and oesophageal mucus confers an intermediate viscosity. It is generally agreed that the viscosity of mucus increases with the alkalinity of the gut fluids (Yonge, 1935; Purchon, 1978).

Yonge (1925) reported that the salivary glands in Patella are "responsible for the entire secretion into the gut, and so must be responsible for its acid reaction"; also that the mid-gut and rectum have "a pH on the alkaline side" and that this is due to "a copious secretion of mucus in these regions". It has now been demonstrated, that gland cells other than those of the salivary glands produce secretions and so the gut pH is not entirely due to the saliva. It is also clear that whilst mucus may be carried into the intestine from previous regions, it is not secreted there. There is an ambiguity in the suggestion that the saliva is acidic, yet once it reaches the intestine it produces an alkaline reaction. Yonge's theory on the role of mucus in controlling pH is incompatible with the histological evidence. The following alternative hypothesis is presented for Patella.

Several factors will contribute to pH, including the chemical nature of the secretions, food and whether or not the epithelium absorbs or secretes particular solutes, which could alter the hydrogen ion concentration. The carboxylated and sulphated acid mucopolysaccharides of the saliva and oesophageal mucus can dissociate in a similar way to those of the glycocalyx (page 325). An acid state

(left side of the equations) will only exist with an excess of hydrogen ions and these must be supplied from a different source; from fatty acids in the food, lysosomes from the digestive cell spherules or by proton transport by the epithelial cells.

The rise in pH from 6.0 in the oesophagus to 6.4 in the stomach, style sac and anterior intestine, corresponds to cytoplasmic bleb release by the columnar cells. The low pH of the digestive gland (5.3) does not reduce the pH in these regions to a similar value during the exit of the liver string. It is possible that the blebs and the secretion of the basophilic cells, buffer the pH in these regions. This rise in pH increases the viscosity of the mucus.

Coinciding with another rise in pH (7.2) in the posterior intestine is the appearance of the clavate cell, the proteinaceous secretion of which cannot be termed mucus. It is probably this secretion that Yonge (1925) confused with mucus. In acid conditions the amino groups of a protein act as a base and capture protons, this is believed to be the mechanism by which the protein secretions of the basophilic, clavate and columnar cells reduce the gut pH.

Yonge (1935) stated that the "colloidal properties of a protein are at a minimum at the isoelectric point, it follows that viscosity should be lowest here". At their iso-electric point, protein molecules have a net neutral charge, away from this point they carry a net negative charge in alkaline conditions or positive charge in acid conditions. The change in charge can induce allosteric changes in the tertiary structure of the molecule or allow cross-bridging between molecules (Denny and Gosline, 1981), both contributing to an increased viscosity.

It might be expected to find that the iso-electric point of the proteinaceous secretions, would be close to the pH of the region of gut where they are released, since it is here that least viscosity is necessary to allow the free flow of the secretion. For example, the

secretion of the clavate cell must have a low viscosity for it to be "painted" onto the faecal rod.

Graham's conclusion (1932) that the clavate cells of Patella are not normal mucous cells is confirmed by the histochemical results presented here. Protein-secreting cells resembling the clavate cells in structure and function have been reported in the intestinal epithelium of other molluscs; Aplysia (Howells, 1942), Cellana radiata (Born) (Balaparameswara Rao, 1975), Dentalium (Taib, 1976), H.cracherodii (Campbell, 1965) and L.stagnalis Say (Carriker and Bilstad, 1946).

The clavate cell is a typical protein-secreting cell, its ultrastucture and histochemistry clearly reflects this and the appearance of a proteinaceous layer enwrapping the faecal rod in section D of the intestine, supports Graham's (1932) view that the secretion acts as a "cement". The continuation of water transport out of the posterior intestine and the reduced hydrogen ion concentration facilitates compaction and cementation.

There is little evidence to implicate the basal gland cell in a similar role. Graham (1932) reported that anterior to section D, the gut is full of particles, but that along this length the faecal rod acquires "peripheral layers of a yellow substance" (the clavate cell secretion), suggesting that cementation does not begin in section C, where basal gland cells initially occur. He reported that "section C, by means of the secretion of the basal gland cells, builds up the matter into a very much more consistent rod", which for reasons discussed later seems unlikely.

