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THE STRUCTURE AND FUNCTION
OF INVERTEBRATE SEPTATE JUNCTIONS

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A thesis submitted to the University of Auckland for the degree of Doctor of Philosophy.
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ABSTRACT:

The structure and function of septate junctions have been studied by ultrastructural techniques in a wide range of invertebrate phyla. Techniques have included conventional and tracer impregnated tissue thin sectioning, and freeze-fracture of both fixed and unfixed tissue. Standard and goniometer stage transmission electron microscopy has been used. Work in this study has been concentrated mainly on the lower invertebrate phyla in which the simplest forms of junction might be seen, and on the higher invertebrate phyla. This latter group is of special interest in the transition region between the invertebrate and vertebrate phyla.

Six new types of septate junction and a form of tight junction have been found in invertebrate phyla and are described. Two of these new septate junctions occur in each of the phyla Coelenterata, Echinodermata and Hemichordata. The tight junction occurs in the phylum Tunicata. Further data has also been obtained on five of the six previously described types of septate junction. The distribution of all twelve types within the invertebrates and their phylogenetic relationships are discussed. Work with the goniometer stage has aided in interpreting junctional structures.

The results have revealed several features of the septate junction that support the idea that it has a mainly occluding function analogous to that of the vertebrate tight junction. In addition, an anastomosing septate junction has been found in the Echinodermata which is postulated to be a structural intermediate between the invertebrate septate junction and the vertebrate tight junction. A hypothesis is proposed suggesting how the septate junction might have evolved into the vertebrate tight junction.

Tricellular junctional regions of most of the septate junction types are noted and briefly described.
1. **INTRODUCTION**

Septate junctions were first described in invertebrates by Wood (1959). Subsequent investigations suggested that their structure might vary in different invertebrate tissues and the term septate junction has been used to describe several junctions whose only common structural feature is a series of septa or bars between apposing plasma membranes. At the time at which this study commenced three types of septate junction were generally recognised to exist in the invertebrate phyla. These were the Hydra type, the pleated type, and the smooth or continuous septate junctions (Staehelin, 1974).

However, only a limited range of invertebrate phyla had been studied, most of the work having been done on the hydrozoan coelenterates, the molluscs, and the arthropods. There was also some doubt as to whether the three known types of septate junction were variations of one junction type, or were different junctions with different functions. Coupled with this was an uncertainty as to the function of the invertebrate septate junction, the most favoured idea being that it had an occluding function between cells analogous to that of the vertebrate tight junction (Flower and Filshie, 1975; Newell and Skelding, 1973; Noirot-Timothee and Noirot, 1973; Oschman and Berridge, 1970; Staehelin, 1974). In addition, earlier works had often been inconclusive in assigning a junction to a specific category because of the restricted range of techniques used. Only when a variety of experiments using conventional thin section, tracer and freeze-fracture techniques have been carried out is adequate information available to decide whether the septate junction present in any tissue fits clearly into one of the three reported categories or even represents a new type.

Study of the literature revealed that each of the three septate junction types appeared only within certain phyla. This allowed the formation of the hypothesis that they were simply variations of the one
junction type and that they might in fact form a developmental sequence from the Hydra type septate junction, through the pleated and smooth septate junctions to the vertebrate tight junction. If such a sequence existed then it would be possible to draw some conclusion as to the function of the septate junction in comparison with the vertebrate tight junction. The aim of this study was therefore to characterise clearly the different types of invertebrate septate junction and to study representatives from as wide a range of invertebrate phyla as possible to ascertain their true distribution and function. Of special interest were those phyla at the lower end of the metazoa in which the first signs of occluding junctions might be seen and those phyla nearer to the transition point between the invertebrate and chordate phyla. This latter group, it was hoped, might show some form of structural link between the invertebrate septate junction and the vertebrate tight junction allowing a correlation in function.

Early on in the study it became apparent that there was a far greater diversity in septate junction structure than first thought and during the course of the study three further variations have been described in the literature. These were the lower invertebrate pleated septate junction (also called the polychaete or annelid septate junction) (Baskin, 1976; Welsch and Buchheim, 1977), the Limulus septate junction (Lane and Harrison, 1978) and the Chaetognatha septate junction (Duvert, in press).

This study has dealt with tissues from most of the main and several minor invertebrate phyla. Conventional thin section, tracer and freeze-fracture techniques have been used whenever possible and as a result a further six variations of septate junction have been found and their structures and distribution described. It has also been possible to add to our knowledge of five of the six previously described variations. Some
of this work is published or is in press (Green, 1978; Green and Bergquist, 1978; Green et al., 1979; Green and Flower, in press). The study has provided new data which is useful in assessing whether the idea of an occluding function of the invertebrate septate junction is correct, and a hypothesis is proposed which suggests a possible sequence by which the septate junction may have evolved into the vertebrate tight junction. The great variety of septate junctions now described permits some discussion on invertebrate phylogenetic relationships. Invertebrate tricellular junctions are noted and discussed briefly.
2. METHODS AND TECHNIQUES

2.1 Introduction

All tissues used in this study of septate junction structure were taken from freshly collected animals found in their natural environments.

Initial experiments were undertaken to determine the effects of varying fixation techniques on the septate junction. A range of seven fixative mixtures was tried with osmolarities between 200 and 1100 milliosmoles. This range included phosphate and cacodylate buffers and glutaraldehyde concentrations of one to six percent (Table 2.1).

Despite wide variation in the quality of cytoplasmic fixation, the septate junctions showed no obvious differences related to the various fixative mixtures used and the work in this thesis has therefore been carried out using two mixtures found to give good fixation of all the tissues studied. One is of a lower osmolarity for fresh water and terrestrial organisms, the other of high osmolarity for marine organisms. Both fixatives chosen are based on cacodylate buffer which allows the addition of lanthanum tracers which precipitate in phosphate or similar buffers.

These two fixatives alone and standard freeze-fracture preparation techniques have been used throughout the study to enable direct comparisons between junctional structures in different tissues. The only exception was the freezing of unglycerinated starfish feet after soaking them in magnesium sulphate (1000 mOsM) to cause relaxation of muscular tissue (see Chapter 3.6, The phylum Echinodermata). This was necessary following repeated unsuccessful attempts with untreated tissue.

All thin sectioning was done using a Porter Blum manual ultramicrotome or a Reichert OMU2 ultramicrotome. Thin sections and freeze-fracture replicas were viewed in a Philips EM301 electron microscope which was fitted with a goniometer stage when required.

Abbreviations used in this thesis are given in Appendix 2.1.
Table 2.1  Fixative Mixtures Tested Initially

<table>
<thead>
<tr>
<th>Name</th>
<th>% Glutaraldehyde</th>
<th>% Formaldehyde</th>
<th>Buffer</th>
<th>Substances used to raise the osmolarity</th>
<th>Final Osmolarity of fixatives (mOsM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Marine Glut/Phosphate (1)</td>
<td>1</td>
<td>-</td>
<td>Sodium phosphate (pH approx. 7.4)</td>
<td>NaCl</td>
<td>1100</td>
</tr>
<tr>
<td>Glut/Cacodylate (1)*</td>
<td>3</td>
<td>-</td>
<td>Sodium cacodylate (pH approx. 7.2)</td>
<td>-</td>
<td>200</td>
</tr>
<tr>
<td>Glut/Form Cacodylate</td>
<td>2.5</td>
<td>2</td>
<td>Sodium cacodylate</td>
<td>-</td>
<td>200</td>
</tr>
<tr>
<td>Marine Glut/Phosphate (2)</td>
<td>6</td>
<td>-</td>
<td>Sodium phosphate</td>
<td>glucose, NaCl</td>
<td>700</td>
</tr>
<tr>
<td>Glut/Cacodylate (2)</td>
<td>1</td>
<td>-</td>
<td>Sodium cacodylate</td>
<td>sucrose</td>
<td>835</td>
</tr>
<tr>
<td>Marine Glut/Cacodylate*</td>
<td>3</td>
<td>-</td>
<td>Sodium cacodylate</td>
<td>NaCl</td>
<td>920</td>
</tr>
</tbody>
</table>

(* indicates fixative mixtures selected and utilised throughout this study)
2.2 Fixation

Marine organisms were dissected in sea-water and their tissues fixed in a marine aldehyde fixative. This consisted of a 3% glutaraldehyde fixative in 0.1M sodium cacodylate buffer with sodium chloride added to raise the osmolarity to approximately 900 mOsm. The buffer also contained 0.05% calcium chloride. Fixation was for one to two hours followed by buffer washing in a 0.2M sodium cacodylate buffer containing 2% sodium chloride and 0.05% calcium chloride. Tissue was then stored in this buffer until required. Tissue for conventionally stained thin sections was post fixed with 1% osmium tetroxide in 0.1M sodium cacodylate buffer containing 2.5% sodium chloride. It was then alcohol dehydrated and embedded in Epon 812. This fixative was based on one used at the University of Claude-Bernard, Lyon, France.

Tissue from fresh water and terrestrial organisms was fixed with 3% glutaraldehyde in a 0.1M sodium cacodylate buffer with an osmolarity of approximately 200 mOsm. This buffer also had 0.05% calcium chloride added. Fixation was for one to two hours followed by washing and storage in a 0.2M sodium cacodylate buffer containing 0.05% calcium chloride. Tissue for conventionally stained thin sections was post fixed for one hour with 1% osmium tetroxide in 0.2M sodium cacodylate buffer, dehydrated in alcohol and embedded in Epon 812.

Sections for positive staining were picked up on copper grids and double stained with uranyl acetate and lead citrate (Venable and Coggeshall, 1965).

2.3 Lanthanum Impregnation

Impregnation of lanthanum tracer was obtained by three methods, the first of these being far less successful than the latter two. The latter two gave good results reasonably regularly but were still somewhat unpredictable.
Method 1: This method is the method of Revel and Karnovsky (1967) with lanthanum added to the post fixation stage only. Fixed and buffered tissue was changed to an s-collidine buffer followed by one hour post fixation with 1% lanthanum nitrate and 1% osmium tetroxide in s-collidine buffer. It was then rapidly dehydrated in acetone and embedded in Epon 812. Thin sections were viewed with no further treatment. This technique was used successfully for the phylum Coelenterata only.

Method 2: This is the method of Shklai and Tavassoli (1977). Tissue was fixed in 3% glutaraldehyde in the appropriate buffer (section 2.2) containing 1% lanthanum nitrate and adjusted to pH 7.8 using 1N sodium hydroxide. Fixation was for more than two hours (two to twelve were used with no variation in results). The tissue was then changed to Millonig phosphate buffer and left overnight at 4°C. The low temperature and phosphate ions both cause precipitation of the lanthanum in the tissue. Post fixation was for one hour in a 0.1M sodium cacodylate buffer containing 1% lanthanum nitrate and 1% osmium tetroxide. Tissue was then rapidly dehydrated in acetone and embedded in Epon 812. Thin sections were viewed with no further treatment.

Method 3: This technique is a variation of method 2, but can be used on previously fixed and stored tissues. It was devised for this work as it is convenient to use when it is not possible to carry out specimen collection followed the next day by embedding, as is often the case when collecting in the field. Tissue was fixed and buffered by either method described in the fixation techniques above (section 2.2) and stored. At a later date when embedding was possible the tissue was transferred to fresh buffer containing 1% lanthanum nitrate for two hours or more. During this time it was shaken regularly or rotated. A Millonig phosphate buffer was then added and the tissue left overnight at 4°C. Post fixation
was as for method 2 above, followed by rapid acetone dehydration and embedding in Epon 812. Thin sections were viewed with no further staining. In some cases during the first lanthanum treatment the lanthanum slowly precipitates. In such cases the buffer and lanthanum solution was changed after one hour and the tissue was left in this fresh solution for the second hour.

2.4 Freeze-fracturing

Freeze-fracturing was by the brass block method of Bullivant (1973) and replication was carried out in a Ladd vacuum evaporator. Fixed tissue was soaked in 30% glycerol in the appropriate buffer (section 2.2) before freezing. Unfixed marine tissue was frozen after soaking for 15-30 minutes in 30% glycerol in sea-water. The only exception was the freezing of unfixed starfish tube feet after soaking them in magnesium sulphate (1000 mOsm) only. This tissue was frozen with no cryoprotection as the muscular tissue of the feet tended to contract in glycerol to such an extent that intra-membrane fractures were rare due to wrinkling of the cell membranes. Unfixed terrestrial tissue was soaked in 30% glycerol in buffer for 15-30 minutes before freezing. All tissues were frozen in liquid freon or, more commonly, subcooled liquid nitrogen before fracturing.

After freezing, tissue was transferred to liquid nitrogen and fractured with a razor blade. While still under the liquid nitrogen it was placed into a brass block holder (Bullivant, 1973) and the whole assembly transferred to an evaporator. The brass block holder allowed the tissue to be kept covered with liquid nitrogen until a vacuum was produced. Shadowing took place at less than $2 \times 10^{-5}$ torr and was with platinum-carbon at $45^\circ$ backed with carbon at $90^\circ$. Replicas were removed from the evaporator and coated with collodion to aid in holding them together during the digestion process. Digestion was with household bleach
(Janal) and/or chromic acid followed by washing with distilled water and then amyl acetate to remove the collodion. Replicas were picked up on single slot copper grids coated with a formvar support film.
**Appendix 2.1**

**Abbreviations:**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>°C</td>
<td>degrees Celsius (centigrade)</td>
</tr>
<tr>
<td>FF</td>
<td>freeze-fracture</td>
</tr>
<tr>
<td>Form.</td>
<td>formaldehyde</td>
</tr>
<tr>
<td>glut.</td>
<td>glutaraldehyde</td>
</tr>
<tr>
<td>M</td>
<td>molar</td>
</tr>
<tr>
<td>mm</td>
<td>millimetre(s)</td>
</tr>
<tr>
<td>mOsM</td>
<td>milliosmoles</td>
</tr>
<tr>
<td>N</td>
<td>normal</td>
</tr>
<tr>
<td>nm</td>
<td>nanometre = 10^{-9} metre</td>
</tr>
<tr>
<td>sp.</td>
<td>species</td>
</tr>
<tr>
<td>TS</td>
<td>thin section</td>
</tr>
<tr>
<td>um</td>
<td>micron = 10^{-6} metre</td>
</tr>
<tr>
<td>%</td>
<td>percent</td>
</tr>
<tr>
<td>°</td>
<td>degrees (of a circle)</td>
</tr>
</tbody>
</table>
3. RESULTS

3.1 General Introduction to Results

This study has revealed that there are at least twelve distinct types of septate junction in the invertebrate phyla. Despite a real diversity of septal types two factors are common to every variation so far studied. The septa always span a 15-18 nm intercellular space and the junctions are always found in a belt around the apical edge of cells which line lumenal spaces and external bounding epithelia.

Gap junctions and spot desmosomes commonly occur below the septate junction, while a belt desmosome is often seen above the septate junction, between it and the lumen or outside edge of the tissue. However, not all septate junctions have a desmosome above them (Filshie and Flower, 1977; Juberthie-Jupeau, 1979; Wood, 1977).

Wherever possible, the description of septate junction structure is a synthesis based on thin section, lanthanum impregnation, and on freeze-fracture observations of both fixed and unfixed tissues. In some cases it has not been possible to carry out all of these techniques on a particular junctional type. The results are organised according to phyla, rather than septate junctional type, as most junctions are specific to one or two phyla. The only exception is the lower invertebrate pleated septate junction which occurs in many phyla, consequently it is listed under its junctional name (Chapter 3.4). Appendix 5.1 shows invertebrate classification as used in this thesis and shows the distribution of the various septate junction types within the different phyla.

Results include a brief description of the tricellular region of each septate junction type wherever possible. The term 'tricellular region' is used in the same way as the term 'tricellular junction' defined by Noirot-Timothee and Noirot (1980).
Their definition reads:
'A septate junction is constituted of elements of two adjacent
cells and appears as a bicccllular junction. However different
and very elaborate structures are set up at the line of contact
between three cells (abutment of three bicccllular junctions);
these special features are designated as tricccllular junctions'.
In this study, no attempt is made to classify the tricccllular
structures as either part of their respective septate junctions or as
a separate junction type.

The terminology used in describing membrane fracture faces is that
of Branton et al. (1975) and on all micrographs of replicas an encircled
arrow head indicates the direction of shadowing.

3.2 The Phylum Porifera

3.2.1 Introduction

Sponge organisation with mobile cells in an unstructured
matrix does not require the constant presence of intercellular junctions
as seen in more complex and structured animals. The transient nature
of virtually all intercellular interactions means that cell junctions
are difficult to locate and to date no structural analogues for gap
junctions and desmosomes, found in all higher phyla, have been located
in the Porifera. Evidence for intercellular communication has been produced
however by electrical coupling experiments (Loewenstein, 1967) and
circumstantial evidence (Bergquist and Green, 1977). Septate junctions
have been reported previously in only one case (Ledger, 1975).

The sponge case none the less is of special interest for two reasons.
In the occasional situations where septate junctions do occur, their
occurrence can be correlated with a specific metabolic activity thus
providing some indication of their function. Further, the Porifera show
a range of occluding structures which vary from relatively simple collagen
coats surrounding a developing embryo to recognisable septate junctions (Green and Bergquist, 1978).

Only two of ten sponge species studied in this work showed structures relevant to this thesis and further information on sponge occluding and communicating systems can be obtained from Green and Bergquist (1978). The rarity with which sponge junctions occur has meant that no lanthanum tracer and freeze-fracture results have been obtained and only positive stained thin section results are available in this case.

3.2.2 Results

Inflatella belli (Class Demospongiae), an Antarctic species, has developing embryos which appear as dense, rounded structures within the adult sponge matrix. Positively stained thin sectioning reveals that these structures are surrounded by a layer of adult cells up to 12 µm wide (figure 1a). This layer is predominantly one cell thick except when cells interdigitate where they join end on end (figure 1a). During early stages of development this adult layer is relatively thin (figure 1b), but gets thicker with time. There is little collagen fibril deposition as is commonly seen around developing embryos in other species (Green and Bergquist, 1978). The embryo lining cells are of adult origin and are joined by extensive 'junctiional' regions which consist of long stretches of parallel membrane between the interdigitating cells (figures 1b, 1c). The junctions show no intercellular modification, even after carbohydrate staining (Green and Bergquist, 1978), but the junctional membranes are a regular 8 - 10 nm apart for their entire length except where the occasional slight inflexion occurs (figure 1b). Lengths of parallel membrane junction up to 25 µm are commonly seen.

In Clathrina sp. (undescribed species, class Calcarea) another type of development is seen. In this species, vitelline platelets are observed in some areas of the mesohyl (figure 2a), presumably as a future food
supply for developing larvae (Green and Bergquist, 1978). In areas where these platelets occur, the choanocytes, normally rounded cells with little intercellular contact, extend laterally and form interdigitating cell extensions (figure 2a). These interlocking extensions are joined by septate junctions (figure 2b) similar in cross section to those seen in other invertebrate phyla (e.g. Staehelin, 1974). The membranes and septa of this species are difficult to stain however, implying a chemical difference in their structure to those of other invertebrates. The septa span a 15 - 18 nm intercellular space, and are seen as straight unmodified bars spanning the gap between the outer leaflets of the adjoining membranes. Where these junctions occur, there is also a dense organised collagen multilayer about 2 μm thick deeper in the mesohyl (figures 2a, 2c). There is effectively an enclosed space formed in the mesohyl between the septate junction joined choanocytes and the collagen layer. It is within this space that the vitelline platelets occur either as free structures or in cells containing large amounts of rough endoplasmic reticulum (figure 2a).
Figure 1:

(a) Adult cells lining an embryo in the marine sponge

*Inflatella belli.* Some embryonic cells (E) are seen in the lower part of this micrograph. The adult layer is predominantly one cell thick except where overlap occurs at junctional areas (arrows) (lead citrate/uranyl acetate stain) x 10,000.

(b) Adult cells surrounding the embryo of the marine sponge

*Inflatella belli.* This is an early stage of development in which the adult cells are thin and elongated. They are however, joined by extensive junctional regions in which the membranes of adjacent cells run parallel and have a regular 8 - 10 nm intercellular space except where slight inflexions occur (arrow). (lead citrate/uranyl acetate stain) x 25,000.

(c) A region of junction between adult embryo investing cells of

*Inflatella belli.* The membranes of adjacent cells are a consistent 8 - 10 nm apart over long stretches. There is no evidence of any intercellular structure. (lead citrate/uranyl acetate stain) x 120,000.
Figure 2:

(a) A thin section of a reproducing *Clathrina* sp. Intracellular vitelline platelets (V), to be utilised in larval development, are being produced in an enclosed space. This space is bounded on one side by choanocytes that have lost their rounded shape and have become laterally extended and interdigitating (arrows). The other side of the space is bounded by a dense organised collagen belt (C) deeper in the mesohyl. (lead citrate/uranyl acetate stain) x 4000

(b) The choanocyte layer of reproducing *Clathrina* sp. showing septate junctions between adjacent cells. The septa are difficult to stain but appear as straight unmodified bars spanning a 12 - 25 nm intercellular space. (lead citrate/uranyl acetate stain) x130,000.

(c) A view of the organised collagen layer seen in the mesohyl of a reproducing *Clathrina* sp.

(lead citrate/uranyl acetate stain) x 31,000
3.2.3 Discussion

In the only other reported instance of septate junctions in sponges (Ledger, 1975), they were located between sclerocytes of *Sycon ciliatum*. Sclerocytes are involved in spicule production, calcareous in the case of *Sycon*. The need for occluding junctions between cells involved in producing spicules is clear since the maintaining of a high ionic concentration separate from the adjoining sea-water permeable matrix would be necessary. In the example of *Clathrina* sp., the septa differ from those seen in *Sycon* (Ledger, 1975) in that they do not have any central modification, but appear as straight bars between the adjoining membranes. The choanocytes with septate junctions between then and the collagen layer lower in the *Clathrina* mesohyl could form an enclosed space for embryonic development. This seals the embryo from sea-water flushing, aids in retaining larval components in a confined area, and retains chemical messages involved in development in the appropriate area. The use of a fluid extra-cellular communication pathway is a common sponge feature (Pavans de Ceccatty, 1974). The occurrence of septate junctions in both *Sycon* and *Clathrina* can therefore be directly correlated with a need for an occluding junction. No other intercellular structures that could fulfill this role have been observed in either species. This is further discussed in Chapter 4.2.

The cells which invest the embryo in *Inflataella belli* have the same function as the choanocytes and collagen layer of *Clathrina*; to protect a developing larva. The simple, parallel membrane type junction of this species is therefore presumed to have the same function as the septate junctions and collagen belt of *Clathrina*. This simple, parallel membrane type junction has also been reported, although in a less extensive form, between exopinacocytes of *Sycon ciliatum* (Jones, 1966; Ledger, 1975) and between embryo investing cells of *Tethya* sp. (Green and Bergquist, 1978).
In the Tethya case the investing cells are backed by a dense collagen layer. In all cases the intermembrane spacing is between 8 and 20 nm and is stable within any one species. These spacings are not greatly different to the 15 nm intercellular spacing observed between membranes of all septate junctions. The regularity of the 8 - 10 nm intercellular spacing of Inflatella in the absence of septa or any other obvious inter-membrane structures implies that some factor other than the septa themselves may determine the intercellular spacing of septate junctions. This possibility is important when attempting to envisage the developmental sequence leading from the septate junctions of invertebrates to the tight junction of the vertebrates (see chapter 4.4).
3.3 The Phylum Coelenterata

3.3.1 Introduction

The phylum Coelenterata has three distinct types of septate junction. The first type, known as the 'Hydra type', found in species of Hydra (Class Hydrozoa, Order Hydrida) has been well described (Danilova et al., 1969; Filshie and Flower, 1977; Hand and Gobel, 1972; Wood, 1959, 1977). It also occurs in Cordylophora lacustris (Order Gymnoblastea) (Overton, 1963), and Phialidium gregarium (Order Calyptoblastea) (Leik and Kelly, 1970), although these two studies do not provide definitive evidence that the junctions seen are precisely similar to that seen in Hydra. In both studies, the septa as seen in positive stained cross sections show some branching modification, a feature so far only noted for Hydra type septate junctions and for some sponge septate junctions (Ledger, 1975). In the case of Hydra type septate junctions the modifications appear as two electron opaque bodies parallel to, and midway between, the two adjoining plasma membranes (Filshie and Flower, 1977; Hand and Gobel, 1972). The density of staining, thickness of a section and the angle a section is cut appears to be critical in elucidating these structures and they are not always readily seen. For this reason, they are not a good feature by which to identify the Hydra type septate junction. They are not particularly clear in Leik and Kelly's work, although they are present; but they are readily visible in Cordylophora (overton, 1963). One recent paper of Polyorchis penicillatus (Class Hydrozoa, order Gymnoblastea) by King and Spencer (1979) claims to show Hydra type septate junctions, but their reasons, mainly that the septa are straight and not pleated, are not convincing.

Despite the thoroughness of some of these previous studies some points about the Hydra type septate junction are still open to doubt. Wood (1977) claimed that in freeze-fracture experiments the septate
junction of epidermal Hydra tissue fractured in such a way that the
majority of particles were on the P face while gastrodermal tissue
fractured with the septate junction particles predominantly on the E
face. Filshie and Flower (1977) did not make this distinction, claiming
the particles were always mainly on the E face. Furthermore, the suggested
models of septal structure proposed by Hand and Gobel (1972) and Filshie
and Flower (1977) show vast differences. In order to resolve these
problems, at least partially, another hydroid Tubularia antennoides (Class
Hydrozoa, Order Gymnoblastea) and the common green Hydra, Chlorohydra
viridissima (Class Hydrozoa, Order Hydrida) were studied by thin section
and lanthanum tracer techniques. In addition freeze-fracturing was
carried out on Chlorohydra, but was aimed mainly at resolving the
differences between the published results of Wood (1977) and Filshie
and Flower (1977). The general freeze-fracture appearance of the Hydra
type septate junction has been well described by these workers.

Study of the class Anthozoa has revealed two new variations of
septate junction which have also been characterised using conventional thin
section, lanthanum tracer and freeze-fracture techniques. One variation
is apparently restricted to epithelial tissue of this class, while the
other is found in endothelial tissue.

3.3.2 Results (class Hydrozoa)

Positively stained thin sections of Tubularia antennoides and
Chlorohydra viridissima epithelia reveal septa spanning a 15 nm inter-
cellular space (figure 3a). Most septa appear as solid bars between the
plasma membranes, but in some instances some modification is visible
(figure 3a). This is usually in the form of two bars crossing the septa
at right angles near their mid point.

Junctional areas of lanthanum impregnated tissue cut tangentially and
viewed at low magnification show septa in negative contrast as long bands
approximately 8 - 12 nm wide (figure 3b). Closer inspection however reveals they consist of a narrow central backbone about 2 nm wide with projections from both sides (figure 3c). The thinness of this central backbone is not readily apparent in cross sections. The clarity of the side projections depends upon the precise orientation of the section to the junction. In true tangential sections they are difficult to see, but if the junction happens to be tilted relative to the section about an axis within the plane of the section and at right angles to the line of the septa, the central narrow core and the projections become more easily discernible (figure 3c). In figure 3c the projections show a centre to centre spacing of 3 - 4 nm and are up to 4 nm long. They are staggered on alternate sides of the central backbone. Goniometer stage observations showed that for clarity the optimum angle of tilt from what was presumed to be a true tangential view was about 30°.

When the section is cut at an angle such that only a relatively short length of junction is visible (i.e. tending towards a cross section) the septa take on an almost 'zig-zag' appearance and the side projections appear quite sharp and distinct (figure 3d). In places where the section is cut such that a septum between two cells, as seen in tangential view, is on the edge of a gap between the lower cell and a third adjacent cell (i.e. adjacent to the tricellular junctional region), what appears to be a series of circles in a chain like structure becomes apparent (figure 3d). In such views where the septa is probably viewed almost side on, and slightly end on, the narrow, straight central backbone is no longer visible. The fineness of these structures borders on the resolution limit of the lanthanum tracer technique and this factor, coupled with superimposition effects makes adequate interpretation difficult.
In the tricellular region itself, a 'zipper' like structure is seen with the bars of the 'zipper' at right angles to the line of the tangentially viewed septa of adjacent cells (figures 3e, 3f). These bars have a centre-to-centre spacing of 7-9 nm and often appear slightly wavy. Closer inspection of photomicrographs often reveals circular structures forming at least part of these bars (figure 3f).

Freeze-fracture of fixed tissue of *Chlorohydra* showed that the majority of junctional particles were on the E face of both gastrodermal (figure 4a) and epidermal tissue (figure 4b).

In *Hydra* it is common to observe in freeze-fracture replicas or tangential sections 30 or more septa running side by side around a cell (figure 3b, or see for example Filshie and Flower, 1977). The septa are spaced 13 - 16 nm apart (centre to centre) in areas where they are most closely and regularly stacked. This means that the side projections of adjacent septa can only be 1 - 4 nm apart at their tips. In contrast, the septa of *Tubularia* do not form the extensive arrays seen in *Hydra* and often occur well spaced and meandering in appearance with large spaces between adjacent septa (figure 3e). At no time were more than 20 septa seen in tangential view around any one cell.

3.3.3 **Results** (Class Anthozoa)

Epithelial tissue surrounding the tentacles of the sea anemones *Isactinia olivacea*, *Actinothoe albocincta* and *Isactinia tenebrosa* (all Class Anthozoa, Order Zoantharia), and gastrodermal tissue from inside the tentacles or gut lining were studied in this class. Two new variations of septate junction were located in these tissues; one around gastrodermal lining cells and the other around superficial epidermis cells. In both types septa span a 15 - 18 nm inter-cellular space (figures 4c, 4d). The septa usually are spaced irregularly. This irregularity and the variety of angles which septa can show in any one section means that good
positively stained cross sectional views are rare. In tangential views
and in freeze-fracture replicas however, the junctions are quite distinct.
In both types the septa are unpleated.