He did not see secretion released from these cells and there is no evidence from the present study. He described "rhombic crystalloid masses" in the lumen of section C and because they stained similarly to the granules in the basal cells, he concluded that they were a "pseudocrystalline form" of the secretion. Neither these or the "fine

ducts" were encountered during the present investigation.

The absence of the ducts could be for one of two reasons. Firstly, because the ducts may be periodically extended to the lumen during the secretory phase or secondly, that the ducts represent the presence of secretion released into the intercellular space above the cells; this could be mistaken for ducts at the histological level. Presuming that the cells produce a "cement", then its release and the formation of a duct should coincide with the presence of the faecal rod in the posterior intestine; sections taken under such conditions failed to show this.

The periodic development of fine ducts or secretion into the intercellular space are very inefficient ways for delivering large volumes of secretion to the lumen to build up the faecal rod. Furthermore, the sparse RER, mitochondria and golgi are suggestive of a low rate of protein synthesis, but one should not discard the possibility that these cells must have previously undergone an intense phase of synthetic activity, resulting in the production of the granules followed by a reduction in the organelles involved. The fact that only the storage phase has been encountered by separate investigators, suggests that the secretory phase is either very rapid or infrequent.

The reputed neurosecretory neurones on which these cells rest, contain granules (75-100nm) resembling known elementary neurosecretory granules in certain neurohaemal organs (sinus gland, neurohypophysis and urohypophysis; Bullock and Horridge, 1965). Exocytosis of the granules into the intercellular space adjacent to the basal gland cells, suggests that they are under neurohormonal control. Processes under such control are those in which the response needs to persist for a considerable time and where speed is not an overriding factor. In gastropods, spermatogenesis, ovulation, oviposition, growth and osmoregulation are all controlled by neurosecretion (Maddrell and

Nordmann, 1979).

Basal gland cells may be gut endocrine cells and the neurosecretion may initiate release of the granules or the synthetic phase of the cell. The search for insulin-like producing cells in the molluscan gut has found reasonable success (Boquist et al., 1971; Davidson, Falkmer and Mehrotra, 1971; Fritsch, Van Noorden and Pearse, 1976; Fritsch and Sprang, 1977; Plisetskaya, Kazakov, Soltitskaya and Leibson, 1978).

The ultrastructural features of these cells in Buccinum are; the lack of microvilli, the presence of a prominent RER and golgi and numerous electron-opaque, membrane-bound granules (150-200nm) (Boquist et al., 1971). In Anodonta cygnaea (L.) and Unio pictorum (L.) similar granules (250-350nm) occur in unspecified epithelial cells and aldehyde fuchsin-positive nerve fibres of the intestine. In M.edulis, the endocrine cells rest on the basal lamina and extend to the lumen by slender necks, the apical surface bears microvilli. There is little RER, SER occurs as cisternal and vesicular forms, ribosomes are free in the cytoplasm and sometimes microtubules are well developed, characteristically, electron-opaque, membrane-bound granules (200-400nm) occur.

Pearse (1974) reported that vertebrate gut hormones are produced by cells which possess the ultrastructural and cytochemical features of the "APUD" (an acronym for Amine and Precursor Uptake and Decarboxylation) cell series. Characteristic features of these cells are low levels of RER, abundance of vesicular SER and free ribosomes, distinct microvilli, the presence of microfibrils and microtubules and osmiophilic membrane-bound granules (100-200nm).

The crystalline styles of Unio and Anodonta have an immunoreactive insulin content, indicating that an insulin-like substance produced by certain intestinal epithelial cells is taken up by the style (Plisetskaya et al, 1978). They suggested that this

substance may be transferred to the stomach and then to the digestive gland where it undergoes proteolysis and the active form is transported into the blood. They reported that injection of glucose into the blood (to induce hypergylcaemia) reduced the number of cells containing insulin-like reactive granules. After 24 hours the blood glucose concentration was reduced to 66% of the initial value. This evidence suggests that the insulin-like hormone stimulates glucose uptake by tissues and that the release of the hormone is induced by a glucose load. Injections of insulin, followed by glucose, increased the glucose concentration of muscle. In these molluscs the insulin-like hormone acts similarly to vertebrate insulin.

As Patella lacks a style it is unlikely that any hormone-like substances released from the basal gland cells would reach the blood via the digestive gland. Perhaps the intestinal epithelial cells could be stimulated to absorb glucose from the gut lumen.

The ultrastructural differences between these gut endocrine cells and the basal gland cells indicates that the latter are not typical gut endocrine cells. Since they retained their typical appearance in starved animals, they are probably not involved in the physiological control of metabolism during starvation. Without further experimental evidence it is not possible to identify their function.

SUMMARY.

1. The original histological investigation of the gut of Patella vulgata (Graham, 1932) has been extended using modern techniques, including electron microscopy, cyto- and histochemistry, liquid scintillation counting and autoradiography.

2. On the basis of anatomy, histology and functional criteria the gut is divisible into six sections: the buccal cavity, oesophagus, stomach, style sac, intestine and rectum. Into the buccal cavity open the ducts of the salivary glands, the stomach receives the digestive gland duct.

3. The epithelium of each of these regions was examined by transmission electron microscopy, the cell types present are described and their possible function discussed.

4. Each section of the gut was also examined by scanning electron microscopy to give a three-dimensional picture of the disposition of the numerous ciliated folds on its walls and to map the distribution and secretory activity of the cell types. Ciliary currents were followed using graphite suspensions.

5. The chemistry of the secretory granules and inclusions of the nine cell types described, were determined by histochemical tests specific for carbohydrates, proteins, lipids, pigments and enzymes.

6. Carbohydrates were demonstrated cytochemically with the low pH phosphotungstic acid, periodic acid-silver methenamine and periodic acid-thiosemicarbazide-silver proteinate techniques, and the enzymes acid and alkaline phosphatase by incubation in buffered lead solutions.

7. The pH of the gut fluids from the different regions of the gut was determined to establish its effects on the viscosity of the secretions released into the gut.

8. The release of neurosecretory material by intestinal nerves closely associated with a particular type of epithelial gland cell, was shown

by incubation in a tannic acid-ringer medium.

9. Several ultrastructural features typical of absorptive epithelia are displayed by the ciliated epithelium of the gut: for this reason experiments were conducted to determine whether the gut could absorb tritiated D-glucose.

10. The effects of inhibitors, pH, sodium ion concentration and different D-glucose concentrations on absorption rates were demonstrated in vitro.

11. Autoradiography was used in an attempt to determine which cell types were involved in glucose absorption.

12. Ferritin feeding experiments demonstrated that some cell types are involved with the pinocytosis of proteinaceous molecules.

13. Phasic activity of the digestive and oesophageal glands was revealed by examining starved limpets and normal animals fixed at hourly intervals of the tidal cycle.

14. The results of this research are discussed and related to previous work on other molluscan species, the emphasis being placed on the significance of ultrastructure in relation to function.

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APPENDICES.

A CONSIDERATION OF POSSIBLE CAUSES OF FAILURE IN UNSUCCESSFUL EXPERIMENTS.

1A). To determine if the limpets were regurgitating their meals, the radioactivity in samples of the experimental sea water (that in which the limpets were kept after feeding) was calculated and compared with the total in the meals administered. Each of the seven limpets were fed an 4.12nMol dose of ^{3}H-glucose, giving a total concentration of 4.12 . 7 = 28.84nMol L^{-1} in the 1L bucket of sea water. Thus, if each limpet regurgitated its entire meal, the experimental samples should reflect this value. The concentration of the samples was 0.4 µMol L^{-1} (table 18) which is 14 times the predicted concentration (0.4 µMol/28.84nMol = 13.87). This clearly represents contamination (possibly the microsyringe used was inadequately rinsed) that would mask the smaller amounts of radioactivity from a regurgitated meal.