Gastrodermal type: This junction has been found inside the tentacles
and the gut of the species noted above. In tangential sections of lanthanum
impregnated material the junctions in tissue of endothelial origin appear
as twin septa separated by about 6 - 7 nm (figures 4e, 4f). In many
places lateral projections can be seen protruding from the side of the
twin septa (figure 5a). Projections are also often seen between the twin
septal halves where they give a cross hatched appearance. The lateral
projections can be up to 6 mm long and are spaced at about 7.5 mm along
the septa (centre to centre). In some areas the lateral projections are
present on only one side of the twin septa. In such cases they can sometimes
be seen on one side of the twin septum in one area and on the other side of
the same twin septum in a different area (figure 4e). In regions where
the lateral projections are present on both sides of the twin septa, they
can be seen to be staggered on the two sides (figure 5a). As these
projections can be identified readily only over short lengths of the septum
at a time they must be either discontinuous or else need to be orientated
precisely within the section for them to be identified. In many sections,
twin septa can be seen running at an angle towards each other and almost
anastomosing (figure 4e). However, in all the sections examined a small
gap was always found between the twin septa and no clear case of anastomosis
has been found.

Wherever the septa are sectioned in such a way that they are tilted
within the section, their appearance can be different to the twin lines
seen in true tangential view. When the axis of tilt is within the plane
of the section, but at right angles to the line of the septa, there is
little change, but when the axis of tilt is within the plane of the section
and also runs along the line of the septa a considerable change in appearance results. With small amounts of tilt the twin septa appearance changes into a blurred single septum (A in figure 5b). As the tilt increases a single narrow septum becomes apparent (B in figure 5b), usually with rather large side projections up to 8 or 9 nm long.

Freeze-fracture replicas of these gastrodermal junctions show that the intramembrane arrangement of particles approximates the twin septal arrangement revealed by lanthanum tracer studies. In tissue glycerinated, but unfixed, freeze-fracture replicas reveal twin rows of particles on the P face of the junction, with the two rows separated by about 7 nm (figure 5c) so matching the separation between the two halves of the twin septa seen in lanthanum tracer studies.

Examination of such replicas reveals that the particles in the two rows are distinctly different. The particles in one row appear much smaller than those of the other row (figure 5c). The small particles are normal rounded ones while the larger particles appear to be elongated in a direction perpendicular to the line of the septa. The small particles are 5 - 6 nm in diameter, whereas the larger ones are about 8 - 9 nm long and about 5 - 6 nm in width. Where the spacings can be identified, particles in both rows have a separation of 7.5 nm along the septa. Although it is difficult to be certain because of the somewhat random orientation of the septa, the row of larger particles tends to be on the side of the septum nearest the apical edge of the cell. The E faces of freeze-fracture replicas in the junctional region show an array of broad shallow grooves (figure 6a). These are occasionally seen to have fine cross striations up to 12 nm long and 7.5 nm apart (figure 6b). The freeze-fracture replicas show the junction consists largely of arrays of relatively short, straight septa (figure 5c).
Freeze-fracture of fixed tissue shows essentially the same features with the particles remaining on the same face as in unfixed tissue; the P face. However, the appearance of the particle arrays is never as crisp and some distortion appears to have occurred during fracturing, presumably because of binding of the particles caused by fixation (figures 6c, 6d). In extreme cases the septa appear as single broad jumbled rows of particles (figures 6c, 6d). The size difference between the twin particle rows seen in unfixed tissue is less evident in fixed tissue. The E face again consists of broad shallow grooves as in unfixed tissue.\footnote{In some replicas of anemone species the twin septa occasionally show a form of anastomosis. This was not however observed in my own replicas and readers are referred to Green and Flower (in press) for a description of this phenomenon.}

Epithelial type anemone septate junction: When tangential sections of lanthanum impregnated ectodermal septate junctions are examined (figures 6e, 7a, 7b), it is immediately obvious that these junctions are different to the gastrodermal septate junctions as most septa present are single 3-4 nm wide structures. Septa are also seen to be long wavy structures rather than short, straight septa characteristic of the gastrodermal septate junctions. As in the gastrodermal septate junctions large lateral projections can be identified along the septa. They appear to be about 7 nm long and have a separation of about 7 nm along the septa (figure 6e). Careful examinations of the sections show that the lateral projections are clearest when present on one side of the septum only (figures 6e, 7a). In some areas projections arise from both sides of the septa (figures 7a, 7b), but in these cases they are less well defined. As in the gastrodermis, the lateral projections cannot be identified along the whole length of the septum. Only occasionally do septa run parallel as twin rows (figure 7b), and then the two septa do not appear to be exactly parallel to each other. Septa sometimes abut one another approximately at right angles (figures 7a,
7b) to give a branching appearance, but do not join completely.

Freeze-fracture replicas of epithelial junctions show that there is an even greater variation between this type of junction and the gastrodermal septate junction than is obvious in the lanthanum tracer studies. On replicas of both fixed and unfixed tissues rows of closely spaced particles are seen on the P face (figures 7c, 7d). These particles vary in size, but most appear to be between 8 and 9 nm in diameter. The particles are not spaced sufficiently regularly to suggest a standard separation, probably due to distortion during the fracturing process. The E face of the junction is characterised by an array of shallow grooves which are very fine and difficult to see (figure 7e). In most replicas the septa run singly, and only occasionally do they run for short distances parallel to each other (figure 7e) in an arrangement which probably parallels the twin septum appearance occasionally seen in lanthanum impregnated tissue tangential sections (figure 7b). In these epithelial septate junctions there is no evidence for a dichotomy of particle size or shape. Even when two septa run parallel to each other, both rows of particles appear the same, there is no sign of elongated particles as seen in the gastrodermal septate junctions.
Figure 3:

(a) A positively stained cross sectional view of a **Hydra** septate junction. Septa span a 15 nm intercellular space and in places a modification is seen in the form of two bars at right angles to the septa near their mid point (arrows). (lead citrate/ uranyl acetate stain) x 155,000

(b) A thin section of lanthanum impregnated **Hydra** tissue revealing the septate junction in tangential view. At this magnification septa are seen as 8 - 12 nm wide bands in negative contrast. In **Hydra** it is common to see large numbers of septa running side by side as in this micrograph. Septa are only 13 - 16 nm apart (centre to centre) where they are most closely stacked. x 150,000.

(c) Lanthanum impregnated **Hydra** tissue thin sectioned to reveal the septate junction in an oblique but tangential view. In this view septa are at an angle within the section such that they are being viewed as if tilted about an axis within the plane of the section and at right angles to their direction. It is possible to see that septa consist of a central backbone only 2 nm wide with projections 3 - 4 nm apart (centre to centre) and up to 4 nm long off alternate sides (arrow) x 200,000
Figure 3:

(d) Lanthanum impregnated Tubularia tissue thin sectioned to reveal the septate junction. Only a short length of each septum is visible and these are being viewed slightly end on (tending toward a cross sectional view). The septal central backbone is almost 'zig-zag' in appearance and the side projections are clear and sharp (arrow). On the edge of the tricellular region where a septum is being viewed slightly end on and also slightly side on, it appears to consist of circular structures in a chain like configuration (double arrow). x 260,000.

(e) The tricellular region of a Tubularia septate junction seen after thin sectioning of lanthanum impregnated tissue. The tricellular region looks like a 'zipper' with cross bars at right angles to the septa on either side. The bars have a 7 - 9 nm centre to centre spacing and appear wavy. Septa in this species are well spaced and meander between cells. x 200,000

(f) A thin section of lanthanum impregnated Tubularia tissue showing the tricellular region. Circular structures (arrows) form at least part of the 'zipper' cross bars. x 280,000.
Figure 4:

(a) A freeze-fracture replica of fixed gastrodermal *Hydra* tissue showing that the majority of septate junction particles are on the E face (E), not the P face (P). x 60,000.

(b) A freeze-fracture replica of fixed ectodermal *Hydra* tissue showing that the majority of particles of the septate junction are on the E face (E), not the P face (P). x 67,000.

(c) A cross sectional view of the anemone epidermal septate junction. The septa are difficult to see in this view, but the junction clearly has a 15 - 18 nm intercellular spacing. (lead citrate/uranyl acetate stain) x 94,000.

(d) A cross sectional view of the anemone gastrodermal septate junction. The junction has a 15 - 18 nm intercellular spacing, but septa are difficult to see (lead citrate/uranyl acetate stain). x 110,000.

(e) and (f) Lanthanum impregnated anemone gastrodermal tissue thin sectioned to reveal the anemone gastrodermal type septate junction in tangential view. Septa are seen to be double structures with the two halves 6 - 7 nm apart. Lateral projections are visible in many areas: often visible off one side of a septum at one point and off the other side at another point on the same septum (arrows on 4e). Septa often abut against another but a small gap is always seen between them (double arrow on 4e). 4e x 120,000; 4f x 96,000.
Figure 5:

(a) The anemone gastrodermal septate junction thin sectioned after lanthanum impregnation. The pegs arising from the septa are clearly visible, being about 6 nm long and spaced 7.5 nm centre to centre along the septum. Where they are visible on both sides of a twin septum, they are staggered on the two sides (arrow). Cross hatching between the two halves of a twin septum is also seen (double arrow). x 235,000.

(b) The anemone gastrodermal septate junction seen in tangential view after lanthanum impregnation. In this view the septa are seen on either side of a gap between two cells (arrow) and are thus seen at a range of angles as if being tilted about an axis within the plane of the section and along the line of the septa. With small amounts of tilt the twin septa appearance changes into a blurred, broad single septum (arrow A). As the angle of tilt increases a single narrow septum becomes apparent (arrow B) with side projections 8 - 9 nm long. x 155,000.

(c) A replica of the P face of freeze-fractured, unfixed anemone gastrodermal tissue showing the septate junction structure. The junctional structures consist of twin rows of particles 7 nm apart. One half of a twin row consists of small 5 - 6 nm diameter rounded particles (arrow), the other consisting of particles elongated in a direction perpendicular to the line of the septum (double arrow). These larger particles are 8 - 9 nm long and 5 - 6 nm wide and tend to be on the lumenal side of a septum. Particles in both rows are spaced at about 7.5 nm along the septa. Individual septa appear to be short and straight. x 120,000.
Figure 6:

(a) A freeze-fracture replica of unfixed anemone gastrodermal tissue showing the E face in an area of the gastrodermal septate junction. The junction is seen as a series of broad shallow grooves. x 110,000.

(b) Unfixed anemone gastrodermal tissue freeze-fractured to reveal the E face in a junctional area. On this replica the grooves of the gastrodermal septate junction are seen to have fine cross striations 12 nm long and about 7.5 nm apart. x 150,000.

(c) and (d) Freeze-fracture replicas of fixed gastrodermal anemone tissue showing the P face in the septate junction area. The junction appears as twin rows of particles, but they are not as well ordered as on unfixed tissue replicas. In extreme cases the septal particles appear as broad jumbled rows (arrows). There is little size variation between the two halves of each twin septum. 6c x 170,000; 6d x 91,000.

(e) A thin section tangential view of an anemone epidermal septate junction after lanthanum impregnation. The septa are single, long and undulating. They are 3 - 4 nm wide and have 7 nm long side projections at 7 nm intervals along the septa. They are clearest when seen off one side only. x 180,000.
Figure 7:

(a) and (b) Tangential views of the anemone epidermal septate junction after lanthanum impregnation. The 3 - 4 nm wide septa can have lateral projections from both sides (arrows), but they appear clearest when off one side only (double arrows). Septa sometimes abut at right angles to one another but do not join completely. They can also run closely together for short distances though not exactly parallel. 7a x 200,000; 7b x 195,000.

(c) A freeze-fracture replica of the P face of fixed anemone epidermal tissue showing particles of the septate junction. The particles are mostly 8 - 9 nm in diameter and irregularly spaced. x 78,000.

(d) A freeze-fracture replica of the P face of unfixed anemone epithelial tissue showing the particle structure of the anemone epidermal septate junction. x 105,000.

(e) A freeze-fracture replica of anemone epidermal septate junction from fixed tissue. The E face has fine grooves (arrows) which are difficult to see. Occasionally septa run closely together as twin rows as seen on the P face of this replica. x 90,000.
3.3.4 Discussion

The phylum Coelenterata has the greatest diversity of septate junctions found in any phylum, having three distinct variations. Each of the three coelenterate septate junctions has unique features when observed in tangential view. All three types have straight or wavy septa as opposed to pleated septa, but the twin septa characteristic of the anemone gastrodermal septate junction are easily recognised. In the case of the Hydra type septate junction the narrow 2 nm central backbone with projections from both sides makes this junction distinct from the anemone epithelial septate junction which has the wider 3 - 4 nm wide septa with large projections predominantly arising from one side at any one place.

Freeze-fracture results further differentiate the three junctional types. The asymmetric twin rows of particles seen in the anemone gastrodermal type septate junction give this junction another unique and prominent identifying feature. In a comparison of Hydra type and anemone epithelial type septate junctions, their differing particle shape (the epithelial junctional particles are more rounded and regular in shape) and fracturing properties distinguish between them. The particles of the anemone epithelial septate junction are seen on the P face of freeze-fracture replicas from both fixed and unfixed tissue, while the majority of Hydra type septate junction particles always occur on the E face, at least after the treatments used in this study.

A dominant general feature of the coelenterate septate junctions is their intricacy. The septate junction is usually envisaged as being a series of 'walls' spanning a 15 nm gap between cells, but in the cases of the Hydra type and anemone gastrodermal type septate junctions at least, this conception appears unfounded. The situation observed in lanthanum tracer impregnated tissue sections of the anemone gastrodermal septate junction suggests that solid septa do not span the 15 nm intercellular space. If they did they would be expected to appear as wide bars as the section was tilted about an axis along the line of the septa and within the plane of the section. Instead they appear initially as a wide blurred line, but then a single narrow line approxi-
imately the same thickness as one side of the twin septum and with longer side projections (a diagrammatic representation of these two possibilities is given in figure 8 to clarify this point). Similarly, the appearance of an almost 'zig-zag' structure and ultimately a chain like structure when Hydra type septa are tilted about an axis within the plane of the section and at right angles to the line of the septa suggests these septa do not form a solid 'wall' between cells either. This feature of the Hydra type septate junction was most apparent when the septa were tilted not only end on to the electron beam, but also slightly side on; that is, tilted simultaneously about two axes. In the case of the Hydra type septate junction the two centre bars with a gap between them seen in some cross section views also suggests that these septa do not form a solid intermembrane wall.

Attempts to visualise the exact make-up of these septa results in complex models being proposed such as those for the Hydra type septate junction (Filshie and Flower, 1977; Hand and Gobel, 1972). Results from this study of the Hydra type septate junction are not explained fully by either of these models. The 'zig-zag' nature of the backbone and the chain-like structures seen when septa are viewed from the side and slightly end on best fits the model of Hand and Gobel who found similar structures in their own thin sections. However, their model does not adequately explain the thin straight central backbone seen in true tangential sections. In this case the model of Filshie and Flower is preferable. An amalgamation of features from these proposed models may in fact provide the best fit to all available data, but at this stage further attempts at modelling would be largely speculation.

Another feature of the coelenterate septate junctions that is of interest is the occurrence of side projections on all three types, even though most predominant when off only one side at a time in the anemone epidermal septate junction. The side projections on the Hydra type septate junctions however, appear most distinct when the junction is tilted about an axis within the plane of the section and at right angles to the line of the septa. Filshie and Flower (1977) found by examining stereo pairs that a tilt of about 30° gave best differentiation of the projections.
Present results utilising the goniometer stage confirm this. A similar enhancement effect was not observed in the anemone epidermal septate junction, but as noted above, larger side projections are apparent on the gastrodermal anemone septate junction when it is tilted about an axis within the plane of the section and along the line of the septa.

Filshie and Flower (1977) suggested that the projections on the Hydra type septate junction could be a staggered arrangement of lateral pegs. This idea does seem to best fit the available data. For the anemone epidermal septate junction present results suggest that each projection seen in tangential view is a single buttress protruding from a septum. The situation in the anemone gastrodermal septate junction is not clear, but projections as in the case of the Hydra type septate junction, seem best explained if they are visualised as pegs rather than buttresses.

An interesting point arises when lanthanum tracer and freeze-fracture results of the anemone gastrodermal junction are compared. In tangential view the septa are relatively symmetrical, the two halves appearing equal except when side projections are more prominent off one side or another. In such cases however, the asymmetry is reversible within the length of a single septum, the projections being most prominent off one side in one area but off the other side in another area. In contrast, freeze-fracture replicas reveal a distinct and continuous asymmetry with one side of the septa having larger particles. Clearly there does not appear to be a direct relationship between intermembrane and intramembrane structures in this case.

Freeze-fracture in the present study has revealed that the majority of particles of the Hydra type septate junction occur on the E face of replicas from both fixed epidermal and fixed gastrodermal tissue. While Filshie and Flower (1977) did not report any difference between epidermal and gastrodermal tissue, Wood (1977) claimed the particles of his replicas
of fixed epidermal tissue were more numerous on the P face, therefore contrasting with the results of the present study. It is difficult to explain why these differences in results have occurred. It is apparent that Wood has used different fixation techniques, fixing for only 10 - 15 minutes in 3% glutaraldehyde and 1% formaldehyde, but this fixation method would have been consistent for both of his Hydra dermal layers. Until more work is done on this junction the difference in results has to go unexplained.

Finally, it is of note that while Tubularia epithelia and Hydra epithelia share the same type of septate junction, the number of septa in each animal varies considerably. Hydra, a fresh water organism, has large numbers of septa with 40 or more commonly seen running side by side around a cell. In contrast, the number of septa seen in Tubularia, a marine organism, was never more than 20 around any one cell. It is possible therefore that the number of septa is variable depending upon environmental conditions (see Chapter 4.2).
Figure 8:  

A diagrammatic representation of the anemone gastrodermal septate junction to demonstrate the expected results that might be obtained as this junction is tilted about an axis within the plane of the section and along the line of the septa. (a) is a representation of what is seen in tangential views. If septa were solid structures spanning the entire intercellular space, then tilting would result in a gradual broadening of the septal image seen as drawn in views A(b) and then A(c). However, the images seen in tangential views of firstly a single, broad, blurred line and then a single narrow line, with lateral projections as the tilt increases are best explained by views B(b) and then B(c) in which septa are not drawn as solid structures. It can be seen that on the basis of thin section studies as presented here, it is more likely that septa are not solid structures spanning the intercellular space, but rather are more complex structures. If the septa are something like those drawn in view B(c) with supporting 'pegs' branching at an angle from the septum, it is conceivable that these pegs might appear longer in certain views as seen in lanthanum impregnated tissue thin sections when the septa are tilted (figure 5b).
3.4 The Lower Invertebrate Pleated Septate Junction

(The Phyla Platyhelminthes, Annelida, Sipunculoidea, Brachiopoda, Nemertina and Bryozoa).

3.4.1 Introduction

The lower invertebrate pleated septate junction was first described by Baskin (1976) in the class Polychaeta (Phylum Annelida) on the basis of his observations of conventionally stained thin sections and lanthanum impregnated tissue thin sections. Baskin's work was followed by a freeze-fracture description of this junction by Welsch and Buchheim (1977). This work dealt with an Oligochaete and there was no supporting thin section evidence that the junction they described was in fact the same junction as that described by Baskin. Furthermore, their work apparently included two separate junction types, or else the different treatments used caused marked differences in the freeze-fracture appearance of the junctions from species to species. They viewed replicas of *Lumbricus terrestris* tissue frozen after it had been soaked for 10 minutes in 30% glycerol, and replicas of *Tubifex* sp. tissue frozen with no cryoprotectant treatment.

The lower invertebrate pleated septate junction as described by Baskin (1976) can also be recognised, on thin section evidence, in the phylum Platyhelminthes (Class Turbellaria, Storch and Welsch, 1977; Class Trematoda, Noiroit-Timothee and Noirot, 1980) and is also probably the same as that found in the nemertean *Lineus ruber* by Vernet et al. (1979). Although these workers claim they have a mollusc-arthropod pleated septate junction, their freeze-fracture replicas are more indicative of the lower invertebrate pleated septate junction (see results section of this chapter for identifying features). This particular junction has been recognised in the present study in several lower invertebrate phyla, following thin section and freeze-fracture
examination. It has therefore been renamed the 'Lower invertebrate pleated septate junction' as opposed to the term 'Annelid Septate Junction' used by Welsch and Buchheim (1977).

In the present work it has been possible to improve the clarify of lanthanum impregnated tissue thin section and to obtain freeze-fracture replicas and thin sections from the same tissue thus allowing better correlation of structures. The junction is therefore described fully and the results compared with those previously published.

3.4.2 Results

The lower invertebrate pleated septate junction has been found in the tissues listed below and is therefore common to several invertebrate phyla; more than any other single septate junction type.

Phylum Platyhelminthes

Class Turbellaria
Order Tricladida

Neopia montana epithelium

Class Turbellaria
Order Polycladida

Pseudoceros sp. epithelium

Phylum Annelida

Class Oligochaeta
Order Terricola

Lumbricus terrestris gut endoderm

Class Polychaeta
Order Errantia

Eulalia microphylla gut endoderm

Phylum Sipunculoidea

Sipunculus mandanus tentacle epithelium

Phylum Brachiopoda

Terebratella inconspicua tentacular fringe
In positively stained thin sections, septa of this junction are seen to span a 15 - 18 nm intercellular space though they are often indistinct. The membranes of the adjacent cells often have a slightly scalloped appearance (figure 9a). In lanthanum tracer impregnated tissue, tangential sections of the junction reveal septa to be pleated structures following a wavy course between cells (figures 9b, 9c). Septa seldom run straight or parallel to each other for any distance and this explains why good positive stained cross sections are difficult to find. The pleating appears more pronounced than in the mollusc-arthropod pleated septate junction (chapter 3.5), but the periodicity of the pleating is 16-22 nm, similar to that reported for the mollusc-arthropod pleated septate junction (figure 9b). The apex of each pleat has a lateral projection which in some sections appears relatively long compared with the sides of the pleats of the central backbone (figure 9c). The side of a pleat is 6 - 8 nm long where they appear clearest, with 3 - 5 nm projections from the apex. It is these projections that give the junction its more pronounced pleating appearance. Where two or more septa run closely parallel the apices of their pleats often align and their projections fuse to form a chain of hexagonal structures 11 - 12 nm across at their widest point (figures 9b, 9c). In freshwater species studied such as Neopia montana (Phylum Platyhelminthes) large numbers of septa become fused in large hexagonal arrays (figure 10a), but in marine species of the same class the number of septa fused together is rarely more than four or five, and fusion is never seen over more than a 0.5 µm long stretch of junction. If the angle of pleating is measured, taking
care to measure the angle without including the side projections, it
is found to vary. Where septa run straight in true tangential view
however, the angle is about 100°-130° (figure 9d). In many sections
septa are seen terminating (figure 9b).

In some sections several dots with a dark central core can be
seen outlined with the lanthanum tracer (figure 9b). These dots
presumably represent hollow pillars between cells, but do not always
occur in conjunction with the septate junction strands. These pillars
are about 7-10 nm in diameter with a 2.5-4 nm diameter central core
where it is visible.

In sections which show the septa most clearly the appearance is
of thin delicate structures about 2 nm wide. Where good lanthanum
penetration has not occurred they often appear as thick structures
difficult to identify as being pleated (figure 9e).

In the tricellular region, the apex of each pleat of a septum
running parallel to the edge of the tricellular junction has extended
lateral projections that span the intercellular gap to join the
projections from a septum on the other side. Depending on the angle of
section, such linked projections or bridges are often seen to have a
circular structure in their centre (figure 10b).

In freeze-fracture replicas the lower invertebrate pleated
septate junction is characterised by particles on the P face of both
fixed and unfixed tissue. Freeze-fracture of fixed tissue shows a ragged
assortment of P face particles varying in size from less than 3 nm to
about 12 nm wide (figures 10c, 10d, 11a). Occasionally two or three
particles appear fused into short rods (figure 11a), but they are
usually individual with a centre to centre spacing up to as much as
40 nm, though usually less than 20 nm. There is no regularity in particle
shape, some appear rounded, others jagged, and particles are never seen
in neat rows, but rather as ragged bands following a wavy course between cells. In places where presumably two or more septa run closely parallel these bands become quite broad, but it is not usually possible to distinguish one septum from its neighbour in such cases (figures 10d, 11a). In some species (e.g. Sipunculus mandanus, Phylum Sipunculoidea) the particles occur more in rows although the size variation remains (figure 11c). The E face of fixed tissue has a complementary assortment of pits and an occasional particle (figures 10c, 11b). The pits, like the P face particles, show an assortment of sizes but are generally smaller than the particle sizes due to the shadow filling them in.

Freeze-fracture of unfixed tissue reveals that there is a more regular appearance to the junction. Particles on the P face are in neat rows and are nearly all a standard 8 - 10 nm in diameter (figure 11d). Some smaller particles, and the occasional larger particle, are still present however. The E face is characterised by a series of distinct grooves or furrows as opposed to pits, and with an occasional particle left adhering in the grooves (figure 11e). The grooves are about 3 - 6 nm wide. Under these conditions the lower invertebrate pleated septate junction is almost indistinguishable from the mollusc-arthropod pleated septate junction (chapter 3.5).
Figure 9:

(a) A positively stained cross sectional view of the lower invertebrate pleated septate junction in the brachiopod Terebratella inconspicua. Septa span a 15 - 18 nm intercellular space and the contributing membranes have a slightly scalloped appearance (arrow). (lead citrate/uranyl acetate stain) x 130,000.

(b) A tangential thin sectional view of the lower invertebrate pleated septate junction seen in an annelid gut after lanthanum impregnation. The pleating of the septa is clear with the side of each pleat being 6 - 8 nm long. When two septa run close together they form crosslinks between alternate pleat apices to form chains of hexagonal enclosures. The 7 - 10 nm diameter pegs with hollow 2.5 - 4 nm diameter central cores (arrows) do not always occur in conjunction with the septate junction and are not considered to be part of it. x 90,000

(c) A tangential thin section view of the lower invertebrate pleated septate junction in an annelid gut cut after lanthanum impregnation. Crosslinking between septa to form hexagonal enclosures occurs in several places. Lateral projections 3 - 5 nm long occur at the apex of each pleat (arrows). The main pleated central backbone of each septum is about 2 nm wide where it is seen most clearly. x 120,000.

(d) A tangential thin section view of a piece of lanthanum impregnated lower invertebrate pleated septate junction from an an annelid gut upon which the angle of pleating has been marked. Care must be taken to measure this angle without being influenced by the lateral projections at the pleat apices. The angle of pleating is usually between 100 and 130°. x 170,000.
Lanthanum impregnated tissue from the bryozoan *Watersipora cucullata* which has been thin sectioned to reveal the lower invertebrate pleated septate junction in tangential view. In this case the lanthanum has not packed tightly around the septa and the pleating is not readily apparent. $x$ 70,000.
Figure 10:

(a) Hexagonal arrays formed by large numbers of lower invertebrate pleated septate junction septa seen in tangential view after lanthanum impregnation in the freshwater platyhelminth Neopia montana. x 110,000

(b) The tricellular region of a lower invertebrate pleated septate junction from an annelid gut outlined with lanthanum tracer. Crosslinks joining the apex of septal pleats on either side of this region have a circular central core (arrows). x 117,000

(c) A freeze-fracture replica of fixed polychaete gut in a junctional region. Both the P face (P) and the E face (E) of the lower invertebrate pleated septate junction have been revealed. The P face characteristically has particles while the E face has a series of pits with the occasional particle left adhering. x 58,000.

(d) A lower invertebrate pleated septate junction from a polychaete gut freeze-fractured after fixation to reveal both membrane faces. The particles of the P face are up to 40 nm apart (centre to centre) though usually less than 20 nm apart. They do not align in neat rows, but occur as ragged bands. Where two or more septa run together (arrow) it is not possible to distinguish between them. The pits on the E face show a similar pattern of ragged bands. x 78,000.
Figure 11:

(a) A replica of the P face of the lower invertebrate pleated septate junction after freeze-fracturing fixed polychaete gut tissue. The particles are irregularly shaped and vary in width from less than 3 nm to more than 12 nm. Some are rounded in shape, others ragged. They do not align in neat rows but occur as ragged bands. Occasional particles appear fused into short rods (arrow). x 130,000.

(b) A freeze-fracture replica of the E face of the lower invertebrate pleated septate junction from fixed polychaete gut tissue. The pits characteristic of this face show an irregular size variation and form ragged bands in a complementary fashion to the particles of the P face of this junction. An occasional particle is left adhering to the E face (arrows). x 130,000.

(c) A freeze-fracture replica of the P face of the lower invertebrate pleated septate junction in fixed sipunculoid tissue (Sipunculus mandanus). In this species the particles appear more in rows than bands but their size variation and ragged appearance remains. x 120,000.

(d) A replica of the P face of unfixed polychaete gut seen after freeze-fracturing. The particles are predominantly 8 - 10 nm in diameter although some smaller and some larger particles are present. The particles align in neat rows. x 124,000.

(e) A replica of the E face of unfixed polychaete gut seen after freeze-fracturing. Distinct grooves or furrows 3 - 6 nm wide are seen with the occasional particle left adhering to this face. x 120,000.
3.4.3 Discussion

The lower invertebrate pleated septate junction is interesting in that it is common to a large number of invertebrate phyla. It is also interesting in that it has many features in common with both the *Hydra* type septate junction and the pleated septate junction seen in the Mollusca and Arthropoda.

This junction has a delicate septal construction when seen in lanthanum tracer impregnated thin sections. It has a narrow 2 nm wide central backbone similar to that of the *Hydra* type septate junction though pleated. The septa also have side projections spaced regularly along their length, a feature shared with all three coelenterate septate junctions (chapter 3.3). This feature is emphasised in the lower invertebrate pleated septate junctions and the three coelenterate septate junctions more than in any other invertebrate septate junction type. In freeze-fracture replicas, the lower invertebrate pleated septate junction has irregularly sized particles with uneven spacing, again a feature in common with the *Hydra* type septate junction. However, the presence of pleated septa and the characteristic seen in freeze-fracture of particles being on the P face rather than the E face differentiates this junction from the *Hydra* type.

The lower invertebrate pleated septate junction is similar to the mollusc-arthropod pleated septate junction (chapter 3.5) in having a pleated septal structure. It also freeze-fractures to give particles on the P face of both fixed and unfixed tissue as does the mollusc-arthropod pleated septate junction. However, the more pronounced pleating of the lower invertebrate septate junction is obvious if lanthanum impregnated tissue thin sections which show true tangential views of the junctions are compared side by side. This difference is apparent despite the 16 - 22 nm periodicity of pleating in the lower invertebrate junction being similar to the 17 - 23 nm reported for the mollusc-arthropod junction (chapter 3.5) and the angle of pleating in these two junctions being similar (about 100 - 130° in true tangential views). The more pronounced pleating of the lower invertebrate pleated septate junction is in fact an illusion created by the side projections at the apex of each pleat. Where crosslinks are formed between two septa that run closely together and parallel, as can occur
in both types of junction, they can become virtually indistinguishable.

These two junctions can be differentiated further by comparison of their respective freeze-fracture replicas. Particles seen on the P face of fixed mollusc or arthropod tissue have an even spacing, a regular size and shape, and align in neat rows rather than in ragged bands (chapter 3.5). The E face of the lower invertebrate pleated septate junction is characterised by complementary pits rather than by the furrows of the mollusc-arthropod pleated septate junction (chapter 3.5). There are no obvious differences when viewing unfixed tissue.

The 7 - 10 nm diameter tubular structures occasionally seen in conjunction with the lower invertebrate pleated septate junction are not unlike those reported to occur in conjunction with the mollusc-arthropod pleated septate junction (Giusti, 1976). These structures reported by Giusti in the gastropod *Cepaea nemoralis* were 9 nm in diameter and had a 2 - 2.5 nm central canal into which lanthanum was able to penetrate. They differ from the pegs seen in conjunction with the smooth septate junction which are only about 3.5 nm in diameter and which have no central pore (Flower and Filshie, 1975). Giusti suggested they may be acting as communication channels in a similar manner to the subunits of the gap junction. As they do not consistently appear in association with the lower invertebrate pleated septate junction they are not considered to play an important role, if any, in relation to the septate junction. This is in contrast to the regular association of pegs with the smooth septate junction (chapter 3.5).

Previously published works on the lower invertebrate pleated septate junction have reported structures which are slightly different to those reported here. Baskin (1976) claimed the junction had an intercellular spacing of 18 - 20 nm, but close inspection of his
micrographs, as well as those obtained in this present study, reveals a 15 - 18 nm intercellular spacing. The differences in measurements could be understood if Baskin measured from centre to centre of the contributing membrane bilayers; the 15 - 18 nm figure would therefore be a truer indication of the actual intermembrane spacing. There is also some discrepancy in freeze-fracture results observed here and those observed by Welsch and Buchheim (1977). Results obtained by these workers on Lumbricus terrestris (Class Oligochaeta, Phylum Annelida) are comparable but at no time were large rounded particles in rows similar to those seen by Welsch and Buchheim in Tubifex sp. (Class Oligochaeta, Phylum Annelida) observed. This could arise because they used uncryoprotected tissue for that part of their work, or, it is possible that Tubifex has yet another type of septate junction. Tubifex is a fresh water species whereas all other species so far studied in the Phylum Annelida have been marine or terrestrial.

Study of the platyhelminths has been particularly useful in allowing comparison of septate junction structure in similar epithelia exposed to different environments, a similar comparison to that made between Hydra and Tubularia epithelial septate junctions (chapter 3.3). The lower invertebrate pleated septate junction was studied in the epithelium of Neopia montana, a freshwater flatworm, and in the epithelium of Pseudoceros sp., a marine flatworm. The only differences observed were in the number of septa present and in the extent of crosslinking between septa. The fresh water Neopia has numerous septa, many of which are crosslinked, while in the marine Pseudoceros, septa are fewer in number and crosslinking is less common. It is possible that the number of septa present is variable in response to environmental stresses (see chapter 4.2). It is noteworthy that many crosslinked septa also occur in the terrestrial Bipalium (Phylum
Platyhelminthes; Storch and Welsch, 1977).

In septa that do form crosslinked pairs or larger arrays, lanthanum tracer has still been able to penetrate into the apparently closed hexagonal chambers formed. Therefore, at least in fixed tissue, the septa themselves or the lateral projections do not form a barrier to the tracer. The lateral projections may not be buttresses crossing the entire intercellular space, but may be only pegs. It cannot be assumed however that the septa themselves cross the entire intercellular space as complete 'walls'; they may form a lattice of finer components. This view is also held by Baskin (1976) on the basis of his own results.

Finally, it is of note that the lower invertebrate pleated septate junction is found in epidermal tissue at least of the Phylum Brachiopoda. The brachiopods have many molluscan features but phylogenetically are an enigmatic group. The occurrence of the lower invertebrate pleated septate junction in this group as well as in 'lower' phyla is interesting when attempting to evaluate the phylogenetic position of the Brachiopoda (see Chapter 4.3).
3.5 The Phyla Mollusca and Arthropoda

3.5.1 Introduction

The Mollusca and Arthropoda are treated together as both have the well known pleated septate junction. In addition the phylum Arthropoda has the equally well described smooth or continuous septate junction (for references see tables 3.5.2, 3.5.3, and 3.5.4). It is not proposed to go into a detailed description of these junctions in the present study as a recent review by Noirot-Timothee and Noirot (1980) covers this. However, it is necessary to clarify some points regarding the structure of the pleated septate junction in order to differentiate it clearly from the lower invertebrate pleated septate junction. Also it is desirable to describe the smooth septate junction briefly in order to allow easier comparison with similar junctions which occur in the Pycnogonida, Merostomata and Collembola (chapter 3.8). The disposition of these two junctions in various tissues is detailed and discussed in this chapter.

Specimens used in this section of work were selected primarily to fill gaps in the published literature. In the Mollusca, for example, specimens from the Classes Amphineura (chitons) and Cephalopoda (squids and octopi) were studied as there are no published results for these classes. In addition the Classes Lamellibranchia (bivalves) and Gastropoda (snails and slugs) were studied to allow direct comparison of methods used in this study with classes studied previously by other workers (for references see table 3.5.2). In the Arthropoda the non-mobile adults of the barnacles and a crab were included. The crab was studied to allow comparison between similar tissues in the classes Crustacea and Insecta. Previous studies of the Crustacea have included a variety of tissues of endodermal origin (table 3.5.4), but the study of tissue of epidermal origin has been restricted to the antennal
gland of the crayfish *Orconectes virilis* (Shivers and Chauvin, 1977). In the Insecta, the cricket midgut was used as previous studies of the order Orthoptera have been limited to tissues of epidermal origin (Lane *et al*., 1977; Szollosi and Marcaillou, 1977). Other previous studies of the class Insecta have included both epidermal and endodermal tissues of at least the orders Blattaria (cockroaches), Diptera (true flies) and Lepidoptera (butterflies and moths) as well as tissues of either epidermal or endodermal origin in several other orders (tables 3.5.3 and 3.5.4).

Tilting stage (goniometer stage) electron microscopic study was carried out on the pleated and smooth septate junctions. This was done in order to prove the concept of reinforcement in the pleated septate junction as proposed by Flower and Filshie (1975) and to show that the non-occurrence of a ladder like view in cross sections of the smooth septate junction is only the result of the angle of a thin section in relation to the septa, not a specific feature of the junction. Species of a glow-worm, cricket and gastropod mollusc were used in these experiments.

A full classification of species used in this chapter is given in table 3.5.1.

3.5.2. Results (The Pleated Septate Junction)

The pleated septate junction spans a 15 - 18 nm intercellular space (figure 17c). In cross section view it is seen as a ladder like structure with septa regularly spaced in most cases (see references tables 3.5.2, and 3.5.3). This spacing between septa is variable, however. In tangentially cut sections of lanthanum impregnated tissue the junction appears pleated (16 - 23 nm periodicity) with 2 - 3 nm wide septa running between cells (figure 12a). This junction characteristically has large numbers of septa, often running parallel
in extensive arrays. Forty to sixty septa is a common occurrence in many tissues (figure 12a). The angle of pleating varies considerably, but where septa are viewed in true tangential view it is about 100 - 130° (figure 12a). The total width of the septa (width from a line drawn linking apices on one side of the septa to a similar line on the opposite side) is never less than about 6 nm (figure 12a).

Often septa form crosslinks, producing chains of hexagonal chambers (e.g. Noirot-Timothee and Noirot, 1973), but this is a far less obvious feature than in the lower invertebrate pleated septate junction (chapter 3.4). Generally, pegging at the pleat apices is not seen, but it is apparent in some views (e.g. Noirot-Timothee and Noirot, 1973). The tricellular region of this junction has similar crosslinks with circular centres (figure 12b) to those reported earlier in the three coelenterate septate junctions and the lower invertebrate pleated septate junction.

In freeze-fracture replicas of fixed tissue the pleated septate junction has regularly spaced particles 16 - 20 nm apart (centre to centre) and about 8 - 12 nm diameter on the P face (figure 12c). There is the occasional smaller or larger particle present. In all cases the particles seen on the P face are in neat rows and are relatively rounded in shape; similar in shape to the majority of free membrane particles. The E face of this junction has a series of complementary fine furrows (figure 12d) which on close inspection seem to be made up of a series of closely placed pits with collapsed walls between each pit. Furrows are about 4 - 6 nm wide and the pits are presumably complementary to the P face particles.

Freeze-fracture replicas of unfixed tissue show little variation from the fixed state. Particles remain on the P face and furrows are seen on the E face.
Table 3.5.1: Classification of Species Studied in the Phyla Mollusca and Arthropoda.

<table>
<thead>
<tr>
<th>Phylum Mollusca</th>
<th>Class Amphineura (chitons)</th>
<th>Eudoxochiton nobilis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Class Cephalopoda (squids and octopi)</td>
<td>Unknown squid species</td>
</tr>
<tr>
<td></td>
<td>Class Lamellibranchia (mollusc bivalves)</td>
<td>Perna canaliculus (mussel)</td>
</tr>
<tr>
<td></td>
<td>Class Gastropoda (snails and slugs)</td>
<td>Maoricrypta monoxyla (limpet)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nerita melanotragus (black sea snail)</td>
</tr>
<tr>
<td>Phylum Arthropoda</td>
<td>Class Crustacea</td>
<td>Elminius modestus</td>
</tr>
<tr>
<td></td>
<td>Subclass Cirripeda (barnacles)</td>
<td>Chamaesipho brunnea</td>
</tr>
<tr>
<td></td>
<td>Subclass Malacostraca (crabs, prawns and woodlice)</td>
<td>Petrolisthes elongatus (crab)</td>
</tr>
<tr>
<td>Class Insecta</td>
<td>Order Orthoptera</td>
<td>Teleogryllus commodus (cricket)</td>
</tr>
<tr>
<td></td>
<td>Order Diptera</td>
<td>Arachnocampa luminosa (glow worm)</td>
</tr>
</tbody>
</table>
Figure 12:

(a) A thin section showing a mollusc-arthropod pleated septate junction in tangential view after lanthanum impregnation. This section from a crab hindgut (*Petrolisthes elongatus*) shows a large number of 2 - 3 nm wide pleated septa. The angle of pleating as marked is about 100 - 130° and the total width of a septum (the space between lines drawn linking pleat apices on either side of a septum) is about 6 nm. x 90,000.

(b) A thin section of the tricellular region of the mollusc-arthropod pleated septate junction in a gastropod (*Nerita melanotragus*) after lanthanum impregnation. Crosslinks in this area are seen to have circular core centres (arrows). x 105,000.

(c) A freeze-fracture replica (P face) of fixed gastropod gut (*Nerita melanotragus*) pleated septate junction. The junctional particles 8 - 12 nm in diameter are relatively rounded and similar to the majority of free membrane particles. Occasional smaller and larger particles are present. The particles are in neat rows with a 16 - 20 nm centre to centre spacing. x 90,000.

(d) A freeze-fracture replica (E face) of fixed gastropod gut (*Nerita melanotragus*) septate junction. The junction is made up of rows of closely placed pits with the walls between then apparently collapsed to give the appearance of furrows or grooves 4 - 6 nm wide. x 135,000.
3.5.3 Results (The Smooth Septate Junction)

The smooth septate junction has a 15 - 18 nm intercellular space and a ladder like appearance when positively stained cross sections are viewed (figure 14e). The appearance of the ladder like structure however depends on the angle of the septa in relation to the thin section (see section 3.5.4).

In lanthanum impregnated tissue, tangential sections reveal broad septa approximately 7 - 8 nm wide (figures 13a, 13b, 13c). Septa generally have an even width along their entire length, are straight edged and follow a straight or slightly curved course between cells. On occasions septa fuse together to form broad intercellular structures (figure 13b) and in many instances a row of 3.5 nm diameter pegs is seen running parallel to septa (figure 13c). The septa appear generally to be solid structures with no visible substructure or side projections although the pegs alongside the septa can appear as short fused side projections if a section is tilted about an axis within the plane of the section and along the line of the septum (Flower and Filshie 1975). Occasionally septa have a more ragged appearance but no obvious repeating structure is apparent in these cases (Skaer et al., 1979).

As the smooth septate junction has been well described by Flower and Filshie (1975) and in subsequent work (table 3.5.4), no further description will be given here. However, it should be noted that freeze-fracture replicas of fixed tissue show a series of short rods on the P face which may become almost continuous in some cases. The E face is predominantly grooved, although with a considerable number of particles in some regions. In unfixed tissue, it is the P face which has the grooves, with few particles, and the E face is characterised by regular rows of particles (Flower and Filshie, 1975; Skaer et al. 1979). Skaer et al. (1979) also report particles on the E face on both fixed and unfixed Musca domestica (housefly) Malpighian tubule preparations.
Figure 13:

(a) A tangential thin section view of the smooth septate junction in the gut of a barnacle (*Elminius modestus*) following lanthanum impregnation. The septa are an even 7 – 8 mm wide and follow a straight or slightly curved path. They appear solid with no substructure. x 130,000.

(b) Lanthanum impregnated smooth septate junction in the barnacle *Elminius modestus* sectioned to reveal the junction in tangential view. The 7 – 8 mm wide septa often fuse to form broad intercellular structures (arrow). x 245,000.

(c) Lanthanum impregnated smooth septate junction in the barnacle *Elminius modestus* sectioned to reveal the junction in tangential view. Pegs 3.5 mm in diameter are seen regularly spaced alongside septa (arrows). x 200,000.
3.5.4 Results (Tilting Stage Electron Microscopy)

Thin sections of positively stained material were examined using the goniometer stage of the electron microscope. Cross sections of junctional areas were aligned by grid rotation so that they could be tilted to various angles while looking along the plasma membranes of the adjoining cells. This allowed the septa of the junctions to be viewed from different angles.

In the case of the smooth septate junction (Malpighian tubule of the New Zealand glow-worm Arachnospira luminosa) septa were positioned so that they appeared as a ladder-like junction in cross section. The section was then tilted along an axis within the plane of the section, and at right angles to the junctional membranes so that a view was taken along the membranes at various angles positive and negative to the original starting direction. Tilting in either direction resulted in a loss of the ladder-like structure and a uniform greyness appearing between membranes. Tilting to angles as much as plus or minus $55^\circ$ never resulted in the reappearance of the ladder structure (figure 14).

In a second type of experiment, septa of lanthanum impregnated and positively overstained cricket midgut were found by tilting to an angle of, for example, plus $40^\circ$ and the section was then tilted back through $0^\circ$ to minus $50^\circ$, a total of $90^\circ$ rotation, without septa reappearing (figure 15). This experiment was repeated several times with angles of up to $120^\circ$, the total tilt possible. Septa were visible clearly only when looking directly down their length from end on.

These experiments were repeated with positively stained Hydra type septate junctions (Chlorohydra viridissima), which also have straight unpleated septa, with similar results (figure 16).

When a cross section of the pleated septate junction was tilted (digestive gland of the gastropod mollusc Maoricrypta monoxyla), a
different result was obtained. In this case tilting causes a loss of the ladder like image to form a uniform greyness between membranes, as was observed in the smooth septate junction. However, continued tilting resulted in a reappearance of the ladder image, usually after tilting approximately 70°, although this angle varied between 50° and 80° for different areas of the junction (figure 17). Due to the angle of the tilt, and the resulting thickness of section being viewed, septa do not appear very sharp, but are none the less obvious. It is thus possible to view the pleated septate junction from at least two different angles and see a ladder like structure (within the tilting range of the apparatus used: - plus or minus 60°).
Figure 14:

A tilting stage series of a positively stained smooth septate junction from the glow worm *Arachnocampa luminosa* seen in cross section. In this series septa were located in a thin section spanning a 15–18 mm intercellular space. The section was then tilted slightly until the septa were seen clearly (14e). The section was then further tilted either side from this point about an axis within the plane of the section and at right angles to the membranes contributing to the junction. Photomicrographs were taken at regular intervals but at no time did septa again become visible. (refer to chapter 3.5.6 for discussion of these results).

Angles of tilt recorded are relative to the plane of the original thin section.

(a) $-30^\circ$  
(b) $-20^\circ$  
(c) $-10^\circ$

(d) $-5^\circ$  
(e) $15^\circ$  
(f) $25^\circ$

(g) $35^\circ$  
(h) $45^\circ$  
(i) $55^\circ$

(lead citrate/uranyl acetate stain). All micrographs x 135,000
Figure 15:

A tilting stage series of a smooth septate junction in a cricket midgut that is viewed in cross section. The junction has been impregnated with lanthanum and the thin section later overstained. The septa are visible in view 15a (arrows) which is a micrograph taken after tilting the section 40° from its original plane. The tilting was about an axis within the plane of the section and at right angles to the junctional membranes. Tilting was then carried out going back from this point through the horizontal point (15e) and then in a negative direction to -50° (15j). At no time did septa again become visible.

(refer to chapter 3.5.6 for discussion of these results)

Angles of tilt are relative to the plane of the original section.

(a) 40°  (b) 30°  (c) 20°
(d) 10°  (e) 0°  (f) -10°
(g) -20°  (h) -30°  (i) -40°
(j) -50°

(lead citrate/uranyl acetate overstain). All micrographs x 200,000
Figure 16:

A tilting stage series of a positively stained *Hydra* type septate junction in *Chlorohydra viridissima*. In this series septa were located in cross section view and the section tilted slightly until they appeared clearest (16f - 16g). The section was then further tilted either way from this point about an axis within the plane of the section and at right angles to the contributing membranes of the junction. Photomicrographs were taken at regular intervals but at no time did the septa again become visible although they can be seen over a wide range of tilt (16d - 16h).

(refer to Chapter 3.5.6 for discussion of these results).

Angles of tilt recorded are relative to the plane of the original thin section.

(a) 50°  (b) 45°  (c) 25°
(d) 10°  (e) 0°  (f) -10°
(g) -20°  (h) -30°  (i) -45°
(j) -60°

(lead citrate/uranyl acetate stain). All micrographs x 175,000
Figure 17:

A positively stained view of a mollusc/arthropod pleated septate junction in cross section. This junction in the gastropod *Maoricrypta monoxyla* has been tilted about an axis within the plane of the section and at right angles to the junctional membranes. At two different angles, 17c and 17f, septa are seen in cross section view (arrows). The angle between the views at which septa are visible is 60°.

(refer to chapter 3.5.6 for discussion of these results).

Angles of tilt recorded are relative to the plane of the original thin section.

(a) 36°  (b) 20°  (c) 12°
(d) -12°  (e) -30°  (f) -48°

(lead citrate/uranyl acetate stain). All micrographs x 155,000
3.5.5 Discussion

The pleated septate junction was first described by Locke in 1965 and since then has been reported in several molluscan and arthropod classes. It varies from the lower invertebrate pleated septate junction mainly in its appearance in freeze-fractured, fixed tissue. The neat rows of rounded particles on the P face contrast with the ragged bands of particles seen on replicas of the lower invertebrate pleated septate junction (chapter 3.4). The mollusc-arthropod pleated septate junction is also different in having furrows on the E face rather than pits. In tangential view the septa of the mollusc-arthropod pleated septate junction do not generally have the side projections from the apices of their pleats as is commonly seen on septa of the lower invertebrate pleated septate junction. These projections give the latter junction a more pronounced pleating appearance. None the less these projections are seen occasionally in the mollusc-arthropod pleated septate junction and are presumably able to be utilised in forming the interseptal crosslinking that can be seen in many views. Gilula (1973) and Staehelin (1974) have both suggested that such crosslinking, forming hexagonal subunits or combs, is an optical illusion rather than a real structure. However, the clear crosslinking seen in the lower invertebrate pleated septate junction reinforces the suggestion that such structures could occur in the mollusc-arthropod pleated septate junction, even if at a reduced frequency.

In the Mollusca the pleated septate junction has been shown to occur in many species on the basis of thin section tangential views or freeze-fracture replicas. Table 3.5.2. is derived from the study of published micrographs, and does not solely accept the authors' own conclusions. In cases where the junction is not clearly recognisable it is not listed. This table includes results from the present work
(those species listed with no adjacent reference). Wherever possible, approximate measurements of the periodicity of pleating have been taken from the original micrographs. These figures depend partially on the angle of a section and the clarity of the septa as seen in tangential view.

In addition the pleated septate junction has been reported in a wide range of arthropod species, mainly in the classes Insecta and Crustacea, as noted in table 3.5.3. Again, species listed with no adjacent reference are from the present study and measurements have been made wherever possible of the periodicity of pleating.

Table 3.5.2 demonstrates that the pleated septate junction occurs in tissue of both epithelial and endothelial origin in the four main molluscan classes. The smaller groups Scaphopoda and Monoplacophora have yet to be studied. The same junction also occurs, mainly in tissue of epithelial origin, in the Classes Crustacea and Insecta of the Arthropoda (table 3.5.3).

An exception to this epithelial localisation is the report of its occurrence in the central nervous system of insects (Lane et al., 1977), where it is apparently restricted to the perineurium. There is no definitive evidence for the existence of the pleated septate junction in endothelial tissues in the Arthropoda. They do however occur in some instances in the Malpighian tubules of the cockroach (Dallai, 1976; Wall et al., 1975), Malpighian tubules most commonly have the smooth septate junction (L.F.B. Green, 1978; Dallai, 1976; Noirot-Timothee and Noirot, 1974; Skaer et al., 1979). The fact that both of these junction types have been located in this tissue, even coexisting (Dallai, 1976), is in keeping with the uncertain embryological origin of the Malpighian tubule (Chapman, 1969).

The periodicity measurements confirm the similarity of periodicity
in the lower invertebrate pleated septate junction (16 - 22 nm, chapter 3.4) and in the mollusc-arthropod pleated septate junction (17 - 23 nm).

The smooth septate junction was first described by Noirot and Noirot-Timothee in 1967 and has since been reported in many arthropod species (table 3.5.4). It was initially called the 'continuous' septate junction or zonula continua, but was renamed the 'smooth' septate junction by Flower and Filshie (1975). Table 3.5.4 is derived from the study of published micrographs. While many workers claim smooth or continuous septate junctions are occurring wherever septa do not readily appear in cross section, this table only lists works in which the junction can be reasonably recognised on the basis of thin section tangential views or freeze-fracture replicas. In addition this table includes species studied in the present work; these are listed with no adjacent reference.

There are no differences in the occurrence of the smooth septate junction when similar tissues of the two major arthropod classes, Insecta and Crustacea, are compared. Apart from the midgut and its immediate appendages (all of endothelial origin), it has only been located clearly in the Malpighian tubules of the cockroach and glow-worm (table 3.5.4), and the posterior caeca of an amphipod crustacean (subclass Malacostraca, Graf, 1978). The smooth septate junction is therefore basically a feature of arthropod tissue of endothelial origin.

The mollusc-arthropod pleated septate junction and the smooth septate junction have the same sort of tricellular structures as reported in the three coelenterate septate junctions and in the lower invertebrate pleated septate junction (chapters 3.3, 3.4). Both junction types have crosslinks with circular centres in their tricellular regions despite the fact that the smooth septate junction has wide solid septa in comparison with the other previously described junction types (Figure 12b for the mollusc-arthropod pleated septate junction; Noirot-Timothee
and Noirot, 1980, for the smooth septate junction).

It is unfortunate that so much work has been done on molluscan and arthropod tissues to the neglect of other phyla. This has been responsible for considerable misunderstanding of the septate junction. It has often been argued that the smooth septate junction and the pleated septate junction are different junctions with different functions (see review by Staehelin, 1974). In fact the large variety of septate junction types now described indicates these two junctions are merely variations of one type, the septate junction.
<table>
<thead>
<tr>
<th>Class</th>
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<th>Species</th>
<th>Tissue</th>
<th>Evidence (freeze-fracture) or Plating in nanometres</th>
<th>Reference</th>
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Notes:
1. Evidence: Freeze-fracture or plating in nanometres.
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Table 3.5.2: The occurrence of the plotted species junction in the phylogeny

(resource: [1976])

Note: The table above is a representation of the occurrence of plotted species junctions in the phylogeny. The table includes columns for the name, common name, order, class, and species of each species. Each species' occurrence is indicated by a reference number.
Note 1: Periodicity measurements not possible as an incorrect magnetization is apparently given.

Table 3.3 (continued)
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3.5.6 Discussion (Tilting stage electron microscopy)

The term 'continuous septate junction' was proposed by
Noirot and Noirot-Timothee (1967) because this junction often appears
in positively stained cross sections to have an intercellular
space completely filled with a dense granular material with no indication
of septa. However, as these workers now note (Noirot-Timothee and
Noirot, 1980), this was an unfortunate label as later studies did reveal
septa in cross section views (Flower and Filshie, 1975; Hudspeth and
Revel, 1971; Satir and Gilula, 1973). The junction was subsequently
renamed the 'smooth septate junction', a reference to the appearance of
the septa when seen in tangential view (Flower and Filshie, 1975).

None the less several workers have separated this junction from the
pleated septate junction according to the appearance or non-appearance
of septa when the junctions are viewed in cross section (Baskin, 1976;
Noirot-Timothee and Noirot (1980) claim that the adjacent membranes of
the smooth septate junction also run exactly parallel whereas in the
pleated septate junction they are often scalloped in cross section views
(see also Caverney and Podgorski, 1975). However, this is not always
an obvious identifying feature and a lack of membrane scalloping does not
mean that the junction being viewed is not a pleated septate junction
(figure 17, for example). A scalloped membrane can also be indicative
of the lower invertebrate pleated septate junction (figure 9a).

Even an ultrathin section of about 45 nm thick is three times the
width between membranes which are spanned by a septate junction. The
angle at which septa pass through a section will therefore influence the
resulting image.

Tilting the smooth septate junction from a point where septa are
clearly visible produces the uniform grey dense intercellular material
as described originally by Noirot and Noirot-Timothee (1967). Conversely, it is possible to tilt sections showing the uniform greyness until septa become visible. Within the range of the apparatus used it was only possible to locate septa at one angle; when they were vertical to the electron beam (i.e. perpendicular to the section). These results are shown diagrammatically in figure 18. Clearly the appearance or non-appearance of septa is simply a result of the angle of the septa within a section and it is not a useful means of classifying septate junction types. Similar results were also noted for the Hydra type septate junction, another straight septum junction.

On the other hand, the appearance of septa in thin sections of the pleated septate junction when it is viewed from at least two angles confirms that reinforcement of images does occur. The angle between points at which septa were clearest varied between 50° and 80°. These measurements correspond approximately to the complementary angle of the 100° to 130° angle of pleating. This has been diagrammatically represented in figure 19. In this diagram the junction is drawn as seen in tangential view with adjacent septa drawn out of phase so that their alternate pleat apices come together. The occurrence of out of phase pleating is a common situation observed in tangential sections, although in phase pleating and intermediates also occur (Noirot-Timothee and Noirot, 1973). It appears to be a favoured configuration as it allows crosslinking to occur.

The diagram has a scale such that 1 mm represents approximately 1 mm. The periodicity of the pleating (drawn as 20 mm) is 20 nm, and the septa are drawn as if about 2 mm wide. The gap between septa is drawn so that crosslinking, if it occurred, would result in symmetrical hexagons. This gap and the angle of pleating may vary in tangential views of the junction however. The angle of pleating is drawn as about
120° and from this it is possible to calculate the total width of the septa to be about 5 nm.

\[
\text{Width} = \frac{\sin (\text{compliment of } 120°)}{\text{Half the periodicity}} = \frac{10 \sin 30°}{2} = 5 \text{ nm}
\]

This corresponds well with the measured width from micrographs of about 6 nm when the thickness of the septa is taken into account (figure 12a). On the basis that the section drawn is about 70 nm thick, it can be seen that only 3½ pleats per septa will occur within the depth of the section.

The diagram shows three possible angles at which septa might be visible in cross sections. A and C represent angles in which reinforcing of pleats occurs, while B is the view obtained when looking along the septa. If 'septa' were visible when looking along their length (view B) and when reinforcing each other (views A and C), three angles at which they can be seen in tilting sections should be recorded. When a section is tilted, the angles between these points should be only 25° to 35°, half the complimentary angle of the pleating. Since the only angles recorded were approximately double this figure (50° to 80°), it means that 'septa' of the pleated septate junction as seen in cross section view are in fact difficult to visualise individually (looking along their lengths). They are more readily seen when viewed as reinforced stacks.

The scale diagram illustrates why this is so. It can be seen that viewing sections with septa perpendicular to the section (view B) will reveal broad septa 6 nm or more wide. Such septa will be difficult to discern due to the relatively small depth of uninterrupted septal structure through which the electron beam must pass compared to the total
depth of the section. A maximum of less than half the depth of the section will consist of septal material. More importantly, contrast between the septa and interseptal spaces will be poor because the septa 'fade in' and 'fade out' as progressively less septal material is viewed toward the upper and lower limits of the 6 nm wide septal strip.

Where reinforcing of septa occurs (views A and C), the depth of septal structure through which the electron beam must pass can be more than half the depth of the section although it may be only about one third of the section depth in some places depending upon where the 70 nm thick section occurs in relation to the pleating. However, view A or C will result in greater contrast between the 'septa' and the interseptal space than would occur in view B. This is because septa are being viewed along most of their length with a sharp cut off between them and the interseptal space (bars on figure 19a).

Furthermore, the width of the 'septa' seen in views A and C can be as narrow as the septal core itself, down to 2 nm where pleats align perfectly. This compares favourably with published results that show septa vary from 2 - 9 nm in width (Noiro-Timothee and Noirot, 1980). The lower half of this range of widths could not be seen when looking down the length of the septa (view B) which have a minimum total septal width of 6 nm. Slight tilting either side of views A or C will reveal slight widening of the septa, but with a proportional loss of contrast in the section. Further tilting of the section will increase the width of the septa seen and the loss of contrast until they are no longer discernable.