If it is assumed that each limpet absorbed the entire meal, then the 4.12nMol dose of ^{3}H-glucose should be recovered from each animal. To calculate the total radioactivity in the gut and blood, a limpet of similar size to the experimental animals, was dissected and the various sections of the gut measured to calculate the gut volume and then weighed. An approximation of 500 µl for the blood volume was obtained (Arason, personal communication). These values are presented in table 18B, they were used to estimate the values in column 4 of table 18A, as shown below using the blood and oesophageal samples from the animal sampled 30 minutes after feeding as examples.

1 µl of blood contained 1.31pMol of ^{3}H-glucose.

The total blood content = 1.31 . 500 = 655pMol.

1mg of oesophagus contained 0.633pMol of ^{3}H-glucose.

The total content = 0.633 . 19mg = 11.99pMol.

Examination of the totals in column 4 of table 18A, indicates that

the gut and blood contain only a fraction of the administered dose. Several deductions can be made: 1. That a lot of the ^{3}H-glucose was regurgitated and this was not noticed because of the contamination of the experimental sea water samples. 2. That a lot of the radioactivity was accumulated by unsampled tissues (foot, gonad, kidney, etc.). 3. That bacteria and unicellular algae sequestered the regurgitated glucose from the sea water. 4. That the sampling and counting techniques were inacurately performed.

The second suggestion is possible if the missing radioactivity can be incorporated into the unsampled tissues within 30 minutes; because 90% of the administered dose was lost from the limpet sampled after this period. To calculate the possibility of this, the absorption rates obtained from the the glucose absortion pH experiments were used (table 24). The normal pH of the oesophageal contents is 6.0, so the absorption rate (3.63nMol $mg^{-1}hr^{-1}$), for this tissue at this pH was chosen. A typical oesophagus weighs 19mg and so in 30 minutes could theoretically absorb 34.48nMol (3.63/2 . 19) of glucose.

From this it is clear that the oesophagus alone could absorb the $\pm$4.12nMol dose within a 30 minute period. The ^{3}H-glucose would then have to be transported into the blood, circulated around the body and accumulated in the unsampled tissues. The inconclusive glucose-flux experiments using intestine did not indicate glucose transport into the blood, but it may occur in the oesophagus, so this idea is plausible.

Bacterial action might account for any breakdown of regurgitated glucose, but the radioactivity would remain in the bucket as metabolic by-products (pyruvic acid, lactic acid, water etc.). If bacteria and algae were assimilating some of the glucose they might prevent it from being sampled. The sea water was vigourously aerated and therefore mixed, leading to a normal distribution of the contents. Randomly taken aliquots would sample this distribution and indicate the

radioactivity present. However, the bacteria or algae may collect on the bucket surface where they would have remained unsampled and the radioactivity they contained, presumed lost.

The dissection and sampling techniques could have led to contamination. Once the efferent pallial vessel and style sac had been pierced with the microsyringe for sampling, their contents could leak onto tissues that were not sampled; this might have led to unaccounted radioactivity.

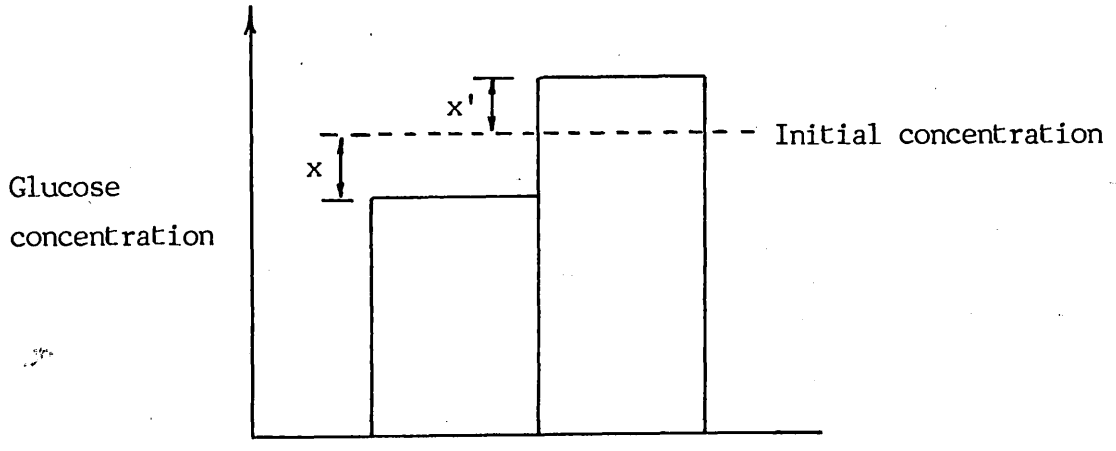
1B). It is clear from figure 25 that a greater fraction of the 4.12 nMol dose was recovered from the limpet sampled 16 hours after feeding; all the graphs attain their maximal peak at this point. This conflicts with the prediction because the anterior sections of gut should have the highest concentrations before the posterior ones and also each section should only peak once, because each limpet was fed a single meal or "pulse". Furthermore, as shown in appendix 1A the oesophagus is capable of absorbing the entire dose and so there would be no glucose left for the latter sections to absorb. There is one factor that has yet to be considered.