Where such reinforcement of septa occurs, septa will appear regularly spaced in cross sectional view. The spacing between septa will be dependent upon the periodicity of the pleating (and hence the angle of pleating) and the distance between septa. This regularity of the septal spacing is a commonly observed feature of the pleated septate junction (Noiro-Timothee and Noirot, 1980).
It is thus interesting that in the original description of the smooth septate junction (Noirot and Noirot-Timothee, 1967) it was claimed no septa were seen in cross sections of that junction while they were readily seen in cross sections of the pleated septate junction. In fact the converse is more correct. When septa like structures do become visible in cross sections of the smooth septate junction they do at least represent individual septa viewed along their length whereas 'septa' seen in cross sections of the pleated septate junction are more likely to represent several reinforcing septa viewed at an angle.
Figure 18:

Figure 18a is a three dimensional representation of a thin section of a smooth septate junction. Even in a very thin section of 45 nm depth as drawn here, the depth of the section is three times the width of the gap between membranes crossed by the septa. The angle at which the septa lie relative to the plane of the section will influence the image seen in the electron microscope. If the septa are at right angles to the plane of the section then they will be viewed along their length in the section (arrows A). In this case the image seen on the microscope screen will be of a ladder like structure as represented in figure 18b. On the other hand if septa are at an angle within the thin section they will be viewed more from the side (arrows B), and the resulting image seen will be of a uniform greyness between membranes as represented in figure 18c. This diagram is further discussed in chapter 3.5.6.
Figure 19:

A diagrammatic representation of a pleated septate junction in tangential view. The scale of this diagram is approximately 1 mm : 1 nm. In cross sections the septa can be viewed from a range of angles but a ladder like image would be possible only in views A, B and C. These views are drawn as if looking along or across the septa from the side of the page. This diagram is fully discussed in chapter 3.5.6.
3.6 The Phylum Echinodermata

3.6.1 Introduction

Previous work on echinoderm intercellular junctions is limited, and probably refers only to epithelial tissue junctions (Gilula, 1973; Wolpert and Mercer, 1963; Wood, 1977). None of these studies have described the structure of the echinoderm septate junctions in any detail.

In the present study two previously undescribed septate junctions have been found in the Echinodermata. The first of these consists of straight double septa and has been found in epithelial tissue of all classes except the Crinoidea which have not been studied. This junction has been called the 'echinoderm double septum septate junction'. The second echinoderm junction is apparently restricted to endothelial tissues and is an anastomosing septate junction where the septa form a mesh-like structure between adjacent cells. This junction has been found in the classes Asteroidea (Starfish), Echinoidea (sea urchins) and Holothuroidea (sea cucumbers).

Freeze-fracture replicas of the echinoderm double septum septate junction in this section of work were made using uncryoprotected tissue. This was necessary as glycerination of the tube feet being studied caused complete contraction resulting in wrinkling of the cell membranes. This in turn causes intracellular rather than intramembrane fractures to occur. Tissue was relaxed in magnesium sulphate (1000mOsm) before freezing.

3.6.2 Results

The Echinoderm double septum septate junction:

The echinoderm double septum septate junction has been studied in the ectoderm of tube feet from Coscinasterias calamaria and Patiriella regularis (Asteroidea), Ophionereis fasciata (Ophiuroidea), Euechinus
chloroticus (Echinoidea) and Stichopus mollis (Holothuroidea).

Cross sections from lanthanum impregnated tissue show septa spanning a 15 - 18 nm intercellular space (figure 20a). Close inspection reveals that each septum is a double structure which crosses the entire intercellular space. Tangential views of the junction reveal septa consisting of straight lines following a course between cells that is generally parallel to each cell apex (figure 20b). Again, closer examination reveals that septa have a double structure, each half of which varies between 2 and 5 nm wide (figure 20c). The two halves can be touching or have a space between them up to 3 nm wide, and as a result the total width of each double septum varies between 7 and 12 nm. There appears to be no correlation between total septal width, the width of the two component halves of a septum and the gap between these halves. In some areas for example the gap between the two parts of a septum can be greatest when the width of the component halves is least (arrow, figure 20c), while in other areas the component halves can be at their greatest width (double arrow, figure 20c). This lack of correlation between these varying factors gives the septa an irregular appearance.

Septa can often be seen to terminate within a tangential field of view, and while they mainly follow a circular course around cells, they often become less orientated at the lower end of the junction (the end away from the lumen) (figure 21a). In some areas this tendency to lose orientation is very marked and the septa appear as a conglomerate of short randomly arranged pieces (figure 21b).

There are no interseptal structures such as the pegs seen between septa of the arthropod smooth septate junction, and no side projections from the septa. In this study it has not been possible to discern structures in the tricellular region of the junction.

The freeze-fracture appearance of the echinoderm double septum
septate junction following fixation is somewhat unique in that the intramembrane features revealed by this technique cannot be correlated with the intermembrane septal structures as seen in thin section tangential view. The freeze-fracture appearance of the junction is of a broad band around the apical circumference of each cell varying between 0.8 and 2.5 μm in width. The edge of this band nearest the lumen is always distinct, the junctional structure starts abruptly (figures 21c, 22a). The lower edge of the junction is also usually seen to end abruptly, (figure 21c, 22a) but in some places junctional particles are seen dispersed in other regions of the cell membrane below the main junctional band itself (figure 21d). These areas presumably correspond to regions seen in tangential views where septa show a random orientation (figure 21b).

The E face of the junction consists of a scattered array of irregularly shaped particles 5 to 30 nm in length (figures 21c, 22a). The particles in no way appear to line up in rows as would be expected after viewing tangential sections of the junction. The P face is characterised by a randomly dispersed band of shallow complementary pits (figures 21c, 22b). Particles left adhering to the P face are generally small (5 - 15 nm), single and rounded, compared with those of the E face. The larger size and longer particles seen on the E face are seldom observed adhering to the P face.

In contrast, replicas of unfixed (and uncryoprotected) tissue reveals extremely prominent junctional structures (figure 22c). The E face again has particles on it, but in unfixed tissue they stand out clearly and align in rows (figures 22c, 23a, 23c). Particles vary between 6 and 25 nm in length and are 6 - 15 nm wide. The P face is characterised by prominent grooves (figures 22c, 23b). Although membrane particles are seen between these grooves there appear to be very few, if any, junctional particles left adhering to this face. The P face is occasionally seen to have
closely packed stacks of short parallel grooves (figure 22c) or short finely cross striated grooves (figure 23b). The cross striated grooves appear to lie randomly, not aligning with the septa. No comparable intermembrane structures were ever seen.

In most cases the junction starts and ends abruptly (figure 23c), with junctional structures (grooves or particles) in rows as expected after viewing tangential views. In some cases however the junctional particles tend to show a slightly random orientation somewhat reminiscent of that seen in fixed tissue replicas (figure 23d). This tendency to apparent randomness is usually more obvious in lower areas of the junction (figure 23d) and probably corresponds to the random orientation of septa in these areas seen in some tangential views (figure 21b).

The echinoderm anastomosing septate junction:

The echinoderm anastomosing septate junction has been found in the pyloric caeca of Coscinasterias calamaria (Asteroidea), the stomach of Evechinus chloroticus (Echinoidea) and the alimentary canal of Stichopus mollis and Trochodota dendyi (Holothuroidea). Endothelial tissue of Crinoidea and Ophiuroidea have yet to be studied.

The echinoderm anastomosing septate junction has the intermembrane spacing of 15 - 18 nm (figure 24a) common to previously described septate junction variations. In cross sections that are positively stained the septa are not clear (figure 24a). This is due to the anastomosing which causes the spacing structure between membranes to be sectioned at many different angles in any one stretch of junction. An anastomosing compartment is on average about 32 nm in diameter and with thin sections of even 50 - 60 nm, more than one compartment at a time is viewed, resulting in septa at many angles to the plane of section. The junction extends downward to about 0.55 μm from the luminal edge of the cells with only a desmosome, seen in thin section, above it (figure 24a).
Using lanthanum tracer the anastomosing structure is seen clearly in tangential section. In some areas the septa run considerable distances without crosslinking (figure 24b), but in most cases the tangential appearance is that of an intricate network pattern of far greater extent than the usual vertebrate tight junction (figures 24c, 24d). The septa, generally 6 - 8 nm wide, have a pleated appearance with an irregular periodicity. This periodicity is difficult to measure, however, because of its irregularity and unevenness (figure 24e). The anastomosing compartments formed by the septa show considerable variation in size, although the majority are 30 - 35 nm wide (figures 24c, 24d). In places bars are seen branching from the septa (figure 24e). The lanthanum tracer has penetrated throughout the junction, including the enclosed spaces of the network system implying that, in fixed tissue at least, the actual septa are permeable to tracers. The tricellular region of this junction appears to consist of fine linking structures with a circular core centre (figure 25a).

In freeze-fracture of fixed material the junction appears as a pattern of mainly individual particles about 7.5 nm in diameter on the P face (figure 25b). The particles are of a regular size and shape. The pattern appears anastomosing, but it would be difficult to recognise as such without previous knowledge of the appearance of the junction in tangential sections. On the E face an occasional particle is left adhering within a fine network of shallow pits and short grooves (figure 25c). The pits have a diameter of about 4 - 5 nm. Networks on the two faces are continuous with rows of particles on the P face seen to line up with rows of pits on the E face where a change of face occurs (figure 25d). Freeze-fracture of unfixed material shows an almost identical pattern; a network of particles 7 - 8 nm diameter on the P face and the E face showing an anastomosing network of fine 4 - 5 nm diameter pits with the
occasional particle left adhering. In the unfixed tissue however, the number of particles left adhering to the E face is very small when compared with the E face of fixed tissue, and the pits are more distinct and clearly separated, not forming grooves as in fixed tissue (figure 25e). In both fixed and unfixed tissue, the particles seen in freeze-fracture presumably correspond with the septa in the intermembrane space.
Figure 20:

(a) A cross section view of an echinoderm double septum septate junction in a starfish tube foot following lanthanum impregnation. The septa are seen in negative contrast crossing a 15 - 18 nm intercellular space (arrows). They are often seen to be double structures crossing the entire intercellular space (double arrow). x 117,000.

(b) A tangential view of an echinoderm double septum septate junction in a starfish which has been impregnated with lanthanum tracer. The septa are seen as straight lines (not pleated) following a course around cells roughly parallel to the cell apices. x 58,000

(c) Lanthanum impregnated echinoderm double septum septate junction in a starfish thin sectioned to reveal the septa in tangential view. The septa have a double structure, each half of which is 2 - 5 nm wide. The space between the two parts varies. They may be touching (small arrow) or up to 3 nm apart (large double arrow). There is no correlation between the width of the gap and that of the component halves of the septa. In some areas there is a large gap but narrow septal components (large arrow) but in other areas a large gap and wide septal components (large double arrow). The total septal width varies between 7 and 12 nm to give septa a slightly irregular appearance at this magnification. x 140,000
Figure 21:

(a) and (b) Lanthanum impregnated echinoderm double septum septate junction thin sectioned to reveal the septa in tangential view. Septa generally follow a course approximately parallel to the cell apex but often become less orientated toward the lower end of the junction (the end away from the lumen) as seen here in figure 21a from starfish tissue. In figure 21b from a thin section of brittle star tissue, the disorientation of the septa is more pronounced, the septa being reduced to a series of randomly dispersed pieces.

(a) x 90,000  (b) x 105,000

(c) A freeze-fracture replica of fixed starfish tube feet epithelial tissue in the area of a double septum septate junction. The E face shows a randomly dispersed array of particles and the P face a complementary array of pits. The junction appears as a broad band starting and ending abruptly. x 70,000

(d) A freeze-fracture replica of the echinoderm double septum junction from fixed starfish epithelial tissue. In this replica the lower end of the junction does not end abruptly, but extends lower down the membrane. The black line indicates the lower limit of the main area of the junctional band. x 90,000.
(a) A replica of the E face of an echinoderm double septum septate junction from fixed starfish epithelial tissue. The junction is seen as a band starting and ending abruptly. Particles are irregularly shaped and vary between 5 nm (arrow) and 30 nm (double arrow) in length. There is no evidence of organisation; the particles do not lie in rows. x 100,000

(b) A replica of the P face of the echinoderm double septum septate junction seen in starfish epithelial tissue following fixation. The junction consists of randomly dispersed shallow pits. Small 5 - 15 nm long particles are left adhering to this face. x 120,000

(c) A replica of a starfish double septum septate junction freeze-fractured without fixation or cryoprotection. The junctional structures are very prominent. The E face has particles aligned in rows and the P face a series of prominent grooves. Occasionally closely packed stacks of short parallel grooves are seen (arrow). x 70,000
Figure 23:

(a) A replica of the E face of a starfish double septum septate junction freeze-fractured without prior fixation or cryoprotection. The particles, aligned in rows, vary between 6 and 25 nm in length and are 6 - 15 nm wide. x 96,000

(b) The P face of a starfish double septum septate junction freeze-fractured without prior fixation or cryoprotection. The junction is seen as a series of grooves with no junctional particles in them. Finely cross striated grooves (arrow) are also seen. These do not line up with the main grooves of the junction. The junction is seen to start and end abruptly. x 105,000

(c) A freeze-fracture replica of a starfish tube foot showing the E face of an echinoderm double septum septate junction frozen without fixation or cryoprotection. This view clearly shows the tendency for the junctional band to start and end abruptly. The particles are all aligned in rows. x 112,000

(d) A P face replica of an echinoderm double septum septate junction from a starfish freeze-fractured with no previous fixation or cryoprotection. In this case the particles of the P face tend to show a more random configuration reminiscent of that seen on fixed tissue replicas. This tendency is more pronounced in the lower regions of the junction. x 105,000
Figure 24:

(a) A positively stained thin sectional view of the echinoderm anastomosing septate junction in a starfish caecum. The junction has a 15 - 18 nm intermembrane space. Due to the anastomosing of the septa they do not appear as clear bars between the membranes. A desmosome occurs immediately above the junction on the lumenal edge. (lead citrate/uranyl acetate stain). x 70,000

(b) A thin sectional view of a lanthanum impregnated anastomosing septate junction in a starfish caecum showing the septa tangentially cut. Septa have a pleated appearance and occasionally run some distance without anastomosing. x 85,000

(c) and (d). Two tangential views of the echinoderm anastomosing septate junction in starfish caeca sectioned after impregnating with lanthanum tracer. The junction, seen here in tangential view, consists of a network of anastomosing pleated septa and is more extensive than the usual tight junction seen in vertebrates. The anastomosing pattern is seen up to 0.55 μm from the lumenal edge of the cell. The compartments formed by the septa are about 30 - 35 nm wide in most cases. (c) x 85,000 (d) x 96,000

(e) A thin section tangential view of lanthanum impregnated echinoderm anastomosing septate junction in a starfish. The pleated appearance of the junction is irregular and uneven, and in places complicated by bars branching from the septa (arrows). x 120,000
Figure 25:

(a) The tricellular region of an echinoderm anastomosing septate junction in a starfish outlined with lanthanum tracer. The crosslinks between septa on either side of this region are seen to have circular core centres (arrows). x110,000.

(b) A freeze-fracture replica of fixed starfish endothelial tissue showing particles on the P face of an anastomosing junction membrane. These particles are regular in size and shape, being about 7.5 nm in diameter and form an anastomosing pattern. This may not be obvious without prior knowledge of the tangential appearance of the septa. x 96,000.

(c) A freeze-fracture replica of fixed starfish endothelial tissue showing the 4 - 5 nm diameter pits on the E face of an anastomosing junction membrane. An occasional particle is left adhering to this face. In places the pits have joined to form shallow grooves (arrow). x 96,000

(d) A freeze-fracture replica of fixed starfish endothelial tissue showing a change of face in the plane of the fracture. Rows of particles on the P face (P) of the anastomosing septate junction align with rows of pits on the E face (E) (arrows). Unfixed tissue shows a similar appearance with particles on the P face aligning with pits on the E face. x 112,000
Figure 25:

(e) Freeze-fracture appearance of the E face of unfixed starfish junctional membrane in an echinoderm endothelium. The 4 – 5 nm diameter pits forming the network are more distinct and separate from one another than those seen on the E face of fixed tissue fractures. Very few particles are left adhering to this face (arrows). x 145,000
3.6.3 Discussion

The only previously published works on echinoderm septate junctions that reveal any detail of their structure are those of Gilula (1973) and Wolpert and Mercer (1963). Gilula shows the freeze-fracture appearance of sea urchin embryo junctions (*Strongylocentrotus purpuratus* - Echinoidea). His replicas show irregularly sized and shaped particles on the E face and are therefore presumably of the echinoderm double septum junction (the echinoderm anastomosing septate junction has particles on the P face of both fixed and unfixed tissue). He also describes an 'en face' thin section view of long bars and so is again probably referring to the epithelial echinoderm septate junction. The particles seen on freeze-fractures by Gilula form rows, rather than being randomly dispersed, and he reports that he has seen grooves on the P face of his replicas. It is probable therefore that he has utilised unfixed tissue for his junctional studies. Wolpert and Mercer (1963) simply show a tangential view of a double septum septate junction in an echinoid after conventional staining. One further mention of echinoderm junctions is that of Wood (1977) who reported that work by himself and Caverney (unpublished) shows that echinoderms (class Echinoidea) have a septate, rather than tight junction.

The present study has however demonstrated that the echinoderm double septum septate junction is present in the Classes Asteroidea, Ophiuroidea, Echinoidea and Holothuroidea, and the echinoderm anastomosing septate junction in the Classes Asteroidea, Echinoidea and Holothuroidea. Both types are thus widespread in the phylum.

Both echinoderm septate junction types have interesting and unique features. The apparent loss of conformity between inter- and intramembrane structures in fixed tissue of the double septum septate
junction is the first definitive evidence that the septa of septate junctions may not necessarily align directly with intramembrane structures. This concept has been suggested previously by Noirot-Timothee et al (1978) but not clearly demonstrated. These workers claimed that in freeze-fractures of the mollusc-arthropod pleated septate junction particle spacing varied within a single row, whereas thin sectioning revealed that the periodicity of septal pleating seen in tangential view did not vary. They felt that their observations did not support structural continuity throughout the entire septate junction, a contrast to gap junction organisation.

It is of special interest that freeze-fracture of unfixed echinoderm epithelium reveals particles in rows presumably aligning with intermembrane septal structures, while in fixed tissue there is a loss of correlation between these structures. This implies that while fixation appears to preserve the basic intermembrane septal structure, it can cause not only dislocation of septa from their associated intramembrane structures, but also movement of these intramembrane structures within the membrane. This fact has far reaching consequences in our interpretation of other freeze-fracture results as it implies that considerable translocation of membrane features is possible during the fixation process.

The echinoderm anastomosing septate junction has the 15 nm intermembrane spacing characteristic of septate junctions, and in freeze-fractures of fixed and unfixed material, particles rather than continuous ridges are seen. However, the anastomosing network is similar in appearance to that of the vertebrate tight junction. This junction is apparently a structural intermediate between the septate junctions of invertebrates and the tight junctions of vertebrates. In view of the frequently cited relationship between the Echinodermata and the Chordata
(see review by Berrill, 1955) it is interesting to note the presence within the echinodermata of such a structural intermediate. Because of the functional importance of this apparent intermediate junctional type, a diagrammatic interpretation of the anastomosing septate junction is given in figure 26.

The anastomosing septate junction has a similar particle structure on freeze-fracture replicas to the pleated septate junction of the molluscs and arthropods; both junctions have particles of regular size and shape (chapter 3.5). Both junctions also have the majority of particles adhering to the P face of both fixed and unfixed material. This similarity of fracturing properties, similarity of particle size and shape, and the pleated appearance of both of these junctions may well indicate a relationship between them. Embryological evidence indicates that the echinoderm and chordate lineage, the Deuterostomia; and the annelid, molluscan and arthropod lineage, the Proterostomia; arose independently from a common coelomate ancestor (Berrill, 1955). The organisms possessing pleated septate junctions and those possessing anastomosing septate junctions could thus share only a remote common ancestry (see chapter 4.3).
Figure 26:

A diagrammatic representation of the echinoderm endothelial tissue anastomosing septate junction. This diagram shows the pits seen on the E face (E) and the particles seen on the P face (P) after freeze-fracturing. It also shows an interpretation of how septa are constructed based on lanthanum tracer impregnated tissue thin sections and positively stained thin sections.
3.7 The Phylum Hemichordata:

3.7.1 Introduction

The Hemichordata is a relatively small phylum, but is of considerable interest phylogenetically when considering the possible transitional states between present invertebrate and vertebrate phyla. The hemichordates are the lowest animal group to possess a notochord and a hollow dorsal nerve cord. No previous studies of junctions in the phylum have been published.

In this phylum two more variations of the septate junction have been found; one of these in tangential views appears complicated and semi-anastomosing. This anastomosing junction which occurs in several tissues of the hemichordate Balanoglossus australiensis is of particular interest in that it exhibits features in common with the echinoderm anastomosing septate junction (chapter 3.6). It is termed here the 'hemichordate anastomosing septate junction' and is apparently restricted to endothelial origin tissue.

The second type of septate junction found in this phylum is a type of stacked, double septum septate junction. This second type is also common in Balanoglossus australiensis where it is apparently restricted to tissue of epithelial origin. This study has yielded little information about this junction, but it is described here because it has similarities to the septate junction described by Duvert (in press) in the intestinal wall of Sagitta setosa (phylum Chaetognatha), a point that is taken up in the discussion section of this chapter.

3.7.2 Results

The hemichordate anastomosing septate junction:

The hemichordate anastomosing septate junction has been found in tissues which line the inside of the collar, genital pleurae and gut, and the outside edge of the probosis of Balanoglossus australiensis. This junction surrounds the apical end of lining cells in these tissues, stretching from just below a desmosome at the luminal
end (figure 27a) to a depth of at least 400 nm (figure 27b). It spans a 15 nm intermembrane space (figure 27a, 27c), but in cross section of positively stained or lanthanum impregnated tissue it is difficult to discern any identifying features due to the anastomosing of the septa (figures 27c, 28a). Because of this anastomosing septa, or parts of septa, are sectioned at many angles within the depth of a thin section.

In tangential views of lanthanum impregnated tissue junctions, septa are seen to consist of a complicated arrangement of circles, semicircles or parts of circles, and short rods (figures 27c, 28a). These structures are often linked so that considerable areas of junction can actually consist of one large complex piece of septum. The circles often have a central core which varies in size from less than 3 nm in diameter up to about 12 nm (figure 28a). The circles themselves, when complete, are all a relatively constant size compared with the variation of size seen in the anastomosing compartments of the echinoderm anastomosing septate junction (chapter 3.6). The inside diameter of these circles is 9 - 20 nm with the septa in general being 6 - 10 nm wide. Occasionally septal pieces only about 3 nm wide are observed (figure 27c). Septa have a rough edged construction which gives them a semi-pleated appearance. There is no regular periodicity to this particular septal modification however (figure 28a). Some junctional areas have a high density of intermembrane structures (A in figure 27c), while some areas appear less dense (B in figure 27c). Other areas of the junctional region can be clear of any apparent intermembrane structures (figure 28a).

It has not been possible to recognise any clear structure in the tricellular region of this junction; it may well be very complex due to the anastomosing structure of the septa (figure 28b).

Freeze-fracture of fixed tissue reveals a dense array of particles on the P face of the replica (figures 28c, 28d). The density of particles
shows variation with an extremely dense particle accumulation on some membranes (figure 28d) and a less obvious density on others (figure 28c). The particles apparently correspond with the intermembrane structures seen in tangential views, with the small clear patches of membrane (where there are no particles) probably corresponding to the areas between the intermembrane septal structures. These clear patches are relatively large compared with the diameter of the intermembrane circle structures, so do not correspond with the centres of these. The areas of lower density particle distribution possibly correspond with areas between cells such as B in figure 27c, whereas the higher density particle distributions might correspond with areas such as A in figure 27c. The particles are a regular size and shape, being rounded and mostly 7 - 8 nm in diameter (figures 28c, 28d).

The E face of the junction has a complementary array of pits with the occasional particle left adhering (figure 29a). The pits show the same arrangement as the particles of the P face with a dense distribution separated by small, clear areas of membrane. Larger areas of clear membrane on the E or P face (e.g. area seen on the E face of figure 29a) appear to correspond to areas between cells similar to that indicated on figure 28a.

The very soft texture of Balanoglossus tissue causes problems in handling, consequently no replicas of unfixed tissue which show junctional detail adequately have been obtained.

The hemichordate double septum septate junction:

The hemichordate double septum septate junction has been found in positive stained thin sections and in thin sections of lanthanum impregnated tissue from the superficial epithelium of the trunk, collar and genital pleurae of Balanoglossus australiensis. It has thus only been found in epithelial origin tissue. It is not as easily found as
the hemichordate anastomosing septate junction, probably because the septa are of light construction and very thin.

In cross section the junction has a 15 nm intercellular spacing with a desmosome between it and the lumen (figure 29b). Septa are difficult to see in cross section views. In tangential view, the junction is seen to consist of stacks of three to eight straight septa in many areas (figures 29c, 30a). Often whole stacks of septa are seen terminating (figure 29c) suggesting that septa may not run for long distances. Single septa can also terminate within a stack (figures 29c, 30a). In other areas the junction consists of a more random distribution of septa, but they are almost inevitably paired (figures 30b, 30c, 30d), even though there may be odd or even numbers of septa seen in the stacked arrangements (figures 29c, 30a). The paired septa have a slightly undulating gap 3 - 6 nm wide between them (figure 30b), but in some places the gap appears thinner where the twin septa have 'pinched' together (figure 30c). In other places the halves of a septal pair flex apart (figure 30b). The slight undulations in the width between septal pairs gives them a faintly pleated appearance in many areas (figures 29c, 30d).

The actual septa, whether stacked or paired, are 2 - 5 nm wide. In most tangential views they appear as solid thin lines (figures 29c, 30b, 30c), but close inspection reveals that they have a substructure which consists of rows of dots about 2 - 4 nm in diameter, although slightly oval with the long axis across the septa (figures 29c, 30d, 30e). Where the dots are discernible as individual structures, probably due to the angle of the section in relation to them, they can give septa a serrated appearance (figure 30e).

The very soft texture of the Balanoglossus tissue caused handling problems and no freeze-fracture replicas of this junction, which occurs around the outer edge of the animal, have been obtained.
Figure 27:

(a) A positively stained cross sectional view of the hemichordate anastomosing septate junction in *Balanoglossus*. The junction spans a 15 nm intermembrane space but it is difficult to discern clear features because the anastomosing septa are cut at many angles within the section. A desmosome is seen between the junction and the luminal edge (lead citrate/uranyl acetate stain). x 90,000.

(b) A thin sectional view of the hemichordate anastomosing septate junction in *Balanoglossus* following lanthanum impregnation. Junctional structures can be identified some 400 nm from the luminal edge. The lumen is just visible in the top left hand corner of the micrograph (arrow). x 30,000

(c) A thin section of *Balanoglossus* endothelial tissue which has been impregnated with lanthanum tracer. The hemichordate anastomosing septate junction is seen in tangential view and consists of a complicated arrangement of circles, semicircles parts of circles and short rods. These structures are often linked so that considerable areas of junction can be one large piece of septum. Septa are generally 6 – 10 nm wide although some thin 3 nm wide pieces are visible (arrows). Some areas of the junction have a high density of junctional structures (A) while others appear less dense (B). Junctional structures span a 15 nm intermembrane space (double arrow). x 90,000
Figure 28:

(a) A thin section of lanthanum impregnated Balanoglossus tissue showing the anastomosing septate junction seen in tangential view. Septa consist of circles, parts of circles and short rods. The circles when complete have a relatively constant size with an inside diameter of 9 - 20 nm. They often have a central core varying from less than 3 nm in diameter (small arrow) to about 12 nm in diameter (small double arrow). The septa are rough edged. Some areas of the junctional membrane are free of junctional structures (large arrow). x 140,000

(b) A tricellular region of the hemichordate anastomosing septate junction outlined with lanthanum tracer. No obvious identifying features are visible and it may well be very complex in this area due to the anastomosing of the adjacent septa. x 130,000

(c) and (d) Freeze-fracture replicas of the P face of the hemichordate anastomosing septate junction in Balanoglossus after fixation. The P face has a large number of regularly sized and shaped particles 7 - 8 nm in diameter. Some areas of the membrane have a high density of these particles (figure 28d) possibly corresponding to a high density of intermembrane structures such as seen in area A on figure 27c. Other areas of the membrane have a lower density of particles (figure 28c) possibly corresponding with intermembrane areas such as B marked on figure 27c.

(c) x 100,000 (d) x 135,000
Figure 29:

(a) A replica of the E face of a hemichordate anastomosing septate junction in *Balanoglossus* freeze-fractured after fixation. This membrane face is seen to have a large number of pits on it with the occasional particle left adhering. Large clear areas (arrow) possibly correspond to intermembrane areas such as that indicated on figure 28a. x 120,000.

(b) A cross sectional view of the hemichordate double septum septate junction in *Balanoglossus* after positive staining. The junction spans a 15 nm intercellular space with a desmosome between it and the lumen. Septa are difficult to find in such cross section views. (lead citrate/uranyl acetate stain). x 100,000

(c) The hemichordate double septum septate junction viewed tangentially. In this thin section of lanthanum impregnated *Balanoglossus* tissue, septa are seen in stacks of three to eight in many areas. Whole stacks of septa are seen terminating (small arrow) suggesting that septa only run for short distances. Single septa can also be seen terminating within a stack (double arrow). In other areas the septa run as pairs (large arrow), often with a slightly pleated appearance. x 65,000
Figure 30:

(a) The hemichordate double septum septate junction viewed in tangential thin section. In this view of lanthanum impregnated Balanoglossus tissue, septa are seen in stacks of three to eight. Single septa can be seen terminating within a stack (arrow). x 120,000

(b) and (c) and (d). Tangential views of the hemichordate double septum septate junction in Balanoglossus thin sectioned after lanthanum impregnation. In these views most septa show a more random configuration than most of those seen in figure 29c and 30a. The septa are almost inevitably paired with an undulating 3 - 6 nm gap between them. In some places the gap appears less where the twin septa have pinched together (arrow on figure 30c) and in other areas the two parts flex apart (arrow on figure 30b). The undulations in the space between the two parts of a twin septum often give it a slightly pleated appearance (figure 30d).

(30b) x90,000 (30c) x 120,000 (30d) x 120,000

(e) Lanthanum impregnated hemichordate tissue in Balanoglossus thin sectioned to reveal the hemichordate double septum septate junction in tangential view. Septa are 2 - 5 nm wide and close inspection reveals they have a particulate substructure. They appear as a row of dots 2 - 4 nm in diameter, although slightly elongated with their long axis across the septa (arrows). The dots tend to give septa a serrated appearance when they are not individually discernible (double arrow). x 120,000
3.7.3 Discussion

The two types of junction found in the Hemichordata are so far unique to this phylum. They are however of interest in that they show close resemblances to septate junctions previously described from phyla which are often held to be closely related.

At first sight the occurrence in Hemichordata of an anastomosing septate junction of the type described with complex circles and rods is difficult to interpret. In the echinoderms an anastomosing septate junction is found that in many features resembles the vertebrate tight junction. It could be expected from conventional phylogenies that the hemichordates would show a similar junctional pattern to that of the echinoderms. However, the hemichordate anastomosing septa do share the same pleated, rough edged appearance reported in the echinoderm anastomosing septate junction. In addition, both junctions share the rounded, evenly sized and regularly shaped particles on the P face of fixed tissue freeze-fracture replicas. It is therefore possible that the only real difference between the two junctions is in the final configuration which the septa take, not in the structural makeup of septa. The occurrence of a junctional configuration in the echinoderms which is similar to that of the vertebrate tight junction and the absence of such a configuration in the hemichordates perhaps gives some weight to the suggestion that the latter do not lie on a direct line of evolutionary descent between echinoderms and vertebrates, but rather represent a separate development from common ancestry (see chapter 4.3).

The hemichordate double septum septate junction varies from the epithelial double septum septate junction reported in the echinodermata (chapter 3.6) in that the component halves of the twin septa are distinct, separate entities rather than appearing as if a splitting of a single septum. It varies from the double septum septate junction of
the class Anthozoa (chapter 3.3) in that the septa have no side projections and are not as straight or evenly spaced as those of the anthozoans. In fact, the hemichordate epithelial junction most closely resembles that described by Duvert (in press) in the intestinal wall of *Sagitta setosa* (phylum Chaetognatha).

*Sagitta* does not have the stacked septal arrangement of the hemichordates double septum junction, but it does have a similar double septal arrangement. In *Sagitta*, septa are about 3 mm wide, the same width as those of the hemichordate junction, and they run in 'loose' or 'tight' formations. When these septa run in a 'tight' formation they are 4 - 5 mm apart, similar to the 3 - 6 mm gap between the paired septa of the hemichordate septate junction. The hemichordate junction in some views also has slightly pleated septa appearance. This pleating is far less prominent and not as regular as it is in *Sagitta*. In places the hemichordate septa flex apart and in this way are reminiscent of the *Sagitta* junction where septa spread apart to form their 'loose' arrangement. The hemichordate septate junction however differs from that of *Sagitta* in that its septa appear to be made of discrete subunits which do not have interseptal modifications. It is notable also that *Sagitta* has a double septum junction in tissue of endothelial origin while *Balanoglossus* has this type of junction in tissue of epithelial origin. The *Sagitta* septate junction is said to coexist with a mollusc-arthropod pleated septate junction (Duvert, in press).
3.8 The Pycnogonida, Merostomata and Collembola:

3.8.1 Introduction

The Pycnogonida (sea spiders), Merostomata (king crabs) and Collembola (springtails) have been grouped in this chapter for two main reasons. All three groups have structural features which ally them with the Arthropoda but their classification is not definitive. The Merostomata and Pycnogonida are often classed as orders of the Arachnida, and the Collembola as an order of the Insecta (e.g. Borradaile et al., 1963). In other texts, the Pycnogonida are classified as a separate phylum while the Merostomata and Collembola are given separate class status within the phylum Arthropoda (e.g. Oates, 1970). More commonly however the Merostomata, Pycnogonida and Arachnida are all given sub-phylum status (Sub-phylum Chelicerata) within the Arthropoda. The Collembola remain as an order within the Insecta (e.g. Clark and Panchen, 1971). Because of this pervasive uncertainty in classification these groups have been lumped here as members of the phylum Arthropoda. Further classification must await synthetic study of features of development, biochemistry and structure.

The three groups all contain primitive arthropod like organisms and relatively little is known of the junctional structures in any of them. Lane and Harrison (1978) gave a full description of the septate junction found in the midgut of Limulus polyphemus (Merostomata), but no epithelial origin tissue studies have been done on this group. In the Collembola the only available data is that of Dallai (1975), again dealing only with midgut tissue. Results on endothelial origin tissue (gut) of the Pycnogonida are given in this chapter.

The septate junctions of all three groups show similarities that justify grouping them together and which separate them from the pleated or smooth septate junctions previously observed in the Arthropoda (chapter 3.5).
3.8.2 Results

Legs from the Antarctic Pycnoconid *Ammothea clausi* were fixed and transferred to buffer in the field. Gut diverticula were later removed for experimental purposes.

In cross section of positive stained or lanthanum impregnated septate junctions, septa can be seen spanning a 15 nm intercellular space (figures 31a, 31b). In tangential views of lanthanum impregnated junction the septa are seen to consist of bands 5 - 8 nm wide following a wavy course between cells, not unlike those of the arthropod smooth septate junction (figure 31b). Closer examination however shows that septa have a secondary sub-unit construction often appearing cross striated (figure 31c) or even to form a delicate chainlike structure (figure 31d). Short rows of 2 - 4 nm diameter pegs are occasionally seen between septa (figure 31c). These appear as thin lines between septa when they are viewed at an angle such that they overlap one another (figure 31e).

In the tricellular regions, circle centred crosslinks are again apparent as in most previously described septate junctions (figures 31b, 31f). They are usually attached to narrow septa only 2 - 4 nm wide on either side of the tricellular region, rather than directly to the 5 - 8 nm wide septa (figure 31f).

Freeze-fracture of fixed tissue reveals rows of particles predominantly on the E face (figure 32a). These particles are irregular in shape and size, their shape being not unlike those of the lower invertebrate pleated septate junction (chapter 3.4), but following more in single rows than ragged bands. The particles vary between about 3 and 14 nm wide and are up to 22 nm long (figure 32a). They are less obvious than most other septate junction particles however and normal 8 - 10 nm diameter membrane particles stand out prominently in comparison
(figure 32a). In many areas the junction is almost indiscernible
(figure 32b). The P face of this junction consists of grooves that
in contrast can be relatively prominent compared with the size of the
E face particles (figure 32d). The occasional particle is left
adhering in these grooves. In other regions however, as on some regions
of the E face, it is difficult to discern any obvious structures and
in these areas the P face appears to consist of occasional indistinct
particles with small pits between (figure 32c).

As the pycnogonids were collected in Antarctica no unfixed
tissue was available for study.
Figure 31:

(a) A positively stained cross sectional view of a pycnogonid endothelial tissue septate junction. Septa are seen spanning a 15 nm intercellular space. (lead citrate/uranyl acetate stain) x 180,000

(b) The pycnogonid septate junction seen in cross section and tangential view after lanthanum impregnation. In this thin section septa are seen in cross section spanning a 15 nm intercellular space (arrows) and in tangential view as 5 - 8 nm wide bands following a wavy course between cells. It is possible to see circular core crosslinks in the junction's tricellular region (double arrow). x 135,000

(c) A tangential thin sectional view of a pycnogonid septate junction following lanthanum impregnation. In this micrograph septa are seen to have a cross striated appearance. Short rows of 2 - 4 nm diameter pegs are occasionally seen between septa (arrows). x 175,000

(d) A tangential thin section view of a pycnogonid septate junction impregnated with lanthanum tracer. Septa in some areas are seen to consist of fine circular structures forming a chain like septum (arrows). x 220,000

(e) A tangential thin section view of a pycnogonid septate junction impregnated with lanthanum tracer. In this view the angle of the section causes the interseptal pegs (as seen in figure 31c) to appear as thin lines between the broader septa. x 112,000
Figure 31:

(f) The tricellular region of a pycnoconid septate junction outlined with lanthanum. In this micrograph the circular cores of the crosslinks are extremely clear. They join onto a thin 2 - 4 nm wide septum on the side of the tricellular region rather than directly onto the normal 5 - 8 nm wide septa. x 180,000
Figure 32:

(a) A freeze-fracture replica of the E face of fixed pycnoconid tissue in the region of a septate junction. The junction is seen as rows of particles irregular in size and shape. The particles are between 3 nm and 14 nm wide and are up to 22 nm long. The junctional particles are less obvious than normal 8 - 10 nm diameter membrane particles which stand out clearly in comparison (arrows). x 110,000

(b) An E face view of a pycnoconid septate junction following freeze-fracture replication of fixed tissue. In this case the junctional particles are very indistinct. x 78,000

(c) A P face view of a pycnoconid septate junction following freeze-fracture replication of fixed tissue in which the junctional structures are almost indiscernible. The occasional particle is left adhering within rows of small pits (arrows). x 145,000

(d) A freeze-fracture replica of fixed pycnoconid endothelial tissue in which P face junctional structures are clearly seen. These P face features consist of grooves, distinct by comparison with the particles seen on the E face of this junction (figure 32a). x 85,000
3.8.3 Discussion

The septate junction found in the gut of the Pycnogonida most closely resembles that of the midgut of *Limulus polyphemus* (Merostomata - Lane and Harrison, 1978) but also seems similar to that of the midgut of *Orchesella cincta* (Collembola - Dallai, 1975). The septate junctions of all three groups when seen in tangential view are quite distinct from the mollusc-arthropod pleated septate junction, but appear superficially to be similar to the arthropod smooth septate junction in that their septa consist of broad 5 - 8 nm wide ribbons. They also show several differences from the smooth septate junction however.

All three junctions freeze-fracture in such a way that the junctional particles of fixed tissue are seen on the E face, not the P face as is characteristic of most fixed tissue smooth septate junction particles (Flower and Filshie, 1975; Skaer et al, 1979). Skaer et al report that the particles of fixed housefly Malpighian tubule fractures are on the E face also). In addition the pycnogonid particles are hard to discern being far less prominent than most other junctional particles. This feature was also noted by Lane and Harrison (1978) in their studies of *Limulus* and is apparent in the photomicrographs of Dallai's (1975) *Orchesella* work. The particles of the smooth septate junction are prominent and easily seen (Flower and Filshie, 1975; Skaer et al, 1979).

Finally, in all three groups, freeze-fracture reveals particles which tend to be individual rather than being fused into short rods or ridges as they are in the smooth septate junction (Flower and Filshie, 1975; Skaer et al, 1979).

The substructure of septa seen in tangential views of the pycnogonid junction, noted by Lane and Harrison in the merostomata
junction and visible in the collembola junction (see figure 6 of Dallai, 1975), though less apparent, is not generally characteristic of the smooth septate junction. However Skaer et al., (1979) claim that the septa of the smooth septate junction when seen in tangential view after lanthanum penetration do have electron lucent subunits with electron dense cores arranged in a linear fashion. These units therefore appear very similar to those of the pycnogonid septa seen in figure 31d. The pycnogonid, merostomata and collembola junctions all have rows of pegs along side septa in some areas of tangential views, although the merostomata also have in addition more complex interseptal embellishments. In this feature these three junctions again resemble the smooth septate junction.

Lane and Harrison (1978) reported that stacks of short septa are seen in Limulus and are a distinct feature of its endothelial septate junction. Often though, their septa are also seen following wavy lines (see their figures 9, 13, 14 and 15 for example) as they do in the pycnogonida and collembola. Closer study of these latter groups might also reveal stacked septal arrangements in some areas.

It is clear that there is considerable room for further study amongst the groups that make up the proterostome line of development. There has been no work done to date on the class Arachnida, and more work on the Pycnogonida to allow better comparison with the merostome work of Lane and Harrison (1978) would be useful. More work on the Collembola might also clarify their phylogenetic position. In addition studies of some of the other minor arthropod classes could be of interest. Studies to date on tissue of epithelial origin of the Thysanura (bristle tails. Noirot-Timothee and Noirot, 1973) show that this group have the mollusc-arthropod pleated septate junction in that tissue, but no endothelial origin tissues have been studied. Study of the Chilopoda
(centipedes - Juberthie-Jupeau, 1979) reveals that this class has
the arthropod smooth septate junction in its endothelial origin tissue,
but no epithelial origin tissues have been studied.

No tissue of epithelial origin of any of the three groups
discussed in this chapter, the Pycnogonida, Merostomata and Collembola
has been studied.
3.9 The Phylum Tunicata

3.9.1 Introduction

It is in the Tunicata that a tight junction rather than a septate junction is first encountered. This tunicate tight junction is of interest in the present study as it has some features which recall more the invertebrate septate junction rather than a typical vertebrate tight junction. Previous description of the tunicate tight junction was given by Lorber and Rayns (1972) who studied tunicate heart tissue (Asciidiella). Other references to tunicate tight junctions (Cloney, 1972; Kalk, 1970) have indicated their existence on the basis of thin sections only.

The present study has revealed features of the tunicate tight junction not previously noted, but which are significant in relation to evaluating the tunicate position as a group of protochordates.

3.9.2 Results

The tunicate tight junction has been located in a range of tissues from Asterocarpa coerulca. It has been found in the epithelia of the mouth (incurrent siphon), stomach, branchial basket, endostyle and atrial cavity.

In positively stained thin sections, the tunicate tight junction appears as a true tight junction when viewed in cross section: the undulating membranes of adjacent cells fusing together in a series of punctate contacts (figure 33a). The junction forms a belt around the apical edge of cells lining an epithelium and has no desmosome above it on the lumenal edge. No desmosomes were ever located below this junction either. Study of lanthanum impregnated tissue shows that the tunicate tight junction can be penetrated readily by this tracer. In cross section view the tracer appears as a broad line with a series of
constrictions where the membranes fuse together (figure 33b). In tangential view the junction appears as a mesh of lines where lanthanum has been excluded when the cell membranes fuse. The joining lines of fusion appear fine, but in fact vary in width between 3 and 14 nm (figure 33c). The lines of fusion are not distinct and sharp but appear 'furry' edged, probably where lanthanum is excluded to differing extents in 'pinched off' areas (figures 33c, 33d). Where the lines appear sharpest, they are also narrowest and the 3 nm measurement of width is more likely to be that of the true fusion line (figure 33c). Occasionally side bars are seen protruding from fusion lines (figure 33c), but these may not necessarily represent true lines of fusion between the adjacent membranes.

Freeze-fracture of fixed tissue reveals an anastomosing network of particles on the E face (figures 34a, 34b). These particles vary in shape from rounded particles about 5 nm in diameter to being short rods 5 - 8 nm wide, but up to 25 nm long. The P face is characterised by an anastomosing network of shallow pits, which vary in length like the E face particles (figure 34c). These pits have a ragged edge appearance, and are often difficult to distinguish clearly. Unfixed tissue reveals complementary anastomosing networks also, with particles again on the E face (figure 34d), but with distinct grooves on the P face (figure 34e). Discrete E face particles are present but the majority are so close together that long stretches of rough topped ridges are formed. These ridges do not have the smooth appearance of the normal vertebrate tight junction (when fixed), but rather appear as rows of fused particles (figure 34d). The grooves of the P face are readily seen and are continuous. Very rarely do they have particles interrupting them (figure 34e).

In both fixed and unfixed tissues the P face pits or grooves are
often along the top of shallow undulations in the membrane (figures 34d, 34e). In such areas the particles of the E face lie along shallow membrane depressions. This feature is far more apparent in unfixed tissue. In both fixed and unfixed tissue also, a change in the fracture plane from one membrane to another to reveal different faces takes place along the line of fusion (e.g. figure 34b).

Positively stained thin sections, lanthanum impregnated tissue thin sections and freeze-fracture replicas all show that the junction can extend some considerable depth from the apices of the cells. The junction can vary from about 0.3 µm deep (figure 33a) to at least 2 µm in depth (figure 33c). In freeze-fracture replicas it is often possible to count 20 or more fibrils between the lumen and the base of the junction. Ten to fourteen fibrils are common (e.g. figures 34a, 34d).
Figure 33:

(a) A positively stained cross sectional view of the tunicate tight junction. The undulating membranes of the adjacent cells fuse together in a series of punctate contacts (arrows). There is no desmosome either above or below the tight junction. (lead citrate/uranyl acetate stain). x 135,000.

(b) A cross section view of a tunicate tight junction, which has been impregnated with lanthanum tracer. The tracer shows the membranes pinch together at a series of fusion points (arrows) but it has however penetrated the entire junction. x 120,000

(c) and (d) The tunicate tight junction seen in tangential view following lanthanum impregnation. The junction is seen as a meshwork of lines where the lanthanum has been excluded when the cell membranes fuse. Fusion lines vary between 3 nm and 14 nm in width although they are clearest when only 3 nm wide (arrow on figure 33c). Occasionally, side bars are seen branching off the fusion lines (double arrow on figure 33c).

(c) x 60,000 (d) x 70,000
Figure 34:

(a) A freeze-fracture replica showing the E face of a tunicate tight junction following fixation. Particles are seen forming an anastomosing network. The particles vary from being rounded and 5 nm in diameter to being short rods 5 - 8 nm wide but up to 25 nm long. The junction extends some depth along the cell membrane and consists of a large number of fibrils.
  x 95,000

(b) A freeze-fracture replica of fixed tunicate tissue showing both faces of a tight junction. The particles of the E face align with a network of pits on the P face (arrows). Where the change of face has occurred, it has taken place along the lines of fusion.
  x 100,000

(c) A freeze-fracture replica of the P face of a fixed tunicate tight junction. Junctional structures consist of an anastomosing network of shallow pits varying in length like the particles on the E face of such replicas (figure 34a). The pits have a ragged appearance and are often difficult to distinguish clearly.
  x 105,000

(d) Unfixed tissue freeze-fractured to reveal the E face of a tunicate tight junction. An anastomosing network of particles is seen with the particles being very close together so as to form long stretches of rough topped ridges. The particles tend to lie in the bottom of shallow membrane undulations. The junction extends some depth down the cell membrane and consists of many fibrils.
  x 78,000
Figure 34:

(e) A freeze-fracture replica of a tunicate tight junction P face made without prior fixation. The junction is seen as a network of grooves lying along the top of shallow membrane undulations. Particles rarely interrupt the grooves (arrows). x 110,000
3.9.3 Discussion

In all tissues so far studied the Tunicates have a tight junction, a feature characteristic of the Chordates. Lorber and Rayns (1972) briefly described the tunicate tight junction as they saw it in heart tissue and decided that in essential features it was similar to the tight junction of mouse small intestine epithelium as described by Staehelin et al. (1969).

This earlier work can now be expanded and two major features of the tunicate tight junction which separate it from the usual vertebrate tight junction have emerged. Firstly, the particles seen on freeze-fracture replicas of tunicate tissue are on the E face of both fixed and unfixed tissue, in contrast to the more common tight junction configuration where most particles are on the P face of fixed tissue and the E face of unfixed tissue (Staehelin, 1973). Secondly, these junctional structures of the E face always consist of discrete particles or short rods as opposed to continuous ridges. This feature is characteristic of only unfixed tissue for all other tight junctions (Bullivant, 1978). Lorber and Rayns show what appears to be continuous ridges on the E face of unfixed tissue (their figure 4), but at no time were similar results obtained under the conditions used in the present study.

On the other hand thin sections clearly show the membranes fusing together with no intercellular space and an anastomosing network is seen in tangential view thin sections and on freeze-fracture replicas. Furthermore, where freeze-fractures change membranes to reveal alternate membrane faces in adjacent cells, the transition always takes place along lines of junctional fusion as it tends to do in other tight junctions (Bullivant, 1978).

In tangential views results are comparable with those of Goodenough
and Revel (1970), though more extensive junctional arrays have been obtained in the present study. A difference is that the tunicate tight junction was readily penetrated by lanthanum with no special pretreatment of the tissue. Goodenough and Revel were able to cause reasonable penetration of lanthanum tracer into mouse liver tight junctions only after pretreatment with acetone. The fact that lanthanum tracer is able to penetrate untreated tunicate tissue is of interest when the depth of the junction is considered. The number of fibrils in the tunicate junction can be very large (twenty or more) in comparison to the 11 - 14 maximum number of parallel fibrils reported by Claude and Goodenough (1973) in 'very tight' tight junctions such as occur in the mouse stomach or amphibian urinary bladder. If their hypothesis that the number of junction strands can be correlated qualitatively to the transepithelial permeability is correct (see also Claude, 1978; McNutt, 1977) then clearly the tunicate junction is vastly different in its sealing efficiency. Other workers (Martinez-Palomo and Erlij, 1975; Mollgard et al., 1976) have not always agreed with Claude and Goodenough, and have shown that some tight junctions do not show such a correlation. The rabbit ileum mucosa for example has a relatively low transepithelial resistance and is permeable to lanthanum, but it has a network of fibrils resembling that of the toad urinary bladder (Martinez-Palomo and Erlij, 1975). However, the tunicate tight junction does not necessarily fit into this latter category either. It has considerably more strands than the rabbit ileum mucosal junction and the osmotic demands of a marine environment on organisms such as the tunicate can not be as great as those exerted by the quite different physiological solutions bathing the two sides of the cells of the rabbit ileum. The need for the extensive tight junctions in the tunicates if they are as efficient as even those of the rabbit ileum is therefore
questionable.

The ready penetration of lanthanum into untreated tunicate tissue despite the large number of fibrils suggests is may not be a fully developed tight junction, but rather a primitive and less efficient form. It seems likely there is a difference in the efficiency of these junctions at the level of the lines of fusion. The separate particle structure of the tunicate junction revealed by freeze-fracture suggests a possible affinity to the invertebrate septate junction and it may be only in the higher chordates that the tight junction has the structure and degree of efficiency normally attributed to it (see also chapter 4.4).
4. DISCUSSION

4.1 General Discussion

At the time that this study commenced three types of septate junction were generally recognised to exist in invertebrate phyla; the Hydra type, the pleated type and the smooth type (Staehelin, 1974). During the period of the study works by Baskin (1976), Welsch and Buchhiem (1977), Lane and Harrison (1978) and Duvert (in press) have introduced three further variations; the lower invertebrate pleated septate junction, the Limulus septate junction and the chaetognath septate junction. The present study describes six more types of septate junction, two in each of the phyla Coelenterata, Echinodermata and Hemichordata. To date therefore twelve certain variations of septate junction have been described in the Invertebrata and more may well remain to be described from phyla not yet studied fully. In the present study for example, the Pycnogonida, Collembola and Merostomata have been grouped together but further study may differentiate their junctional structures. Several interesting phyla, such as the Pogonophora, Phoronida, Tardigrada and Ctenophora for example, have yet to be studied at all; these may reveal more variations of septate junction structure.

It is of interest that most septate junction studies have concentrated on the three first recognised types (see reviews by Gilula, 1978; Noirot-Timothee and Noirot, 1980; Staehelin, 1974). There has been a tendency to view these junctional types as different junctions with different functions rather than as structural variations of a single junction type, the septate junction. This comment is especially true in relation to the smooth and mollusc-arthropod pleated septate junction which can occur together in some tissues (Dallai, 1976). Skaer et al (1979) and Lane and Harrison (1978) consider that the smooth septate junction is
not analogous to the mollusc-arthropod pleated septate junction since they can occur concurrently. Noirot and Noirot-Timothee (1967) in their original description of the smooth septate junction suggested that this junction might be a feature of regenerating tissue, but as noted by Skaer et al (1979), it also occurs in the Malpighian tubules of several arthropod species where there is little tissue turnover. Following the present study, in which such a large variety of structural forms of septate junction have been found, it is more reasonable to view all forms as being just structural variations of the one junction type. The great majority of invertebrate phyla have two variations of septate junction, one apparently restricted to epithelial origin tissue, the other to endothelial origin tissue. The only exceptions are those phyla which possess the lower invertebrate pleated septate junction, the phylum Mollusca and the class Hydrozoa of the phylum Coelenterata. Viewed in this way the occurrence of both the smooth and pleated septate junctions in the Arthropoda is not remarkable. The concurrent occurrence of these junctions in insect Malpighian tubules (Dallai, 1976) could merely reflect the uncertain embryonic origin of this tissue type. It should be noted however that the chaetognath septate junction is said to coexist with the mollusc-arthropod pleated septate junction (Duvert, in press).

Another problem resulting from previous concentration upon few invertebrate phyla is confusion in terminology. Despite an attempt to clarify terminology (Flower and Filshie, 1975), more recent workers (Dallai, 1976; Lane and Harrison, 1978) still prefer to discuss the 'septate junction' and the 'continuous junction' rather than use the terms 'pleated septate junction' and 'smooth septate junction' proposed by Flower and Filshie. This was due initially to the original 'continuous
junction' terminology of Noirot and Noirot-Timothee (1967), but it persists because of the tendency to view the two types as separate junctions having different functions. When studying earlier works a reader must consequently be careful to determine how the author is applying terminology. Some authors discuss the 'septate junction' meaning all variations of it (e.g. Flower and Filshie, 1975; Noirot-Timothee and Noirot, 1980), other discuss the 'septate junction' meaning only only the Hydra or pleated types (Lane and Harrison, 1978). In this thesis the term 'septate junction' is used as defined in the introduction; as a general term covering all structural variants.

The methods used in the present study have been generally successful but care must be taken in drawing conclusions from results at the level of such a study as this. Freeze-fracture results with the echinoderm double septum septate junction strongly suggest that artifact is possible. While previous studies have shown that cryoprotection treatment of unfixed tissue can cause a degree of artifact (McIntyre et al., 1974), it has not been generally recognised that fixation can cause the translocation of intramembrane structures to the extent noted in echinoderm epithelial tissue. Freeze-fracture of uncryoprotected, unfixed tissue presumably gives results as close to natural condition as presently possible. The fact that under these conditions intramembrane echinoderm double septum septate junction structures align in rows, as do the septa when seen in tangential view, suggests that this is a likely natural state. The completely different view seen after fracturing fixed tissue is significant as it appears that fixation has resulted here in a high degree of intramembrane translocation, which is not explicable as plastic deformation. There is no evidence to suggest a significant disordering of the intermembrane septa themselves during fixation. They are in straight lines as would
be expected from freeze-fracture replicas of unfixed tissue.

The degree to which freeze-fracture replicas reflect real features of a junction can also be questioned when results of Wood (1977) or Skaer et al (1979) are considered. Wood claimed that the Hydra type gastrodermal septate junction fractured with reserved polarity to that which occurred in epidermal tissue. His results have not been confirmed by the present study or that of Filshie and Flower (1977), they might however be a result of his particular experimental conditions. Skaer et al noted that the smooth septate junction of Musca domestica Malpighian tubules fractured with the majority of particles on the E face of both fixed and unfixed tissue, this junction usually fractured in fixed tissue with the majority of particles on the P face. There is no indication from thin section studies as to why this difference exists. Furthermore, as noted by these workers, there are no structures seen in freeze-fracture replicas that are analogous to the interseptal pegs characteristic of this junction type. In the present study the symmetry of the anemone double septum septate junction when seen in tangential view is in contradiction to the asymmetry seen in freeze-fractures of this junction type. This lack of direct correlation in structures has also been noted in the mollusc-arthropod pleated septate junction (Noirot-Timothee et al, 1978) and the chaetognath septate junction (Duvert, in press) in which the spacing of the intramembrane features cannot be correlated with any intermembrane features such as the periodicity in the pleating of these junctions. This contrasts with the anemone double septum septate junction where the spacing between intramembrane particles was similar to the 7.5 nm spacing of the septal pegging and the spacing between particle rows (centre to centre) equalled the 7 nm space between the two halves of the septa.

There is evidence to suggest that lanthanum impregnation of tissue can
result in a larger intermembrane spacing in the region of a septate
defense (Duvert, in press; Skaer et al., 1979). Skaer et al. claim the
smooth septate junction has a 14 - 17.5 nm intercellular spacing in
positively stained cross sectional views but a 17 - 21 nm intercellular
spacing after lanthanum impregnation. While this feature was not
noted in the present study it can not be ignored as a possible source of
artifact in a work such as this which has relied on this technique to a
large extent in describing junctional structures. However, all junctions
in this study have been viewed in as near to similar conditions as
possible and there is no reason to suggest that observed differences do
not reflect real differences in structures.

In all the septate junction types studied the septa span a 15 - 18 nm
interecellular space in positive stained cross sectional views. This is
true despite the large variety of junctional types now recognised.
However it appears that in several cases septa are not solid structures
crossing this space. This is especially true when considering the delicate
structures of the lower invertebrate junctions. This feature was noted
by Hand and Gobel (1972) in the Hydra type septate junction, by Baskin
(1976) in the lower invertebrate pleated septate junction and in the
present study in the anemone double septum septate junction. In addition
the ability of lanthanum tracer to penetrate the enclosed spaces
formed by the anastomosing echinoderm and hemichordate septate junctions
implies that these septa do not form solid 'walls' either, at least
following fixation. The septate junction fractures in a quite different
manner to tight junctions. In the tight junction a change of membrane
face occurring during freeze-fracture always follows the line of the junctional
strands. Even in the echinoderm anastomosing septate junction this feature
is not observed, a change of face occurs independently of the septa,
implying that even though septate junction particles occur within the
membrane they do not represent as great a barrier to intramembrane fractures
as do the ridges of the tight junction. It is possible that the particles seen on freeze-fracture replicas of septate junctions only represent discrete anchoring points for the septa and do not make up any substantial part of their structure. In contrast the particles or ridges of vertebrate tight junction replicas supposedly represent portions of the junction proper within the membrane (Bullivant, 1978).

It is difficult to differentiate between the various septate junction types on the basis of thin section, cross sectional views only. This is possibly due to workers naturally attempting to view septa in true cross section. Baskin (1976) noted that slightly oblique cross section views revealed structures in the lower invertebrate pleated septate junction not seen in true cross section. It is possible that future work may simplify the identification of septate junction types, but at present good tangential views and/or freeze-fracture replicas of both fixed and unfixed tissues are usually necessary.

4.2 The function of the invertebrate septate junction:

The function of the invertebrate septate junction has been extensively discussed ever since its original description by Wood (1959). A full discussion of its function is given by Noirot-Timothee and Noirot (1980) but it is necessary to go over this ground to some extent as several additional relevant points have become apparent in the present study.