The rate of flow of the gut contents through the alimentary canal directly controls the period during which a region of gut is exposed to the ^{3}H-glucose and thus ultimately affects absorption. If the meal could be cleared past the style sac within the first 30 minutes, this would explain the low glucose concentration of the gut fluid samples. Graphite suspensions fed similarly can reach the style sac in 20 minutes, so it is feasible for a liquid meal to be rapidly cleared from the gut. Once cleared, the sea water in the bucket would be contaminated with radioactivity and could be continually flushed through the gut. Consequently, the limpets were no longer exposed to a single pulse of radioactivity, but were exposed to it for a period equal to the time each animal spent in the bucket. The animal sampled 32 hours after feeding had the opportunity to absorb the greatest

amount of glucose, but this did not occur.

A proportion of the ^{3}H-glucose remains unaccounted for, the probability is that: it was either present in the sea water but masked in the contaminated samples or that it was sequestered by an unsampled component of the closed system.

2). It was anticipated that the glucose-flux experiments would yield data indicating a net flux of glucose from the luminal to the haemocoelic compartment, leaving a residual quantity of glucose in the tissue. The expected results would produce a post-incubational luminal glucose concentration (x) less than the initial one and consequently the post-incubational haemocoelic concentration (x') would rise.



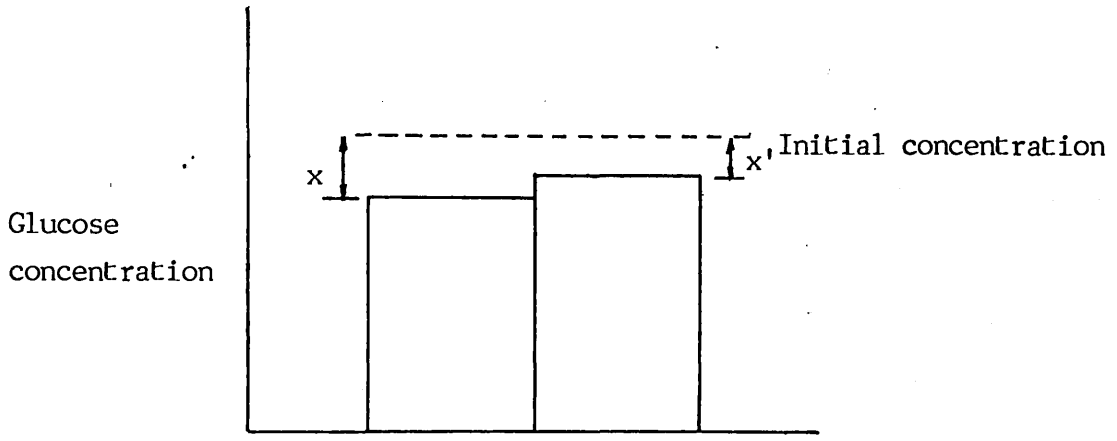
Some of the glucose would remain in the tissue so that,

$$x > x' \quad . \; . \; . \; .1.$$

and therefore,

$$x = x' + \text{residual tissue glucose} \quad . \; . \; .2.$$

As the actual luminal and haemocoelic post-incubational glucose concentations were less than the pre-incubational concentrations, this suggests that the tissue was absorbing glucose from both compartments (figs 26-29). This does not seem to be a sound physiological possibility.