The septate junction, in all its forms, must serve some mechanical role in intercellular adhesion. In most invertebrate tissues there is a general lack of spot desmosomes (Noirot-Timothee and Noirot, 1980), a belt desmosome above the septate junction is the only adhesive structure present. This desmosome is very short however and, in the cases of Hydra tissue and most smooth septate junction containing tissues, is absent altogether (Wood, 1977; Noirot-Timothee and Noirot, 1980). The concept
that the septate junction has such a role, even if it is a secondary
one, has been proposed by several workers (Baskin, 1976; Noirot-Timothee
addition, it has been proposed that the septate junction may maintain
topographical relationships between cells as they undergo changes in
shape during contraction and relaxation (Hand and Gobel, 1972) or that
it may give tissues structural rigidity (Dallai, 1976; Staehelin, 1974).
However, as pointed out by Noirot-Timothee and Noirot (1980) and
Staehelin (1974), any role such as these may be supposed to be purely
passive as there are no filamentous structures associated with the
septate junction as there are with the various desmosomes (Staehelin,
1974). Furthermore, the tight junction of the Tunicata also exists
in tissue with a lack of desmosomes and must therefore also have some role
in adhesion, but its primary function presumably remains that of occlusion
as with all tight junctions (Staehelin, 1974).

It has in the past been suggested that the septate junction might
serve in intercellular communication or ionic coupling (Bullivant and
Loewenstein, 1968; Gilula et al, 1970; Giusti, 1976; Loewenstein, 1973;
Loewenstein and Kanno, 1964; Rose, 1971; Weiner et al, 1964). This seems
unlikely now that gap and septate junctions are known to coexist (Flower,
1971; Gilula and Satir, 1971; Hudspeth and Revel, 1971; Oschman and
Berridge, 1970; Rose, 1971). Further, the model proposed by Gilula et al,
(1970) in which the pleated septate junction was thought to form
intercellular channels is not consistent with morphological data now
Caveney and Podgorski showed that in the tissue they studied (epidermal
cells of the larval beetle Tenebrio molitor) gap junctions alone could
account for the high degree of electronic coupling recorded. Noirot-
Timothee et al (1978) have demonstrated that there is no structural
continuity throughout the entire thickness of a septate junction and it cannot therefore form an intercellular channel. There is in fact no convincing evidence that septate junctions have any role in intercellular communication, the gap junction adequately fulfills this role in both vertebrate and invertebrate tissues.

The position which septate junctions occupy in tissues is analogous to that of the vertebrate tight junction, the function of which is to act as a seal or occluding barrier to reduce paracellular permeability between epithelial cells (Bullivant, 1978; McNutt, 1977; Staehelin, 1974). The idea that septate junctions might have a similar, mainly occluding, function is now preferred by most workers (Filshie and Flower, 1977; Flower and Filshie, 1975; Lord and DiBona, 1976; Mills et al, 1976; Newell and Skelding, 1973; Noirot-Timothee and Noirot, 1973; Noirot-Timothee et al, 1978; Oschman and Berridge, 1970; Skaer et al, 1979; Szollosi and Marcaillou, 1977; Wood, 1977). In the few invertebrate tissues studied physiologically septate junctions have been shown to reduce paracellular permeability to tracers such as lanthanum, horse radish peroxidase and ruthenium red (Hand and Gobel, 1972; Lane and Treherne, 1972; Newell and Skelding, 1973; Szollosi and Marcaillou, 1977). The junctions have also been shown to present a high resistance to ionic flow through the extracellular space from the outside in as shown by electrophysiological experiments (Jophson and Macklin, 1967, 1969; Loewenstein and Kanno, 1964).

The main argument against the concept that the septate junction has an occluding function has been that tracers penetrate readily. It appears that interseptal spaces can be penetrated not only end on, where septa terminate within the junction (Flower and Filshie, 1975), but that the septa themselves may be permeable to certain tracers. In
this study the penetration of lanthanum into the enclosed spaces of the
anastomosing hemichordate and echinoderm septate junctions reinforces
this. None the less, as noted by Flower and Filshie (1975) and
subsequently by Noirot-Timothee and Noirot (1980), a succession of
septa would form a complex compartmentation of the intercellular space
forming a long and tortuous pathway between cells. Hence the intercellular
diffusion of substances will be hindered.

The vertebrate tight junction has also been shown to be leaky to
lanthanum tracers in some cases (Martinez-Palomo and Erlij, 1975) and
even relatively complex tight junctions have some permeability to ion
flow (Claude, 1978; Claude and Goodenough, 1973). In the present study
the tunicate tight junction was penetrated readily by lanthanum despite
being very extensive, the tracer filling the compartments formed by the
anastomosing junction. In this feature the invertebrate septate junction
and the tunicate tight junction are quite comparable.

Penetration of lanthanum into a junction is unpredictable. Often
adjacent junctions within a thin section will reveal vastly different
degrees of penetration. The environment of a tissue type is also
critical; tissues in a region of high mucus content or active secretion
for example are difficult to impregnate. This irregularity of
impregnation makes it difficult to compare various junction types or tissues,
or even various treatments of a single tissue. This feature was noted
by Szollosi and Marcaillou (1977) who reported that 'penetration of
tracers under experimental conditions is in no way an image of what
really happens in vivo; it only indicates that there is a possibility
for entry of large molecules into certain parts (of the gonad) and a
restriction of that entry into other parts'. Despite this, they concluded
that the septate junction in insect testes was the basis of a blood-
testis barrier.
One point apparently ignored in discussing the degree of lanthanum penetration into the septate junction is that the reverse argument must also apply. Green and Bergquist (1978) noted in their discussion of the simple parallel membrane junction of the sponges that the 'ability of Tethya junctions to 'hold' intercellular tracers such as lanthanum shows they must reduce paracellular flow'. The ability to 'hold' tracers is common to all the septate junctions. By this it is meant that a tissue soaked in a tracer such as lanthanum can be penetrated throughout all its lumen, intercellular spaces and passages by that tracer. Often, after dehydration and embedding, the only places in which any quantity of the tracer remains is between the septa of the septate junctions. The tracer has been washed out of all other intercellular spaces although there is often evidence, in the form of some small amounts still remaining, that the tracer has been there. Clearly the flow of dehydrating and embedding fluids across the junctional area itself is considerably less than elsewhere in the tissue. The septa must therefore be acting as a barrier to this flow.

In the present study several factors have further indicated that the main function of the septate junction is one of occlusion, to reduce paracellular flow. In the Porifera, the occurrence of the septate junction in reproducing Clathrina sp. suggests an occluding function. In this sponge there is no other structure that could prevent excessive sea water flushing of the developing larva and its components. This has been discussed by Green and Bergquist (1978) and a similar example has been noted by Ledger (1975). Ledger in his study of Sycon ciliatum found septate junctions between sclerocytes, sponge cells involved in spicule production. Again no other structures were present that could have accounted for the necessary occlusion of sea water from the micro-environment where calcite deposition was occurring and ionic concentration
presumably critical.

The clear variation in the number of septa in some tissues is also of note. The large arrays of septa in the fresh water *Hydra* epithelium compared with the few septa of the marine *Tubularia* epithelium, both hydrozoan coelenterates, was very marked. Similarly, the fresh water triclad platyhelminth *Neopla montana* had extensive arrays of septa in epithelial tissue compared with the number of septa present in the same tissue of the marine polyclad platyhelminth *Pseudoceros* sp. In both examples it is reasonable to assume that the osmotic stresses of a marine environment on an organism will be considerably less than a fresh water one and that the number of septa within a tissue reflects this difference. The relationship between the osmotic characteristics of an environment and the number of septa present further suggests that the septate junction serves an occluding function.

When studying the structures of the various septate junction types two points are of interest. In the lower invertebrate pleated septate junction the crosslinking of septa can be taken to further indicate that the junctions serve an occluding function. The crosslinking would serve to restrict, at least partially, the leakage of material between septa by further increasing the complexity of the intercellular compartmentalisation. Crosslinking will reduce the openness of at least one of the paths by which tracers supposedly penetrate the septate junction; namely by leakage between septa crossing from one layer to the next where septa terminate within the junction (Flower and Filshie, 1975; Filshie and Flower, 1977). In the echinoderms the anastomosing septate junction is of special interest. This junction is a structural intermediate linking the common strand type septate junction with the anastomosing configuration of the vertebrate tight junction. The occurrence of such an intermediate supports the proposition that the two
junctions have similar functions (mainly occlusion) and that the various forms of septate junction are invertebrate precursors of the tight junction.

It should be noted however that in accepting a general occluding function for the various forms of septate junction, there is a danger of oversimplifying the true situation. There is evidence from this study that occlusion is not simply achieved by the formation of a passive structural barrier. It was seen in the lower invertebrate pleated septate junction that the septa might be able to repel such tracers as lanthanum since this tracer was sometimes unable to pack tightly around the septa despite good penetration into the junctional area. This could occur if the septa were ionically charged, a feature such as this would further aid in reducing paracellular flow. Hand and Gobel (1972) have also suggested that septa might bear a weak electrical charge in which case a junction of thirty or more septa would present a considerable barrier to many substances. Baskin (1976) claimed that if septa are constructed of glycoprotein, as suggested by Noirot-Timothee and Noirot (1973), then these components, or perhaps certain amino acids such as proline, could possibly order water molecules in such a way that junctional permeability would be reduced considerably. Skaer et al (1979) believe that the chemical nature and charge characteristics of material trapped between septa may confer specificity on the junctions' occluding ability. They claim that unpublished work by Gupta, Berridge and Prince showed that the septate junctions of Calliphora Malpighian tubules were permeable to the anion sulphate, but not the heavy metal cation barium. The precise permeability properties of any septate junction could therefore depend on the combination of the chemical nature of the matrix held between septa, the chemical characteristics of the septa themselves and upon the physical structure
of the septa.

Finally, it should be mentioned that some workers claim to have located tight junctions in certain insect tissues. Lane et al (1977) and Lane (1978) report a form of tight junction in some insect central nervous systems and Lane (1979) also reports a similar junction in cockroach rectal pads. Furthermore, Toshimori et al (1979) describe a type of tight junction in the cyst envelope of the silkworm testis. In all cases the structures described are similar with ridges of closely packed particles on the F face of membranes and grooves on the E face. They do not however form extensive anastomosing arrays and the ridges are rarely very long, but rather the junction consists of short narrow networks. Furthermore, no evidence is available showing that a change of face occurs along the lines of fusion during freeze-fracturing as it does in true tight junctions (Bullivant, 1978). Other workers believe that these structures are not true tight junctions and find it difficult to believe that they might have an occluding function (Green et al., 1979; Noirot-Timothee and Noirot, 1980; Wood, 1977). It is none the less possible that they are a form of tight junction. It may be that the annelid, molluscan and arthropod lineage (the Proterostomia) has developed a simple tight junction in parallel with that of the chordate lineage (the Deuterostomia).

4.3 Invertebrate phylogenetic relationships:

The large number of septate junctions now recognised on the basis of the present work permits some discussion of invertebrate classification and evaluation of suggested phylogenetic relationships. Electron microscopic studies of animal tissue can yield useful results in this area as the technique can give more information than is available from gross morphological studies, but does not seem to run in to the complex problems of intrageneric or even intraspecific variation so far
revealed at the biochemical level of study. In conjunction with other data employed in phylogenetic study, morphological, palaeontological, developmental and biochemical, electron microscopy of junctions may prove useful in classification.

The first question to be answered in relation to the septate junction is: What parts of a junction are to be considered main structural identifying features or what parts are simply variations of little importance? In cross sectional views of different septate junctions there is little variation; all have septa spanning a 15 - 18 nm intercellular space. In tangential views the most obvious differences are in the shape of the septa themselves, whether they are double, single, straight, pleated or anastomosing. In addition the width of the septa and whether or not they have side projections may well be relevant. In freeze-fracture studies the shape and size of junctional particles revealed is important and the face to which particles adhere is also significant.

There is however some doubt as to the importance that can be placed on the side to which freeze-fracture particles adhere. Wood (1977) claims results that differ from those obtained in the present study when viewing the same Hydra type septate junction and Skaer et al (1979) claim to have found fixed tissue smooth septate junction particles predominantly on the E face of one tissue in contrast to the normal situation of particles on the P face (Flower and Filshie, 1975; Skaer et al 1979). However in the work of Dallai (1976) in which he found smooth and mollusc-arthropod pleated septate junctions occurring concurrently it is noteworthy that one of the junctions, the smooth septate junction, showed reversed polarity between fixed and unfixed tissue while the other, the pleated septate junction, did not. Clearly the reversal or non-reversal of polarity in this case was a feature of the junction type, not
of a membrane or a tissue as a whole. In this section therefore, it is considered that the side of a freeze-fracture to which junctional particles adhere is a feature of the junction and is important as an identifying feature.

A summary of the freeze-fracture properties of the twelve septate junction types located to date, the tunicate tight junction and the usual vertebrate tight junction (Staehelin, 1973) is given in Table 4.3.1. Table 4.3.2 summarises the appearance of these junctions in thin section tangential views of lanthanum impregnated tissues. Appendix 5.1 gives a simplified outline of high level invertebrate classification as used in this study and shows where the different types of junction have been found so far.

The discussion of relationships between the various junctional types is in three parts; the Coelenterates and lower invertebrates, the Proterostomia (the annelid, molluscan and arthropod lineage) and the Deuterostomia (the echinoderm and chordate lineage). The deuterostomes and proterostomes are considered to have arisen independently from a common coelomate ancestor (Berrill, 1955). The phyla which make up each group as discussed in this section are indicated in appendix 5.1.
Table 4.3.1: Summary of the Freeze-fracture Characteristics of the twelve types of invertebrate Septate Junction, the Tunicate Tight Junction and the Vertebrate Tight Junction:

<table>
<thead>
<tr>
<th></th>
<th>Fixed tissue</th>
<th>Unfixed tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P Face</td>
<td>E face</td>
</tr>
<tr>
<td>Hydra type septate junction</td>
<td>Mostly grooves or pits. Some particles</td>
<td>Mainly particles of irregular size and shape. Some grooves or pits</td>
</tr>
<tr>
<td>Anemone epithelial septate junction</td>
<td>Rows of closely spaced, evenly sized particles</td>
<td>Fine shallow grooves</td>
</tr>
<tr>
<td>Anemone double septate junction</td>
<td>Twin or broad jumbled rows of particles</td>
<td>Broad shallow grooves</td>
</tr>
<tr>
<td>Lower invertebrate pleated septate junction</td>
<td>Ragged particles of varying size and shape in bands rather than rows</td>
<td>Pits of varying size and shape in bands</td>
</tr>
<tr>
<td>Mollusc-arthropod pleated septate junction</td>
<td>Rounded particles of even size and shape</td>
<td>Grooves or pits</td>
</tr>
<tr>
<td>Smooth septate junction</td>
<td>Mainly particles or rods</td>
<td>Mainly grooves, some particles</td>
</tr>
<tr>
<td>Limulus septate junction</td>
<td>Grooves</td>
<td>Rows of particles, often indistinct, or stacks of short particle rows</td>
</tr>
<tr>
<td></td>
<td>Fixed tissue</td>
<td>Unfixed tissue</td>
</tr>
<tr>
<td>--------------------------------</td>
<td>------------------------------------------------------------------------------</td>
<td>-------------------------------------------------------------------------------</td>
</tr>
<tr>
<td></td>
<td>P face</td>
<td>E face</td>
</tr>
<tr>
<td>Chaetognath</td>
<td>Ridges with rows of particles on top</td>
<td>Grooves with rows of particles at the bottom</td>
</tr>
<tr>
<td>double septum septate junction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Echinoderm</td>
<td>Random array of pits in a broad band around cells. Some particles of varying size</td>
<td>Wide scattered array of irregularly shaped particles in a broad band around cells</td>
</tr>
<tr>
<td>double septum septate junction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Echinoderm</td>
<td>Evenly sized and shaped particles in an anastomosing pattern</td>
<td>Anastomosing network of pits</td>
</tr>
<tr>
<td>anastomosing septate junction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hemichordate</td>
<td>No results available</td>
<td>No results available</td>
</tr>
<tr>
<td>double septum septate junction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hemichordate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>anastomosing septate junction</td>
<td>Dense array of evenly sized and shaped particles</td>
<td>Dense array of pits, occasional particle</td>
</tr>
<tr>
<td>Tunicate tight junction</td>
<td>Anastomosing network of shallow pits</td>
<td>Rounded particles and short rods in an anastomosing network</td>
</tr>
<tr>
<td>Vertebrate tight junction</td>
<td>Anastomosing network of continuous ridges</td>
<td>Anastomosing network of grooves</td>
</tr>
<tr>
<td>(Staehelin, 1973)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Table 4.3.2</strong> Summary of the Main Thin Section Tangential View Features of the Twelve Types of Invertebrate Septate Junction:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>---------------------------------------------------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Hydra type septate junction</strong></td>
<td>Narrow, straight backbone septa with fine projections from both sides.</td>
<td></td>
</tr>
<tr>
<td><strong>Anemone epithelial septate junction</strong></td>
<td>Wide, straight septa with prominent projections, clearest when off one side only.</td>
<td></td>
</tr>
<tr>
<td><strong>Anemone double septum septate junction</strong></td>
<td>Wide, straight, double septa with component halves evenly spaced. Side projections.</td>
<td></td>
</tr>
<tr>
<td><strong>Lower invertebrate pleated septate junction</strong></td>
<td>Narrow backbone, pleated septa with fine projections from the apex of each pleat.</td>
<td></td>
</tr>
<tr>
<td><strong>Mollusc-arthropod pleated septate junction</strong></td>
<td>Narrow backbone, pleated septa</td>
<td></td>
</tr>
<tr>
<td><strong>Smooth septate junction</strong></td>
<td>Broad, straight septa, often with pegs between them.</td>
<td></td>
</tr>
<tr>
<td><strong>Limulus septate junction</strong></td>
<td>Broad, straight septa. Fewer pegs than in the smooth septate junction and septa often have a sub-structure appearance.</td>
<td></td>
</tr>
<tr>
<td><strong>Chaetognath double septum septate junction</strong></td>
<td>Narrow, double septa, pleated with narrow spaced and wide spaced configurations.</td>
<td></td>
</tr>
<tr>
<td><strong>Echinoderm double septum septate junction</strong></td>
<td>Straight, double septa of irregular width and with uneven spacing between the component halves.</td>
<td></td>
</tr>
<tr>
<td><strong>Echinoderm anastomosing septate junction</strong></td>
<td>Wide, anastomosing septa with rough edge appearance and side branches. Anastomosing pattern like that of the vertebrate tight junction.</td>
<td></td>
</tr>
<tr>
<td><strong>Hemichordate double septum septate junction</strong></td>
<td>Narrow, paired septa or stacks of three to eight septa. Septa often have a sub-structure appearance.</td>
<td></td>
</tr>
<tr>
<td><strong>Hemichordate anastomosing septate junction</strong></td>
<td>Wide, anastomosing septa with rough edge appearance and side branches. Anastomosing pattern of circles, parts of circles and rods.</td>
<td></td>
</tr>
</tbody>
</table>
The Coelenterates and lower invertebrates:

In the phylum Coelenterata all three septate junction types present the Hydra type and the two anemone junctions, have straight septa with side projections. However, the triploblastic invertebrate phyla, those immediately above the coelenterates, which possess the lower invertebrate pleated septate junction, have a junctional configuration more closely related to the Hydra type septate junction than to either of the anemone types. Both the Hydra type junction and the lower invertebrate pleated septate junction have narrow 2 - 3 mm wide central backbone septa with fine side projections and both have irregularly shaped and sized particles as revealed by freeze-fracture of fixed and unfixed tissues. In contrast the broad septa of the two anemone septate junctions and their more rounded and regularly shaped freeze-fracture particle appearance is quite distinct. In addition the type of double septum seen in the anemone double septum septate junction is certainly not apparent in any higher invertebrate groups. The main difference between the Hydra type septate junction and the lower invertebrate pleated septate junction lies in the pleating of the lower invertebrate junction and the fact that these two junctions fracture with the majority of junctional particles on opposite faces in both fixed and unfixed tissue (the E face for the Hydra type, the P face for the lower invertebrate pleated septate junction).

The lower invertebrate pleated septate junction is common to many phyla and the range spans the groups usually considered as the point of division between the Deuterostome and Proterostome lineages. The phyla which have this type of junction include the Annelida which are on the proterostome line, but it also includes the Sipunculoidea which are claimed by some workers to have given rise to the echinoderms (Nichols, 1967), that is on the deuterostome line. Other phyla so far found to have the lower invertebrate pleated septate junction are the Platyhelminthes, Bryozoa,
Nemertina and interestingly the Brachiopoda which have several molluscan structural features.

It appears therefore that on the basis of septate junction structure the hydrozoan coelenterates lie closest to the origin of the phylum Platychelminthes (or their ancestors) than do the Anthozoa. The platychelminths have in turn given rise to two main evolutionary lines of development. The first of these, the Proterostomia, includes the annelids and brachiopods, the second, the Deuterostomia, includes the sipunculoids and echinoderms. The relationships of the Bryozoa and Nemertina remain questionable.

The proterostome lineage:

Considering the proterostome lineage further the next major phylum above the annelids on this evolutionary line is the Mollusca which exhibits a mollusc-arthropod pleated septate junction. This junction, like the lower invertebrate pleated septate junction, has a narrow central backbone and is pleated, although side projections are less obvious. The two junctions freeze-fracture with the majority of particles on the P face in both fixed and unfixed tissue replicas. The particles of the mollusc-arthropod pleated septate junction however are rounded and regular in size and shape, being about 8 - 10 nm in diameter. The mollusc-arthropod pleated septate junction also occurs as its name suggests, in the Arthropoda, but in this phylum is restricted to tissue of superficial epithelial origin. The other junction found in the arthropods is the smooth septate junction which is quite distinct to the pleated septate junction. It has broad straight septa and unfixed tissue freeze-fractures with the particles on the opposite face of those of the pleated septate junction. However the particles though elongated predominantly into rods are still rounded in shape and about 8 - 10 nm wide. The other groups in the Proterostomia, the Merostomata, Pycnogonida,
and Collembola, have septate junctions which are very similar to the smooth septate junction. The septa are straight and broad in structure although they show some divergence from the smooth septate junction in freeze-fracture appearance.

The deuterostome lineage:

Within the Deuterostomia the Chaetognatha are reported to have both a double septum septate junction and the pleated septate junction, which is characteristic of mollusc and arthropod tissues, occurring concurrently (Duvert, in press). However in this report identification of the pleated septate junction rests only on thin section tangential views and it is more than likely that the junction being viewed is in fact the lower invertebrate pleated septate junction. This would place the Chaetognatha on a line of development not very far removed from the Sipunculoidea. It is interesting that the Chaetognatha have a paired septate junction as do both the hemichordates and the echinoderms. The septa of the hemichordate double septum septate junction are very similar in some views to those of the Chaetognatha in that they are similarly paired. In all these phyla the double septa are made of thin component parts when comparison is made with the anemone double septum septate junction. The anastomosing septate junctions of the Echinodermata and Hemichordata are remarkably similar. Both have broad septa with rough edges and side branches and both have rounded particles revealed on the P face of fixed tissue freeze-fracture replicas. The only real difference is in the final configuration the septa take. In this feature the anastomosing network of the echinoderm septate junction appears more closely related to that of the vertebrate tight junction than does the hemichordate junction. On this basis it is not unreasonable to consider the hemichordates a side shoot development and not on the main line of deuterostome development. Certainly the occurrence of an
intermediate structure linking the invertebrate septate junction
and the vertebrate tight junction in the phylum Echinodermata suggests
a close affinity between this phylum and the phylum Chordata.

The particle structures seen in freeze-fracture replicas of the
echinoderm and hemichordate anastomosing septate junctions are very
similar to those of the mollusc-arthropod pleated septate junction.
All these junctions fracture with the particles on the same side for
fixed and unfixed tissue (the situation in unfixed hemichordate tissue
is yet to be studied) and all have rounded 8 - 10 nm diameter particles.
This may be a conservative feature reflecting a common remote ancestry
among the phyla which possess the lower invertebrate pleated septate
junction.

In the tunicates a true tight junction occurs, but it fractures
with particles adhering to the E face of both fixed and unfixed tissue
in contrast to the usual tight junction pattern of fixed tissue
particles adhering to the P face (Staehelin, 1973). In addition the
junctional structures consist of short rods and particles rather than
continuous ridges and in this feature it is more like a septate junction
than a tight junction. The particles seen on the E face of fixed tissue
tunicate replicas are quite like those seen on the P face of echinoderm
anastomosing septate junction replicas though they are closer together.

The work in this thesis has enabled a rough outline to be laid
down. Future studies on some of the minor phyla may well be very
rewarding if it is possible to show similarities between their septate
junction structures and any of those described here. Some care is
required in such studies however. Vernet et al (1979) claim that the
nemertine Lineus ruber has a pleated septate junction and therefore
belongs to the higher groups of proterostomes. The present study has
shown that the nemertine septate junction is in fact a lower invertebrate
pleated septate junction and that there is no junctional evidence for classifying this group as proterostomes.

The phylogenetic relationships described in this section are diagrammatically represented on the following page.
A diagrammatic representation of the invertebrate phylogenetic relationships implied in this chapter.

(The lengths of the linking lines drawn have no significance)
4.4 The relationship between the invertebrate septate junction and the vertebrate tight junction:

A major objection to the proposition that septate junctions have an occluding role as discussed in chapter 4.2 has been their apparent structural differences in comparison to the vertebrate tight junction. Lane writes (personal communication) - "If you consider that the septate junctions have an occluding role and hence are the structure from which the vertebrate tight junctions have evolved, how do you account for the 15 - 20 nm intercellular cleft in septate junctions in the complete absence of such a space in tight junctions? How could two diverse structures be so intimately linked in evolutionary terms?"

In addition to this problem has been the fact that the tight junction forms an anastomosing network of junctional structure whereas the septate junction generally consists of unbranched septa.

In this section a simple argument is presented as a hypothesis by which the change from a pleated septate junction to the tight junction could have occurred. It is contended that this change did not need to involve major structural alterations to the junctions involved.

The hypothesis proposed is based on data obtained in the study of two widely varying phyla, the Porifera and the Echinodermata. The Porifera (chapter 3.2) were noted to have a simple parallel membrane type junction carrying out what appears to be an occluding function. This type of junction has been noted previously in the sponges (Jones, 1966; Ledger, 1975) but not as extensively as those reported in this study in the Antarctic sponge Inflatella belli. The important feature of this junction is its amazingly regular intercellular spacing in the absence of any septa; the intercellular spacing is controlled by some other factor. This implies that in the septate junctions the 15 - 18 nm intercellular space is not controlled by the junction, the septa
merely conform to a 'preordained' intermembrane spacing. This is not surprising when the wide variety of septal types are considered, but all having the same 15 - 18 nm intercellular spacing. The loss of the intercellular cleft in the change from the septate to the tight junction would simply involve a modification of this 'membrane spacing factor'.

In the phylum Echinodermata endothelial origin tissue septate junction (chapter 3.6) there exists the anastomosing network characteristic of the vertebrate tight junction. The problem of forming an anastomosing network, presumably more efficient than straight septa between which substances can leak, has therefore already been overcome within the invertebrate phyla.

The process of evolving from the septate junction to the tight junction might therefore have involved a modification or loss of the membrane spacing factor in an anastomosing septate junction, hence removing the need for the septa. The membranes of the adjacent cells could then flex together and the intramembrane junctional components protruding from the membrane, previously insertion points of the intercellular septa, could then fuse in a tight junction like structure. This would involve a change from a long 'in register' joint to a short 'side by side' joint which fits the supposed structure of the vertebrate tight junction (Bullivant, 1978) (see diagram below). The appearance of a particulate structure seen on freeze-fracture replicas of the tunicate tight junction fits this pattern almost exactly. It is only in the higher chordates that further components are added to the junction to form the more continuous, and presumably more efficient, ridges of the usual vertebrate tight junction.
4.5 **Tricellular regions:**

Tricellular 'junctions' in invertebrates were first noted by Noirot-Timothee and Noirot (1973). In the present study no attempt has been made to describe these structures to any extent other than to note their occurrence and general appearance.

The most notable thing about the invertebrate tricellular regions is their lack of variability in comparison with the many septate junction types. Noirot-Timothee and Noirot (1980) claim the tricellular regions that unite three pleated or three smooth septate junctions are completely similar and that structures recently described by Duvert et al (in press) in the Chaetognatha were also comparable. In this study it has been possible to locate tricellular regions in most of the tissues studied and all have shown a similar type of structure with a circular core crosslink joining the 'limiting strands' (Noirot-Timothee and Noirot, 1980) of the adjacent cells. Although there may be some variation in the diameter of the central cores or in the fine structure of these regions they appear far less variable than their related bicellular junctions. As noted by Noirot-Timothee and Noirot (1980) further study of the tricellular regions may well provide more information on the evolutionary relationships between the different types of septate junction and the various invertebrate phyla.
There is no reason to assume that the function of the tricellular region is any different to that of the septate junction (chapter 4.2). They appear to be analogous to the chordate tricellular junctions that occur in conjunction with the tight junction (Staehelin, 1974; Staehelin and Hull, 1978).
Appendix 5.1: Invertebrate Classification (as used in this thesis) and the Distribution of the Various Types of Septate Junction within the Invertebrata.

- **PORIFERA**

- **COELENTERATA**
  - Hydra type septate junction.
  - Anemone double septum septate junction.
  - Anemone epithelial septate junction.