So, $x + x' =$ residual tissue glucose .....3.

Figures 26-29 show that the values x and x' are measured in mMols and the residual tissue glucose in nMols, so equation 3 is not true. There is a quantity of glucose that is not accounted for. Three suggestions are presented to explain the results.

Firstly, the preparations could have leaked at either end or through an unnoticed discontinuity in the epithelium. Viability of the preparation technique was demonstrated beforehand by filling intestinal preparations with a 5 μCi ml^{-1} labelled solution and incubating them in ASW, which was later sampled. The samples gave counts of 400-500 min^{-1}, whereas leakage would have given counts in the order of 20000 min^{-1}. It is of course possible that some of the 32 preparations were leaky, but this alone could not alter the glucose concentrations because both solute and solvent could diffuse through any discontinuities to reach equilibrium. A leaky preparation would prevent differing concentrations developing across the epithelium and would have no effect on the loss of glucose.

If sampling errors were responsible for the results, then they must have been repeated with all 32 preparations, suggesting an inherent fault in the technique. The same technique, however, gave viable results from the net water movement experiments.

The net water movement experiments demonstrated a flow from the lumen to the blood and this could have a drastic effect on the small

volume of the luminal compartment. The luminal volume was approximately 10% that of the haemocoelic one, so any concentration or volume change will be most noticable as a change in the luminal samples. Simultaneous solute and solvent movements from the lumen could have the following effects on its glucose concentration. If the rates of solvent and solute movement are equal, the concentration will remain constant, but the fluid volume in the luminal compartment will decline. The concentration will rise if the water flow out of the lumen is greater than that for glucose and will fall if the reverse is true. The latter situation may explain why in 26 of the 32 preparations, the post-incubational luminal concentration was less than that of the haemocoelic compartment. But this does not account for the missing glucose which must be attributed to experimental error, as the half-life of tritium is 12.5 years, any loss due to this factor is insignificant.

3). Several reasons can be proposed for the lack of silver grains on the autoradiographs. One is that the tissue did not absorb sufficient quantities of the ^{3}H-glucose to give a positive autoradiograph. A second possibility is that the glucose diffused out of the tissue during a stage prior to exposure. For this to occur, the tissue blocks would have had to melt and to remain in this state long enough to allow diffusion; for the complete removal of residual glucose from the tissue, it would have had to come into contact with a solution to wash away the labelled molecules. As the blocks were immersed in liquid nitrogen until required for sectioning and then transferred directly to the cryostat chamber, this is unlikely.

The emulsion may have been rendered insensitive by its dilution. Bogoroch (1972) recommended dilution with glycerol because it reduces background fog resulting from the drying stresses of the gelatin on the silver halide crystals. She also suggested the use of a 1:1 dilution for quantitative optical autoradiography, so its use should

enhance the results.

If the sections melted on the emulsion during the picking up procedure, the ^{3}H-glucose could have diffused out of the sections. This should give a non-specific distribution of silver grains across the sections and emulsion. Another possibility is that the latent image was incorrectly developed or that the developed silver grains were extracted during a subsequent stage. Recomended development and staining procedures were used under the specified conditions, so these effects should not have occured. Sections approximately 10 μm were used because thinner ones could not be cut, this factor should only reduce the resolution of the autoradiograph providing the ^{3}H-glucose had a homogeneous distribution in the sections.

This leaves the duration of the exposure (4 weeks) as an explanation for the results, although McLean (1970), Mathers (1972) and Stewart and Bamford (1976) obtained results with exposures of 1-4 weeks, 8 days and 24 days respectively. However, they did use entirely different techniques to that used in the present study for diffusable substances and the last two authors exposed their autoradiographs at 4 °C. Since radioactive decay is unaffected by temperature, the -20° C exposure temperature used here would only reduce chemical fogging. The exact cause of the negative results remains unknown.