- **PLATYHELMINTHES**

- **NEMERTINA**

- **BRYOZOA**
  - Lower invertebrate pleated septate junction

- **ANNELIDA**

  - (Deuterostomia)
  - (Proterostomia)

  - **SIPUNCULOIDEA**
  - **BRACHIOPODA**

  - **CHAETOGNATHA**
    - Chaetognath double septum septate junction
### Appendix 5.2: Species list of animals studied

<table>
<thead>
<tr>
<th>Phylum Porifera</th>
<th>Class Calcarea</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Clathrina sp.</td>
</tr>
<tr>
<td></td>
<td>Sycon sp.</td>
</tr>
</tbody>
</table>

|                | Class Demospongiae |
|                | Inflatella belli   |
|                | Halichondria moorei|
|                | Ulosa sp.         |
|                | Spongia reticulata|
|                | Tethya sp.        |
|                | Latrunculia brevis|
|                | Polymastia granulosa|

<table>
<thead>
<tr>
<th>Phylum Coelenterata</th>
<th>Class Hydrozoa</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Chlorohydra viridissima</td>
</tr>
<tr>
<td></td>
<td>Tubularia antennoides</td>
</tr>
</tbody>
</table>

|                     | Class Anthozoa |
|                     | Isactinia olivacea |
|                     | Isactinia tenebrosa |
|                     | Actinothoe albocincta |

<table>
<thead>
<tr>
<th>Phylum Platyhelminthes</th>
<th>Class Turbellaria</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Neopia montana</td>
</tr>
<tr>
<td></td>
<td>Pseudoceros sp.</td>
</tr>
</tbody>
</table>

| Phylum Nemertina | unnamed sp. |

| Phylum Bryozoa | Watersipora cucullata |

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<tr>
<th>Phylum Annelida</th>
<th>Class Oligochaeta</th>
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<td>Lumbricus terrestris</td>
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|                        | Class Polychaeta |
|                        | Eulalia microphylla |
Phylum Sipunculoidea
  Sipunculus mandanus

Phylum Brachiopoda
  Terebratella inconspicua

Phylum Mollusca
  Class Amphineura
    Eudoxochiton nobilis
  Class Cephalopoda
    Unknown species
  Class Lamellibranchia
    Perna canaliculus
  Class Gastropoda
    Maoricrypta monoxyla
    Nerita melanotragus

Phylum Arthropoda
  Class Crustacea
    Elminius modestus
    Chamaesipho brunnea
    Petrolisthes elongatus
  Class Insecta
    Telegrylus commodus
    Arachnocampa luminosa

Sub-Phylum Pycnogonida
  Amмоthea clausi

Phylum Echinodermata
  Class Asteroidea
    Coscinasterias calamaria
    Patiriella regularis
  Class Echinoidea
    Evechinus chloroticus
  Class Ophiuroidea
    Ophionereis fasciata
(Appendix 5.2 continued)

Class Holothuroidea
   Stichopus mollis
   Trochodota dendyi

Class Enteropneusta
   Balanoglossus australiensis

Class Ascidiae
   Asterocarpa coerulea
REFERENCES


VARIATIONS OF SEPTATE JUNCTION STRUCTURE IN THE INVERTEBRATES

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Three main variations of the invertebrate septate junction are now generally accepted: the Hydra type, the pleated septate and the smooth septate junctions (1). A junctional study of many members of a wide range of invertebrate phyla using thin section, lanthanum tracer and freeze-fracture techniques has however revealed at least eight distinct septate junction types, including two anastomosing septate junctions in the higher invertebrate phyla.

In the Cnidaria three forms of septate junction occur. The Hydra type found in Hydrozoa (Fig 1), a pegged junction seen in the epidermal cells of Anthozoa and a ladder-like junction seen in the endodermal cells of Anthozoa. The pegged Anthozoa junction consists of septa with distinct short pegs branching at right angles mainly from one side (fig 2). Where two septa run close together, the pegs may form crossbars linking them. The ladder junction has a pegged double septum with crossbars linking the two parts of each septum (fig 3). From the Platyhelminthes up to epidermal, and certain endodermal tissues of the Arthropoda, the classical pleated septate junction is seen (1), although thin lanthanum tracer impregnated sections show that the fine structure is more complex than previously thought. The pleated septate junction in fact consists of a zig-zag septum with pegs from each apex (fig 4). In some cases pegs from adjacent septa join to form a honeycomb pattern. Thicker sections reveal a normal pleated appearance. The remaining Arthropod tissues have a smooth septate junction (fig 5). In Echinoderm epithelium, septa have an indistinct twin line appearance revealed by lanthanum tracer (fig 6) and a similar freeze-fracture appearance in glutaraldehyde fixed tissue to the smooth septate junction, though with reversed polarity of faces. In endothelial tissues however, an anastomosing septate junction is seen with the same 15-18 nm intercellular spacing reported for other septate junctions. The anastomosing network of septa is similar in tangential tracer view to that seen in freeze-fracture of the vertebrate tight junction. The walls of the network have a pleated structure (fig 7). The Hemichordata show a second type of anastomosing septate junction, again with a 15-18 nm intercellular space. In this case the junction consists of an array of tightly packed circles and joining crossbars. The circles have small pegs radiating from them (fig 8). In the lowest vertebrate phylum, the Tunicata, a variation of the tight junction is seen. This junction is leaky to lanthanum and has a particle distribution similar to that of the smooth septate junction as seen in freeze-fracture of glutaraldehyde fixed material. The junction however appears as an anastomosing network like that of the tight junction and the membranes are seen to fuse in thin section.

The large number of septate junctions now recognised provides a further means to phylogenetically classify many tissues. The junctions are considered to have the same occluding function as the tight junction, their various forms showing a sequence of development with the two anastomosing junctions forming a link between the vertebrate tight junction and the invertebrate septate junctions.

Fig 1. Hydra type septate junction 140000x

Fig 2. Anthozoa pegged septate junction 140000x

Fig 3. Anthozoa ladder septate junction 100000x

Fig 4. Pleated septate junction 95000x

Fig 5. Smooth septate junction 110000x

Fig 6. Echinoderm double septum junction 72000x

Fig 7. Echinoderm anastomosing junction 76000x

Fig 8. Hemichordate circle junction 90000x

All figures show thin sections of lanthanum tracer impregnated tissue.
CELL MEMBRANE SPECIALISATIONS IN THE PORIFERA

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Introduction

Cell junctions are difficult to locate in the Porifera due to the transient nature of virtually all intercellular interactions. None the less, cell junctions do exist, and knowledge derived from the study of these is applicable to higher organisms. Sponge cell junctions are apparently only formed when required for a specific purpose and this makes them particularly useful in attributing functions to particular types of junction. Two junctional types have been investigated in this paper, the gap junction which has a role in intercellular communication, and the septate junction thought to have an occluding function.

Our work has provided further evidence of intercellular communication in sponge larvae, and has revealed a number of occluding systems associated with developing reproductive bodies in various species. The systems range from a dense collagen belt through to recognisable septate junctions. Communication and coordination systems in sponges are discussed, as are features of sponge occluding systems both with reference to their development, and to the function of the septate junction which is characteristic of all other invertebrate phyla.

Materials and Methods

Four New Zealand species of Demospongiae and one species of Calcarea were collected during their reproductive periods. A further species of Demospongiae was collected in Antarctic waters during its reproductive period. Larvae from Halichondria moorei and Ulosa sp. were fixed for electron microscopy after their release from the adults which were held in laboratory aquaria. Reproductive bodies from Tethya (new sp.) Infistella bollii (Antarctic species) and Spongia reticulata were fixed within pieces of adult tissue. Tissue of Clathrina sp. was fixed in early stages of embryogenesis.

Fixation in all cases was carried out for one hour in 3% gluteraldehyde in 0.2 M sodium cacodylate buffer, followed by one hour post-fixation in 1% osmium tetroxide. Tissue was then alcohol dehydrated and embedded in Epon 812. Thin sections were double stained with 1:1 uranyl acetate—absolute alcohol and lead citrate (Venable and Coggeshall, 1965). Lanthanum impregnation in tissue embedded for thin sectioning was achieved by the method of Shakhlet and Tevesxooli (1978) and carbonate staining on thin sections was by the use of thiosemicarbazide and silver proteinate (Thierry, 1967). All thin sections were viewed in a Philips EM301 electron microscope.

Freeze fracture was by the method of Buillivant (1973) using 30% glycerol as a cryoprotectant. Tissue was digested from the carbon platinum replica using chromic acid. Scanning electron microscopy was carried out in an saturated mercuric chloride / 1% osmium tetroxide 'instant' fix (Parducz, 1967) followed by acetone dehydration and carbon dioxide critical point drying. Specimens were viewed in a Jeol JSM U3 electron microscope.

Results

Thin section electron microscopy of Spongia reveals a well organised and dense multilayered collagen coat up to 5.5 µ thick surrounding the developing embryo (Fig. 1). Many bacteria are seen trapped in this coat, and free glycogen rosettes are seen in large quantities inside the area surrounded by the coat (Fig. 1). In Tethya a collagen coat is also present, but it is reinforced by an outer, single layer of adult cells. The collagen coat is generally denser than normal matrix collagen (Fig. 2, 4). In pieces cells in the surrounding layer do not meet fully (Fig. 2), but have collagen between them, while in other places stretches of cell overlap occur (Fig. 3). In the latter case a form of simple cell junction occurs and is comparable with that found between pinacocytes of Sycon ciliatum (Lederer 1975). Lanthanum that has seeped into these junctions, which consist of lengths of parallel membrane, has remained throughout dehydration and embedding (Fig. 4), but carbonate staining shows no intercellular matrix within the junctions (cf. Fig. 8). In places the membranes flex together to come into close apposition (Fig. 3).

In Infistella, a more complex situation is seen in which the developing embryo is surrounded entirely by a single layer of adult cells up to 12 µ deep (Fig. 5). During earlier stages of embryonic development, this layer is much thinner (Fig. 6). There is little collagen fibril deposition, but the cells are joined by long junctional regions (Fig. 5), far more extensive than any previously described in the Porifera. In this species the junction is again of the simple type consisting of long stretches of parallel membrane with no intercellular matrix of any extent apparent (Fig. 7), even after carbonate staining (Fig. 8). In both this species and in Tethya, junctional membranes are about 10 nm apart where they run parallel (Fig. 7). This regular spacing is maintained over long distances in Infistella.

In Clathrina another type of development is seen. In this species layered vitelline platelets
are observed in some areas of the mesophyl (Fig. 9), presumably as a future food supply for developing larvae. These platelets are probably protein as the cells that produce them have large amounts of rough endoplasmic reticulum (Fig. 10). In areas where these platelets occur, choanocytes, normally rounded cells with little intercellular contact, extend laterally and form interdigitating cell extensions (Fig. 10). These interlocking extensions are joined by septate junctions similar in cross section to those seen in other invertebrate phyla (Steinhil, 1974) (Fig. 11). These junctions have an intermembrane spacing of 12-15 nm. Deeper in the mesophyl there is a dense organized collagen multilayer about 2 μ thick (Fig. 10, 12). The platelets are in effect held in an enclosed space bounded on one side by choanocytes joined by septate junctions, and on the other side by a collagen fibre mat.

Halichondria and Ulloa larvae go through a free stage after release from the parent sponge. They have an organized columnar epithelium with one cillum per cell (Berquist and Green, 1977). Larvae viewed in a scanning electron microscope exhibit remnants of metachronal rhythm passing in diagonal waves along the organism, despite some shrinkage having occurred during fixation (Fig. 13). Despite intensive investigation using thin section and freeze fracture techniques no obvious membrane modifications that could provide the means of intercellular communication necessary to maintain coordinated ciliary beat have been found.

Figure 1. The multilayered collagen cost up to 6.5 μ thick surrounding a developing Spongia embryo. The cost reduces water flushing, traps bacteria (B), and seals in components such as intercellular glycogen rosettes (C) required for larval development. (Uranyl acetate/lead citrate).

Figure 2. Adult cells and collagen (C), which is denser than that of the normal adult matrix (M), surrounding a Tethya embryo. In this case the cells do not form a junction, but have collagen between them. (E: embryonic cells) (Carbohydrate stain).

Figure 3. A junction between two adult cells lining a Tethya embryo. The embryo lies beneath the collagen layer (C). In some places the junction between the cells is of a simple parallel membrane type with a 10 nm intercellular spacing (arrow), while in others the membranes flex together (double arrow). (Uranyl acetate/lead citrate).

Figure 4. Lanthanum tracer trapped in a junctional area (arrow) of the adult cell layer surrounding an embryo in Tethya (C: Collagen layer; M: Adult matrix).

Figure 5. The adult cell layer surrounding an embryo in Inflatale. This layer becomes up to 12 μ thick with extensive intercellular junctions of the simple parallel membrane type (arrow). It is mainly one cell deep except where cell overlap occurs at junctions. (Uranyl acetate/lead citrate).
Figure 6. An early stage in the organization of the cell layer surrounding the larva in *Inflateda*. At this stage the extensive cells junctions have begun to develop, but the cell layer is still very thin. (Uranyl acetate/lead citrate)

Figure 7. The simple parallel membrane type junction seen in cells enclosing an *Inflateda* embryo. There is little intercellular material, but the 10 nm spacing between cells is remarkably constant over long distances. (Uranyl acetate/lead citrate)

Figure 8. A simple parallel membrane type junction in *Inflateda* after carbohydrate staining. The stain fails to show an intercellular matrix in the junctional region. A similar result is seen in *Jasaya* junctions. (Carbohydrate stain)

Figure 9. Vitelline platelets in the mesophyll of *Clathrina*. They are made up of layers and are probably protein stores for future embryonic development. (Uranyl acetate/lead citrate)

Figure 10. *Clathrina* preparing for reproduction. The choanocytes (CH) have extended laterally and interdigitate. Mesophyll cells with large amounts of rough endoplasmic reticulum have begun vitelline platelet formation (arrow) and a dense collagen belt (C) has been laid down. (Uranyl acetate/lead citrate)

Figure 11. Septate junctions between choanocytes of *Clathrina*. Septa (arrows) span a 12-15 nm intercellular spacing. (Uranyl acetate/lead citrate)

Figure 12. The mesophyll collagen layer of a reproducing *Clathrina*. The collagen belt is multilayered and about 2 μ thick. (Carbohydrate stain)

Figure 13. A scanning electron micrograph of a *Halichondria* larva. Remnants of metachronal rhythm in the larva’s cilia are seen passing diagonally down the organism. (Parducz fix)
Discussion

Intercellular coordination and communication

Pavans de Cessartey (1974) postulated three types of intercellular coordination pathway in sponges. These are fluid extracellular coordination pathways, mobile cellular coordination pathways involving transitory cell contacts, and fixed tissue coordination pathways. Fixed tissue pathways involve some form of structural intercellular communication link. Loewenstein (1967) was able to measure direct electrical communication between sponge cells and this remains the only evidence to date for the existence of direct intercellular communication channels. The fixed tissue coordination pathway described by Pavans de Cessartey involved relatively slow reactions compared with those measured in higher metazoans. Contractile events in sponges, for example those operating in oscular contraction, last at least 30 seconds and information spread seems to involve a time of 4-6 minutes to cover several centimeters (Pavans de Cessartey, 1974).

The fact that metachronal rhythm exists in sponge larvae when each cilium arises from a separate cell, suggests strongly that a rapid intercellular communication channel exists between larval epithelial cells. While it is possible that dynamic forces caused by ciliary action may trigger adjacent cells in front and behind each other, it seems untenable that such a mechanism could act efficiently enough in a lateral direction to maintain ciliary coordination. Since the rhythm in cilia is rapid, the fixed tissue coordination pathway as proposed by Pavans de Cessartey appears inadequate to explain its operation, assuming always that the rate of oscular contraction is dependent on the rate of message conduction through a cell network. A 'contraction message' may pass rapidly between cells, but the actual process of fibre contraction within each cell could be slow. However, where waves of contraction do occur (Cason, 1966; Pavans de Cessartey, 1969; Prosser, 1967; Ralsouw, 1971) it appears more likely that the initiation of contraction in a cell causes dynamic forces which trigger contraction in the next cell in line. No actual transfer of information through a connecting junction is necessary in such a case. If this is so, then there are four sponge coordination pathways, two of which are fixed tissue pathways. The first of these involves intercellular response to dynamic pressures brought by adjoining cells, suggested by the slower sponge wave contractions, and the second is a rapid intercellular communication pathway suggested by the larval ciliary coordination and the intercellular electrical readings. To date the structural specialization associated with such a rapid channel has not been resolved. Thin sectioning alone does not provide definitive results and freeze fracture work on fixed and unfixed sponges in our laboratory has revealed no obvious membrane modifications that could be involved in communication. In all higher metazoans, the gap junction is involved in communication (Staehlin, 1974).

Loewenstein (1967) found that a lack of calcium ions in his cellular bathing medium prevented the onset of intercellular coupling between sponge cells. An analogue effect is observed in all gap junctions where a removal of calcium ions from the extracellular medium causes an uncoupling of the junctions (Rose and Loewenstein, 1976). In a calcium free medium sodium ion extrusion from within a cell is depressed, and the resulting build up of sodium causes the release of calcium from the mitochondria (Cerafoli et al., 1974). Such a build up of free intracellular calcium ions in turn causes an uncoupling of gap junctions (Delezee and Loewenstein, 1976; Peracchia, 1978; Rose and Loewenstein, 1975, 1976). The inability of sponges to form communicating junctions in the absence of calcium ions, and the uncoupling of gap junctions in similar circumstances is interesting. It implies that the strong effect of calcium ion exclusion on communicating junctions in one way or another, appears to be universal, even though the detailed morphological structures of such junctions may vary.

Occluding systems

Sponges have several structures that serve an occluding function, acting to close off certain areas of tissue from excessive fluid dilution or flushing. In all cases the structures observed can be associated with the need to maintain a protecting environment as in embryonic development, or the need to maintain a certain ionic environment as in epipodite secretion (Ledger, 1975). In some species (e.g. Spongia) larval development occurs within a relatively simple collagen envelope. The fibres surrounding the embryo offer some protection from sea water flushing and aid in retaining larval components in a confined area. Any chemical messages involved in development could also be retained in the appropriate area. The fluid extracellular pathway as proposed implies that the macromolecules of the sponge matrix can control diffusion of such substances, and offer physical resistance to such diffusion (Pavans de Cessartey, 1974).

In Tethys, adult cells form a layer around the embryo, combining with the collagen fibres to make a more effective seal. This tendency is emphasised in Inflataella where an extensive adult layer with complex junctions surrounds the embryo. Although junctions of the type seen in Inflataella have been reported elsewhere (Ledger, 1975; Jones, 1966) and occur in Tethys, the junctional regions are not as extensive. In all cases that junctions have been reported, an intermembrane spacing of between 10 and 20 nm occurs. This spacing corresponds well with the 15 nm spacing found between membranes in all other occluding junctions in the invertebrates (Staehlin, 1974), despite the occurrence in the latter of bridging septa which are often complex in structure (Green, 1978). The regularity of this spacing in the absence of septa implies that some membrane factor other than the septa themselves determines the intercellular spacing in septate junctions. The septa must then adapt to this determined spacing. This could explain the remarkably constant intercellular spacing of septate junctions despite the great variation in actual septal construction (Green, 1978).
In the simple junctions observed in sponges there is some variation in the extent to which intercellular substances are visible. In some cases they can be quite dense (Ledger, 1975), while in others they are difficult to see or appear absent. The extensive infletelle junctional areas for example, show little intercellular modification even after carbohydrate staining. The extent to which demonstration of intercellular material reflects an irregular occurrence, or is an abnor mal condition altered by fixation and dehydration is debatable. None the less, the apparent simplicity of sponge junctions may be misleading. The ability of Tethya junctions to "hold" intercellular tracers such as lanthanum (Fig. 4) shows that they must reduce paracellular flow.

In Clathrina a further stage in junctional development is seen. Here septae bridge a 12-15 nm intercellular space between choanocytes. The septate junctions operate in association with an oriented collagen deposition. In sponges, only between the spherocytes of Hycon ciliatum (Ledger, 1975) have septate junctions been reported as the sole means of intercellular occlusion. In the coelenterates however, and all higher phyla, the latter pattern is the only one present. The development toward the septate junction which can be discerned in the various occluding patterns observed in relation to specific activities such as reproduction and spicule secretion, strongly backs the interpretation that septate junctions serve an occluding function.

Overview of the sponge case

Sponge organisation with mobile cells in an unstructured matrix does not require the constant presence of intercellular junctions as seen in more complex and structured animals. However, when necessary, the sponges are clearly capable of organising communicating and occluding systems of various degrees of complexity. These are the structural precursors of higher metazoan junctions. Some features characteristic of sponge junction organisation such as the intercellular spacing of septate junctions, and the calcium ion dependence of communicating junctions, persist in all higher animals. If as Pavas de Cecatty suggests, sponges can be considered as "witnesses of the prehistory of the nervous system", then it can also be said that they have witnessed the development of intercellular junctions. The range of occluding systems observed in the Porifera lends some weight to such a suggestion.

Summary

Occluding systems and occluding junctions in the Porifera are described. These range from a dense collagen belt through to recognisable septate junctions. Evidence is also presented for rapid intercellular communication in sponge larvae.

Communication and coordination systems in sponges are discussed, as are features of sponge occluding systems, both in reference to their development, and to the structure and function of the septate junction characteristic of all other invertebrate phyla.

Sommaire

Les systèmes à occlusion et les jonctions à occlusion sont décrits chez les Porifères. Ceux-ci ont d'une manière dense de collagène à des jonctions septées reconnaissables. Il y a également évidences d'une communication intercellulaire rapide chez les larves d'éponges.

Les systèmes de communication et de coordination chez les éponges sont discutés, ainsi que les caractéristiques des systèmes à occlusion des éponges, ayant trait à la fois à leur développement et aux structure et fonction de la jonction septée caractéristique des autres embranchements invertebrés.
References


An Anastomosing Septate Junction in Endothelial Cells of the Phylum Echinodermata

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As part of an extensive study of the structure and interrelationships of the various forms of septate junction, several species of the Echinodermata have been investigated using conventional thin-section, lanthanum tracer, and freeze-fracture techniques. In the endothelial tissues of this phylum, a new type of junction has been identified: an anastomosing septate junction. It has the usual 15- to 18-nm spacing between membranes characteristic of a septate junction, and occurs in the same relative position around the apical circumference of cells lining an outside or luminal edge. However, the septa form an anastomosing pleated network, which in tangential view is not unlike that seen in the freeze fracture of tight junctions in vertebrates, though generally it is more extensive. The finding of this structural intermediate between septate and tight junctions adds weight to the idea that both of these junctional types may have the same occluding function.

Several variant types of septate junction have been described in invertebrates (Baskin, 1976; Staehelin, 1974) and more are being found as further tissues are examined in tangential section after lanthanum tracer-impregnation or by freeze-fracture techniques (Green, 1978). This diversity is not unexpected in the invertebrates. This paper describes a new type of septate junction that has been found in the endothelial tissues of some members of the Echinodermata. This new junction is of special interest since it has both septate and tight junction features, and hence appears to be a structural intermediate. The occurrence of such an intermediate adds weight to the idea that both septate and tight junctions may share an occluding function.

MATERIALS AND METHODS

The pyloric cecum of the starfish Coscinasterias calamaria, the lining of the alimentary canal of the sea cucumber Stichopus mollaris, and the stomach of the sea urchin Echinus chloroticus were dissected from the animals and fixed in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer for 2 hr at room temperature. The specimens were washed in several changes of buffer, postfixed in 1% osmium tetroxide for 1 hr, dehydrated in an alcohol series, and embedded in Epon 812. Thin sections were stained with uranyl acetate and lead citrate (Venables and Coggeshall, 1965) before viewing in a Philips EM301 electron microscope.

Lanthanum incorporation was achieved by the method of Shaklai and Tavassoli (1977) in which 1% lanthanum nitrate was added to a fixative of 3% glutaraldehyde in 0.1 M sodium cacodylate buffer. Fixation was for 1 hr followed by a change to a phosphate buffer at 4°C for 24 hr. Postfixation was in 1% osmium tetroxide with 1% lanthanum nitrate added. Tissue was then rapidly dehydrated in acetone, embedded in Epon 812, sectioned, and viewed with no further staining.

Freeze fracturing was by the method of Bullivant (1973) using glutaraldehyde-fixed material in 30% glycerol made up in the buffer and unfixed material soaked in 30% glycerol in sea water for 15 min.

RESULTS

The anastomosing septate junction has an intermembrane spacing of 15-18 nm (Fig. 1) which is similar to that reported for earlier-described septate junctions (Staehelin, 1974). In cross-section and after staining with uranyl acetate and lead citrate the septa are not clear. This is due to the anastomosing which causes the spacing structure between membranes to be sectioned at many different angles in any one stretch of junction (Fig. 1). An anastomosing compartment is on average about 32 nm in diameter and with thin sections of even 50-60 nm, more than one compartment at
a time is viewed, resulting in septa at many angles to the plane of section. The junction extends downward to about 550 nm from the luminal edge of the cells, with only a desmosome, seen in thin section, above it (Fig. 1).

Using lanthanum tracer the anastomosing structure is seen clearly in tangential section. In some areas the septa run considerable distances without cross-links (Fig. 2), but in most cases the tangential appearance is that of an intricate network pattern of far greater extent than the usual vertebrate tight junction (Figs. 3, 4). The septa have a pleated appearance with an irregular periodicity which is always less than the 21-nm periodicity characteristic of the pleated septate junction as seen in molluscs and arthropods (Staehelin, 1974; Noirot-Timothee and Noirot, 1973). This periodicity is difficult to measure, however, because it is irregular and uneven (Fig. 5). The anastomosing compartments formed by the septa show considerable variation in size, although the majority are 30–35 nm wide (Figs. 3, 4). In places, bars are seen branching from the septa (Fig. 5). The lanthanum tracer penetrated throughout the junction, including the enclosed spaces of the network system implying that, in fixed tissue at least, the actual septa are permeable to tracers (Figs. 3–5).

In freeze fracture of fixed material the junction appears as a pattern of mainly individual particles about 7.5 nm in diameter on the P face (Fig. 6) (Branton et al.). The particles are a regular size and shape. The pattern appears anastomosing, but it would be difficult to recognize it as such without previous knowledge of the appearance of the junction in a tangential lanthanum-stained preparation. On the E face an occasional particle is left adhering within a fine network of pits and grooves (Fig. 7). The pits have a diameter of about 4–5 nm. Networks on the two faces are coincident, with rows of particles on the P face seen to line up with rows of pits on the E face where a change of face occurs (Fig. 8). Freeze fracture of unfixed material shows an almost identical pattern: a network of particles 7–8 nm in diameter on the P face, and the E face showing an anastomosing network of fine 4- to 5-nm diameter pits, with the occasional particle left adhering. In the unfixed tissue however, the number of particles left adhering to the E face is very small when compared with the E face of fixed tissue, and the pits are more clearly separated, not forming grooves as in fixed tissue (Fig. 9). As in fixed tissue, rows of particles on the P face line up with pits on the E face where a change of face occurs. In both fixed and unfixed tissue, the particles seen in freeze fracture presumably correspond with the septa in the intermembrane space.

**DISCUSSION**

Previous work on Echinoderm junctions has referred to epithelial, rather than endothelial, junctions (Gilula, 1973; Wolpert and Mercer, 1963; Wood, 1977). In the present work the anastomosing junction has been observed only in endothelial tissues. It has been seen in the pyloric ceca of the starfish *Coscinasterias calamaria* (Asteroidea), the stomach of the sea urchin *Echinus chloroticus* (Echinoidea), and the alimentary canal of the sea cucumber *Stichopus mollis* (Holothuroidea). It has therefore a wide occurrence in the Echinodermata. In epithelial tissue of these groups, and of the brittle stars (Ophiuroidea), a septate junction similar in some ways to the arthropod smooth septate junction occurs (Green, 1978).

Like other septate junctions and the tight junction of the vertebrates, the anastomosing septate junction occurs around the apical circumference of cells facing the lumen of the organ. It has an intermembrane spacing similar to that of recognized forms of the septate junction (Staehelin, 1974), and in freeze fracture of fixed and unfixed material, particles rather than continuous
ridges are seen. This is a pleated septate rather than a tight junction feature. However, the junction has an anastomosing network as does the vertebrate tight junction. A diagrammatic interpretation of the junction is seen in Fig. 10. This junction is therefore structurally an intermediate between the septate and tight junctions. In view of the frequently cited relationship between the invertebrate Echinodermata and the Chordate phyla (see review by Berrill, 1955), it is interesting to note the presence within the Echinodermata of such a structural intermediate between the smooth and pleated septate junction types found in the invertebrates and the tight junction which characterizes vertebrate tissues. Some restraint is, however, required in appraising this structural intermediate. This anastomosing junction cannot be interpreted as the only transition between septate and tight junctional organization. It may be one of several transitional types, or a separate development from a primitive common structure. Work now in progress has revealed a complex form of anastomosing septate junction in the Hemichordate *Balanoglossus australiensis* (Green, 1978).

The anastomosing septate junction has a similar particle structure in freeze fracture to the pleated septate junction of molluscs and arthropods; both of these junctions have particles of a regular size and shape (Staehelin, 1974). Both of these junctions also have the majority of particles adhering to the P face in freeze fracture of both fixed and unfixed material. Smooth septate, *Hydra*-type septate, and tight junctions all show changes in the polarity of particle attachment to the two faces when the fracture appearance of fixed and unfixed tissue is compared (Filshie and Flower, 1977; Staehelin, 1973) (Table 1), although this change is less pronounced in the *Hydra*-type junction (Filshie and Flower, 1977). In this respect therefore the anastomosing junction appears to be most closely related to the pleated septate junction. The fracturing difference between fixed and unfixed states shown for some junctions may however, not be an important factor in terms of the relationships between different types.

On the other hand, the similarity of fracturing properties, the similarity of particle size and shape, and the pleated appearance of this junction may well reflect a relationship between the anastomosing and pleated septate junctions. Embryological evidence indicates that the echinoderm and chordate lineage (the Deuterostomia) and the annelid, molluscan, and arthropod lineage (the Proterostomia) arose independently from a common coelomate ancestor (Berrill, 1955). The organisms possessing pleated septate junctions and those possessing anastomosing septate junctions could thus share a remote common ancestry.

In his original description of the septate
junction in *Hydra*, Wood (1959) suggested that this junction might be involved in intercellular adhesion and control of intercellular permeability. The subsequent discovery of the pleated septate junction, which has a honeycomb appearance in certain planes of section, led some workers to attribute a communicating or ion coupling function to this and other forms of septate junction (Bullivant and Loewenstein, 1968; Gilula *et al.*, 1970; Giusti, 1976; Loewenstein, 1973; Loewenstein and Kanno, 1964; Rose, 1971; Weiner *et al.*, 1964). This seems unlikely now that gap and septate junctions are known to coexist (Flower, 1971; Gilula and Satir, 1971; Hudspeth and Revel, 1971; Oschman and Berridge, 1970; Rose, 1971).

Tight and septate junctions have never been shown to coexist clearly in the same tissue (Wood, 1977). It seems unlikely that structures described by Lane (1978) as tight junctions in invertebrates can actually have a true occluding junction. The structures only consist of short rods and do not extend as a zone around the cells. Furthermore, these structures have only been found in the Insecta, where they are mainly confined to nervous tissue. The absence of coexisting tight junctions, and the fact that the septate junction occupies a similar cellular position in invertebrates to the tight junction in vertebrates suggest that the septate junction is the invertebrate equivalent to the vertebrate tight junction. The function of the tight junction is to act as a seal or occluding barrier to reduce paracellular permeability between epithelial cells (Bullivant, 1978; McNutt, 1977; Staehelin, 1974). The idea that septate junctions have mainly an occluding function is now pre-


Within most tangential fields of view in pleated and smooth septate junctions, the septa can often be seen terminating (Flower and Filshie, 1976; Noiro-Timothéée and Noirot, 1976). Flower and Filshie (1976) suggested that “a random termination of septa within these junctions would lead, because of the extent of these junctions, to a very long, tortuous, maze-like pathway through the junction. Such a pathway could allow access for tracers and yet still present a high electrical impedance and a relatively high impedance to the passage of most small molecules.”

A feature noted in the anastomosing septate junction is the fact that the lanthanum tracer is able to penetrate into the “closed” spaces of the network, implying that in this junction at least, the septal walls themselves are also permeable to this tracer. Nonetheless, the fact that septa may terminate or be leaky to tracers is not considered a major objection to the concept of septate junctions having an occluding function, as will now be explained. The vertebrate tight junction in some cases has been shown to be leaky to lanthanum tracers (Martinez-Palomo and Erlij, 1975), and even relatively complex tight junctions have some permeability to ion flow (Claude, 1978; Claude and Goodenough, 1973). In the few invertebrate tissues that have been studied, septate junctions have been shown to reduce paracellular permeability of tracers such as lanthanum, horseradish per-

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**FIG. 6.** A freeze-fracture replica of glutaraldehyde-fixed tissue showing particles on the P face of an anastomosing junction membrane. These particles are regular in size and shape, being about 7.5 nm in diameter, and form an anastomosing pattern. This may not be obvious without prior knowledge of the tangential appearance of the septa. (In this and all subsequent micrographs of freeze-fracture replicas, the encircled arrowhead indicates direction of shadowing.) × 100 000.

**FIG. 7.** A freeze-fracture replica of glutaraldehyde-fixed tissue showing the 4- to 5-nm diameter pits of the E face of an anastomosing junction membrane. An occasional particle (arrows) is left adhering to this face. In places the pits have joined to form shallow grooves (double arrows). × 100 000.
Fig. 8. A freeze-fracture replica of glutaraldehyde-fixed tissue showing a change of face in the plane of the fracture. Rows of particles on the P face (P) of the anastomosing junction are seen to line up with rows of pits on the E face (E) (arrows). Unfixed tissue shows a similar appearance with rows of particles on the P face lining up with rows of pits on the E face. × 130,000.

Fig. 9. Freeze-fracture appearance of the E face of unfixed junctional membrane. The 4- to 5-nm diameter pits forming the network are more distinct and separate from one another than those seen on the E face of fixed material. Very few particles are left adhering to this face (arrows). The P face of unfixed tissue is similar to that of fixed tissue. × 110,000.

Fig. 10. A diagrammatic representation of the anastomosing septate junction. This diagram shows the pits seen on the E face (E) and the particles seen on the P face (P) after freeze fracturing. It also shows our interpretation of how the septa are constructed based on lanthanum tracer-impregnated tissue-thin sections and uranyl acetate/lead citrate-stained thin sections.

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TABLE I

THE DISTRIBUTION OF INTRAMEMBRANE PARTICLES ON THE E AND P FACES IN THE VARIOUS FORMS OF
SEPTATE JUNCTION, AND IN THE TIGHT JUNCTION, AFTER FREEZE FRACURING

<table>
<thead>
<tr>
<th>Fixed tissue</th>
<th>Unfixed tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hydra type</strong>&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>(Filshie and Flower, 1977)</td>
<td></td>
</tr>
<tr>
<td>P face</td>
<td>E face</td>
</tr>
<tr>
<td>Some particles, most often pits or grooves</td>
<td>Mainly particles, some grooves or pits</td>
</tr>
<tr>
<td>Pleated</td>
<td>Grooves or pits</td>
</tr>
<tr>
<td>(Filshie and Flower, 1977)</td>
<td></td>
</tr>
<tr>
<td>Smooth</td>
<td>Grooves or pits</td>
</tr>
<tr>
<td>(Flower and Filshie, 1975)</td>
<td></td>
</tr>
<tr>
<td>Anastomosing</td>
<td>Grooves</td>
</tr>
<tr>
<td>Tight</td>
<td>Pits</td>
</tr>
<tr>
<td>(Staehelin, 1973)</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Table I shows this distribution for both fixed and unfixed tissues. (The table shows a slightly simplified overview. Faces listed as having particles for example, have a majority of particles, but may also have some pits or grooves.)

<sup>b</sup>Wood (1977) claims a reversal of faces occurs between Hydra-type gastrodermal and epidermal junctions. Filshie and Flower (1977) did not make this distinction however.

oxidase, and ruthenium red (Hand and Gobel, 1972; Lane and Treherne, 1972; Newell and Skelding, 1973). They also have been shown to have high resistance to ionic flow through the extracellular space from the outside in as shown by electrophysiological experiments (Josephson and Macklin, 1967, 1969; Loewenstein and Kanno, 1964). Even though tracers, ions, or molecules can penetrate through septal walls, or travel between septa, their rate of penetration must be severely limited compared to an open space. Adjustment to leakiness of this order could be within the normal physiological capacity of the organism.

Certainly the occurrence of an anastomosing septate junction which appears to be a structural intermediate between the tight and septate junctions supports the proposition that the two junctions have similar functions (occlusion and attachment) and that the various forms of septate junction are invertebrate precursors of the tight junction.

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TWO NEW SEPTATE JUNCTIONS IN THE
PHYLUM COELENTERATA

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SUMMARY

Freeze-fracture of fixed and unfixed tissue, lanthanum tracer and conventional thin-section
studies have revealed 2 new types of septate junction in the class Anthozoa, phylum Coelen-
terata. These new junctions have the 15-18-nm intercellular spacing of all other described
septate junctions and are found around the apical circumference of cells lining a lumen or
outside edge. However, in freeze-fracture replicas and tangential views of lanthanum-
impregnated tissue, they are seen to be quite different from other known septate junction
types. One of the new junctions is found in endothelial tissue such as that lining the gut or
the inside of the tentacles. In tangential view it is seen to consist of relatively short, straight,
double septa, again with lateral projections. In freeze-fracture of unfixed tissue, the junction
consists of double rows of particles on the P face, the particles of one row being rounded,
those of the other being elongated at right angles to the line of the septum. This dichotomy in
particle size is unexpected, as the 2 halves of the septa as seen in tangential view are symmetrical.
In freeze-fracture of fixed material the particle arrays remain on the P face and appear similar
to those of unfixed material, but never as clear. In fixed tissue, some distortion has occurred
and in extreme cases septa appear as a single broad jumbled row of particles. In this double
septa junction, the rows of particles seen in freeze-fracture are occasionally seen to anastomose
with a septum dividing into 2 and a third row of particles aligning with the 2 new septa to
form their double particle rows. In both fixed and unfixed tissues, the E face of the junction
consists of wide, shallow grooves.

The second of the new junctions occurs in epithelial tissue, such as around the outer edge
of sea-anemone tentacles, and consists of long wavy septa with lateral projections. In views
where these projections appear longest, they arise predominantly from one side of the septa.
In freeze-fracture of both fixed and unfixed tissue, this junction appears as rows of closely
spaced particles on the P face. Occasionally rows of particles are seen on the E face, but usually
this face is characterized by shallow grooves. In some aspects these 2 new junctions have
features in common with the Hydra type junction also found in the Coelenterata. In all 3
types septa are relatively straight, rather than pleated, and there are lateral projections on
the septa.

INTRODUCTION

Septate junctions, which are thought to be the epithelial sealing junctions of
invertebrates, were first described by Wood (1959). Subsequent investigations based
on conventional staining studies (Overton, 1963; Wiener, Spiro & Loewenstein, 1964;
Locke, 1965; Gouranton, 1967; Messier & Sandborn, 1967; Bulivant & Loewenstein,
1968; Danilova, Rokhlenko & Bodryagina, 1969; Leik & Kelly, 1970) suggested
that their structure might vary in different invertebrate tissues. Although many of these interpretations seem to have been due to the different fixation and staining techniques used, the diversity of septate junction structure has since been confirmed by the use of lanthanum tracer and freeze-fracture techniques (Gilula, Branton & Satir, 1970; Hudspeth & Revel, 1971; Hand & Gobel, 1972; Noirot & Noirot-Timothée, 1967; Baskin, 1976; Dallai, 1976; Green, 1978), and a number of types of septate junction have now been well established (Noirot & Noirot-Timothée, 1967; Satir & Gilula, 1973; Staehelin, 1974; Flower & Filshie, 1975; Baskin, 1976; Welsch & Buchheim, 1977; Wood, 1977; Filshie & Flower, 1977; Lane & Harrison, 1978) although the details of their structures may still be open to argument.

Such investigations have shown that studies utilizing both lanthanum tracer and freeze-fracture techniques are necessary to define adequately the inter- and intra-membrane structures of the septate junction. Furthermore, more information can be gathered from freeze-fracture experiments if tissue both unfixed and fixed before freezing is examined, as large differences sometimes occur in the appearance of septate junctions when they are subjected to these different prefreezing treatments (Flower & Filshie, 1975; Filshie & Flower, 1977). Only when such a variety of experiments have been carried out is adequate information available to decide whether the septate junction present in any new tissue fits into one of the previously reported categories or represents a new variation. Earlier junction work has often been inconclusive in assigning a junction to a specific category, due to the range of different techniques utilized. The present investigation reports on 2 new types of septate junction which have been discovered in the phylum Coelenterata. They have been characterized using thin-section, lanthanum tracer and freeze-fracture techniques.

MATERIALS AND METHODS

Sea anemones of a number of species collected locally were used: *(Gonactinia olivacea, Actinothoe altecineta, Igastinia tenebrosa* and *Culacehs muscosa,*

Tissue was dissected from the tentacles and gut lining so as to obtain both epithelial and gastrodermal tissue. For sectioning studies, tissue was fixed in a 6% glutaraldehyde in a buffered solution. Buffering solutions consisted of either seawater buffered to pH 7.4 with

Fig. 1. Thin section of lanthanum-impregnated anemone gastroderm showing a tangential view of the gastrodermal septate junction. The septa appear as double lined structures with lateral projections, usually clearest off one side at a time (arrows). Projections are also visible between the 2 halves of the septa giving a cross-hatched appearance. The 2 halves of the septa appear symmetrical except where projections appear longer on one side or the other. × 190,000. Inset, lanthanum-impregnated gastrodermal junction showing a length of septa with lateral projections about 6 nm long clearly visible on one side (arrow). The projections are spaced at about 7.5 nm along the septa. × 215,000.

Fig. 2. Thin section of lanthanum-impregnated gastrodermal septate junction. The septa have been cut at an angle. Some evidence for rows of dots running parallel to the septa can be seen (arrows). They are more easily recognized if the micrograph is viewed at an angle along the line of the septa. × 145,000.
Coelenterate septate junctions

0.1 M cacodylate; or 0.1 M cacodylate buffered to pH 7.4 and NaCl or sucrose added to give osmolarities equal to that of seawater. Material for positive stained sectioning was post-fixed in a 1% OsO₄ and thin sections were stained with uranyl acetate and lead citrate (Venables & Coggeshall, 1963). Lanthanum incorporation into tissue was achieved by the method of Shaklai & Tavassoli (1977). The tissue was acetone dehydrated and embedded in the usual manner without positive staining.

Material for freeze-fracturing was either fixed in glutaraldehyde in buffered solution for 1 h before being placed in 25% glycerol in pH 7.4 buffer or placed directly into a 25% glycerol-seawater solution for 15–30 min before freezing. Specimens were frozen in freon 12 at 150 °C and freeze fractured either by the method of Buvillant (1973) or in a Balzers BA300 machine as described by Moor & Muhlethaler (1963).

RESULTS

The class Anthozoa of the phylum Coelenterata has been found to have 2 distinct, and unique, variations of the septate junction. Both variations viewed in cross-section show septa between cells spanning a 15-nm intercellular space as is characteristic of other described septate junction types (Stachelin, 1974) (Fig. 3 inset, Fig. 12 inset). One variation is apparently restricted to epithelial cells such as those surrounding the sea anemone tentacle, and the other is found between endothelial lining cells such as those in the gastroderm, or those lining the inside lumen of the tentacles. Both junctions consistently appear in thin-section and freeze-fracture studies at the apical end of the cells in which they are found. They therefore form a belt around the apical circumference of these cells in a similar manner to previously reported septate junction types (e.g. Stachelin, 1974).

Gastrodermal tissue

In tangential section of lanthanum-impregnated material the junctions of endothelial tissue appear as twin septa separated by about 6.7 nm (Fig. 1). In many places lateral projections can be seen protruding from the side of the twin septa (arrowed in Fig. 1 and inset). Projections are also often seen between the twin septal halves giving a cross-hatched appearance. The lateral projections can be up to 6 nm long and are spaced at about 7.5 nm along the septa. In some areas the lateral projections

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Fig. 3. Lanthanum-impregnated gastrodermal junction showing septa viewed at different angles where the junction has been sectioned near a gap between two cells. The double septa are clearly visible when the section is tangential to the junction (small arrow), but with(d small amount of tilt about an axis running within the plane of the section and along the line of the septa, they appear as single wide, blurred lines (arrow a). As the angle of tilt increases the septa appear sharp again, but as a single line structure with long side projections up to 5 or 9 nm long (arrow b), × 175,000. Inset, a uranyl acetate/lead citrate stained cross-section of the gastrodermal type anemone junction showing the septa in cross-section view and the 15–18-nm intercellular spacing. × 80,000.

Fig. 4. Freeze-fracture replica showing a general view of the P face of an unfixed gastrodermal septate junction. The junction appears as a series of double lines of particles. The rows of particles are asymmetrical with those on one side appearing larger; the larger particles being consistently on the same side of any one septum. In this and all subsequent freeze-fracture micrographs, the encircled arrow in the bottom left corner indicates the direction of shadowing. × 145,000.
Coelenterate septate junctions

are present on only one side of the twin septa. In such cases they can sometimes be seen on one side in one area and on the other side of the same twin septum in a different area (arrows, Fig. 1). In regions where the lateral projections are present on both sides of the twin septa, they can be seen to be staggered on the 2 sides. As these projections are readily identified only over short lengths of the septa at a time, they must either be discontinuous or need to be oriented quite precisely within the section for them to be identified. At times rows of dots can be seen between septa (Fig. 2). This is clearer if the photograph is viewed at a glancing angle along the line of the septa. Whether or not these are connected directly to the lateral projections of the septa is not clear. In many sections, twin septa can be seen running at an angle towards each other and almost anastomosing (see Fig. 1). However, in all the sections which we have examined a small gap is always found between the 2 twin septa and no unambiguous case of anastomosis has been found.

Wherever the septa are sectioned in such a way that they are tilted within the section, their appearance can be different to the twin lines seen in true tangential view. When the axis of tilt is within the plane of the section, but at right angles to the line of the septa, there is little change, but when the axis of tilt is within the plane of the section and also runs along the line of the septa a considerable change in appearance results. With small amounts of tilt the twin septal appearance changes into a blurred single septum (a in Fig. 3). As the tilt increases a single narrow septum becomes apparent (b in Fig. 3), usually with rather large side projections up to 8 or 9 nm long.

Freeze-fracture replicas of these gastrodermal junctions show that the intramembrane arrangement of particles approximates the twin septal arrangement revealed by lanthanum tracer studies. In tissue glycerinated, but unfixed, freeze-fracture replicas reveal twin rows of particles on the P face of the junction, with the

Fig. 5. Freeze-fracture replica of a P face of unfixed gastrodermal junction. The twin rows of particles are approximately perpendicular to the shadowing direction. It can be seen that one row of particles, that nearest the shadowing source, is smaller than the other (arrow). × 150 000.

Fig. 6. Freeze-fracture replica of the P face of the gastrodermal type septate junction showing that the particle size difference between the twin rows of each septum is still evident when the row of larger particles is closest to the shadowing source (arrow). Unfixed tissue. × 150 000.

Fig. 7. A freeze-fracture replica of the gastrodermal junction in which the rows of particles of the P face lie approximately along the direction of shadowing. The difference between the 2 rows making up each septum is most evident in this orientation, with the larger particles appearing elongated into short rods. Unfixed tissue. × 150 000.

Fig. 8. Freeze-fracture of an E face of a gastrodermal junction. An array of broad shallow grooves can often be seen on these faces. There is little difference between fixed and unfixed tissue. × 85 000.

Fig. 9. A replica of the E face of the gastrodermal junction in which fine crossstriations (between arrows) can be identified across the broad, shallow grooves. The separation of these striations is identical to the separation of particles in the twin rows seen on the P faces of freeze-fracture replicas, being about 7.5 nm apart. × 130 000.
2 rows separated by about 7 nm, so matching the separation between the 2 halves of the twin septa seen in lanthanum tracer studies (Fig. 4).

Examination of such replicas reveals that the particles in the 2 rows are distinctly different. When the rows of particles are approximately perpendicular to the shadowing direction, it can be seen that the particles of one row appear much smaller than those of the other. This difference can be observed when either the small particle side of the twin row or the large particle side is closest to the shadowing source (Figs. 5, 6). When the rows of particles are approximately parallel to the shadowing direction, however, the difference between them is even more marked (Fig. 7). In this situation the small particles are most easily seen to consist of normal 'round' particles, while the larger particles appear to be elongated in a direction perpendicular to the line of the septa. The small particles are 5-6 nm in diameter, whereas the larger particles are about 8-9 nm long and about 5-6 nm in width. Where the spacings can be identified, particles in both rows have a separation of 7-5 nm along the septa. Although it is difficult to be certain because of the somewhat random orientation of the septa, the row of larger particles tends to be on the side of the septum nearest the apical edge of the cell. The E faces of freeze-fracture replicas in the junctional region show an array of broad, shallow grooves (Fig. 8). These are occasionally seen to have fine cross striations up to 12 nm long and 7.5 nm apart (Fig. 9).

In the freeze-fracture replicas this junction seems to consist of an array of relatively short rows of twin septa (see Fig. 4). Often these twin rows appear to be well ordered over small areas. In such regions numbers of septa run parallel to each other for some distance and often a number of such areas can be identified, separated by areas of less-ordered structure. In other replicas, the twin septa sometimes show a form of anastomosis, and in localized areas this can be very common (Fig. 10). Whether this type of appearance should be considered true anastomosing is not clear, as individual particle rows do not anastomose. Instead, the 2 parallel rows of particles making up a given septum branch away from each other, while a third row of particles becomes continuous with both the branching rows so that 2 new twin septa are formed at the fork (Fig. 10). In some instances all 3 septa coming away from such an anastomosis terminate, but in many instances further anastomosing occurs so that a complex network of twin septa is built up. As far as can be ascertained, considerable anastomosing seems to occur towards the apical edge of the junction, but in this region

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**Fig. 10.** Freeze-fracture of an unfixed gastrodermal junction showing a form of anastomosis of the septa. Careful examination of the septa around the point of anastomosis indicates that rows aa, bb, and cc' are continuous. Row aa are large type particles and row bb are small type particles. Row cc' seems to change type at the anastomosis, thus conserving the asymmetry of these septa. Thus at c, the row appears to consist of small particles, and at c' of large particles. × 145,000.

**Fig. 11.** A freeze-fracture replica of the P face of fixed gastrodermal tissue in the junctional region. In fixed tissue the twin rows of particles are less well defined, and appear in places as single jumbled rows (at particles (upper right hand corner). The size difference between particles in the twin rows, easily visible in unfixed tissue, is not as clear in fixed tissue. × 125,000.
Coelenterate septate junctions

details become confusing because of the dense network of septa. When an anastomosis does occur, at least one row of particles must change type if the typical asymmetrical particle pattern of the twin septa is to be maintained. This appears to occur as is seen in Fig. 10. In this figure the row of particles marked a, a are of the large type and the row of particles marked b, b are of the small type. As these 2 rows do not appear to change type as they pass the anastomosis, the row marked c, c' should change type. The area marked c does appear to consist of small particles while the area marked c' consists of large particles. It should be noted that in many instances where septa run towards each other at an angle, anastomosis does not appear to occur, the twin septa stopping just short of each other.

Freeze-fracture replicas of fixed tissue show essentially the same features, with the particles remaining on the same face as in unfixed tissue; the P face. However, the appearance of the particle arrays is never as crisp and some distortion appears to have occurred during fracturing, presumably because of binding of the particles caused by fixation (Fig. 11). In extreme cases the septa appear as single broad jumbled rows of particles. The size difference between the twin particle rows seen in unfixed tissue is less evident in fixed tissue (Fig. 11).

Epithelial tissue

When tangential sections of lanthanum-impregnated epithelial septate junctions are examined (Fig. 12), it is immediately obvious that these junctions are different from the gastrodermal septate junctions, as most septa present are single structures. Septa are also seen to be long wavy structures, rather than the shorter, straight septa characteristic of the gastrodermal junctions. As in the gastrodermal junctions, large lateral projections can be identified along the septa. They appear to be about 7 nm long and have a separation of about 7 nm along each septum. Careful examination of the sections show that the lateral projections are clearest when present on one side of the septum only. In some areas projections are seen off both sides of the septa (Fig. 13), but in these cases they are less well defined. As in the gastrodermal septa, the lateral projections cannot be identified along the whole length of the septa. They can, however, be visible for a far greater proportion of the septal length than are the

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**Fig. 12.** Tangential view of an epithelial type septate junction impregnated with lanthanum tracer. The septa are seen to consist of long wavy structures with lateral projections. These projections appear longest and clearest when they are visible on only one side of the septum. × 220,000. Inset, uranyl acetate/lead citrate stained cross-section of the epithelial type anemone junction showing the septa in cross section and the 15-18-nm intercellular spacing. × 105,000.

**Fig. 13.** Tangential view of lanthanum-impregnated epithelial type junction with the lateral projections off the septa less visible than in Fig. 12, but off both sides of the septa in most areas, although where they are clearest they are predominantly off one side. Septa run parallel in places, but do not form the perfectly parallel double septa characteristic of the gastrodermal septate junction. A septum is seen abutting on, but not fusing with another (arrow). This gives a branching effect, but the join has never been observed to be complete. × 220,000.
lateral projections on the gastroduodenal septa. Only occasionally do the septa run parallel as twin rows (Fig. 13). Even then the 2 septa do not appear to be exactly parallel to each other. Septa sometimes abut on one another approximately at right angles (arrowed in Fig. 13) to give a branching appearance, but do not join completely.

Freeze-fracture replicas of epithelial junctions show an even greater variation between this type and the gastroduodenal junction than do the lanthanum tracer studies. On replicas of both fixed and unfixed tissues rows of closely spaced particles are seen on the P face (Fig. 14). These particles vary somewhat in size, but most appear to be between 8 and 9 nm in diameter. The particles are not regularly enough arranged to suggest a standard separation, probably due to distortion during the fracturing process. Occasionally rows of particles can be seen on the E face, but they are usually fragmentary (Fig. 15) and in most replicas the E face of the junction is characterized by an array of shallow grooves. In most replicas the septa run singly, and only occasionally do septa run for short distances parallel to each other (arrowed in Fig. 14) in an arrangement which probably parallels the twin septa appearance occasionally seen in tangential lanthanum-impregnated sections (Fig. 13). In these epithelial septate junctions, there is no evidence for a dichotomy of particle size or shape. Even when 2 septa are seen running parallel to each other, both rows of particles appear the same, with no sign of elongated particles as is seen in the gastroduodenal septate junctions. No evidence of anastomosing has been observed in freeze-fracture replicas of the epithelial septate junctions.

DISCUSSION

The term septate junction (or septate desmosome) has been used to describe a number of invertebrate structures in which adjacent cell membranes are held apart at a constant separation of 15 nm and a series of septa or bars join the 2 membranes together (see reviews by Satir & Gilula, 1973, and Stachelin, 1974). Although septate junction-like structures have been reported in vertebrates (Stachelin, 1974), they do not appear in general to be closely related to the invertebrate septate junction, which appears to perform a bonding and sealing function in most invertebrate tissues. The only exception to this general statement is the septate junction-like structures reported by Connell (1978) in canine testis. Although there are many differences, the junction described by Connell also has many similarities to the gastroduodenal junction described in the paper, including a tendency to form paired septa. However, since Connell's

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Fig. 14. Freeze-fracture replica showing both faces of an unfixed epithelial type septate junction. The particles mostly appear on the P faces in both fixed and unfixed tissue. Only occasionally do the particle rows run parallel to each other (arrow). × 73,000.

Fig. 15. Freeze-fracture replica of a similar area of unfixed epithelial type junction to that seen in Fig. 14. Normally the E faces of the junction appear as grooves as can be seen in some areas of this micrograph. Occasionally however, short rows of particles can also be found on E faces (arrow). × 67,000.
junction seems to have many characteristics of a tight junction as well as some septate junction features, its status with respect to invertebrate septate junctions is unclear.

Many different interpretations of invertebrate septate junction structure, based on conventional staining techniques, have been proposed since their initial discovery by Wood (1959). It was difficult to be sure whether these proposals represented real differences in structure or just resulted from variations induced in preparation, and it was only with increased use of tracer and freeze-fracture techniques that the variability of septate junction structure has become better defined. Thus, using a variety of techniques a number of types of septate junction have now been well characterized. These are the pleated septate junction (Locke, 1965; Danilova et al. 1969; Gilula et al. 1970; Gilula & Satir, 1971; Flower, 1971; Noirot-Timothée & Noirot, 1973) found in molluscs and the epithelia of arthropods; the smooth (or continuous) septate junction (Noirot & Noirot-Timothée, 1967; Dallai, 1970; Hudspeth & Revel, 1971; Flower & Filshie, 1975) found in endothelial tissues of arthropods; Hydra type septate junctions (Wood, 1959; Danilova et al. 1969; Filshie & Flower, 1977; Wood, 1977); a variation of the smooth septate junction reported in the Merostomata (Lane & Harrison, 1978) and a variation in the pleated septate junction found in the polychaetes (Baskin, 1976; Welsch & Buchheim, 1977). The presence of 2 different types of septate junction in epithelial and gastrodermal tissue of the same animal has previously been reported only in arthropod tissue where the pleated and smooth septate junctions are present, but also occurs in Echinoderms (Green, Bergquist & Bullivant, 1979).

In both types of junction reported in this paper the septa have lateral projections on their sides. These projections are usually more readily visible in the epithelial type junction, suggesting that this type of junction may have larger projections than the gastrodermal junction. Certainly in the gastrodermal type junction these lateral projections are only easily seen along small lengths of the septa. This would indicate either smaller projections in this type of junction or the possibility that there could be several projections in the depth of the septa. This latter possibility could provide an explanation of why any slight misalignment of the junction in a section would lead to the inability to resolve the lateral projections, as a slight tilt of several superimposed projections would quickly lead to overlap with adjacent projections and so to loss of any recognizable repeating structure in the image.

Thus the intermembrane structure of these 2 septate junctions is very similar to that of the Hydra type (Filshie & Flower, 1977; Wood, 1977) also found on the Coelenterata. In all these 3 types of junction septa are relatively straight or only slightly pleated and there are lateral projections on the septa. The main difference as seen in sectioning studies between the epithelial type septate junctions reported here and the Hydra type is the presence of lateral projections on only one side of the epithelial type septa in regions where they are most clearly delineated. The main difference between the gastrodermal type junction and the Hydra type junction is of course the twin septal structure seen in the gastrodermal type. Another difference between the Hydra type and the 2 types of junction reported here is the fact that
lateral projections have only been easily seen in the present study when the junctions are more or less tangentially sectioned. Although such projections could be seen in this orientation in *Hydra* septa they were much more readily seen in septa which pass through a section at an angle of about 30° (Filshie & Flower, 1977). A similar enhancement effect has not been observed in epithelial junctions during the present study, although the gastrodermal junction does show apparently larger side projections when viewed at an angle such that the twin septum appears as a single septum. It was suggested (Filshie & Flower, 1977) that the situation in *Hydra* could be explained in terms of a staggered arrangement of lateral pegs. The present results, for the epithelial junction at least, in contrast, can be satisfactorily explained if each lateral projection is a single buttress protruding from the septa.

*Freeze-fracture* results further differentiate clearly the 2 junctions described here from other septate junction types. The asymmetrical twin rows of particles seen in the gastrodermal type junction is a unique and prominent identifying feature. In the epithelial type junction, particle arrays seen in freeze-fracture resemble to a large extent those described in *Hydra* type septate junctions, but they occur mainly on the P face, while the particles of the *Hydra* type junction are seen mainly on the E face for fixed and unfixed tissue (Filshie & Flower, 1977). The epithelial junction particles are never seen as regularly shaped, or spaced, as those of the pleated septate junction (Stachelin, 1974), or the echinoderm anastomosing junction (Green, Bergquist & Bivallant, 1979). They are also never seen as short rod-like structures such as occur in the smooth septate junction (Stachelin, 1974) or the meristomata junction (Lane & Harrison, 1978) and finally they are generally more closely spaced, even though irregularly, than the particle arrays characteristic of the annelid septate junction (Welsch & Buechheim, 1977).

Parallel arrays of septa on the apical surface of the lateral membranes are common in most types of septate junction where they may cover several microns of lateral cell membrane. In most cases the junctional network normally becomes more open and apparently disorganized towards the basal end of the junction. In the anemone gastrodermal junction, the array of septa does not appear to be as extensive as it is in most septate junctions and the septa are found in all orientations throughout the junction, although they are similarly more tightly packed toward the apical edge. In some areas therefore, this junction is very reminiscent of the open ladder-like networks present in many tight junctions (Stachelin, 1974).

It is interesting to note in the gastrodermal junction that despite a perfect symmetry between the 2 halves of each septa as seen in tangential lanthanum-impregnated sections, an asymmetrical situation is seen in freeze-fracture studies. The situation within the membrane of rounded particles corresponding to one side of the septum, and rod-like particles to the other, does not reflect the exact situation as seen between the membranes. Furthermore, this junction also shows a type of anastomosing in some of the freeze-fracture replicas, a feature previously only associated with tight junctions. Somewhat surprisingly we have been unable to find similar anastomosing in sectioning studies. Although it is possible that we have failed to observe the intermembrane anastomosing in sections and that in fact it does occur, the large number of
sections examined would argue against this possibility. Thus our observations suggest that the intermembrane septa do not anastomose, although the intramembrane structures do so in some cases. It should however, be emphasized that in most cases when septa are seen approaching each other obliquely in freeze-fracture replicas, anastomosis does not occur, the septa stopping just short of each other, exactly as is observed in sections.

Another point of interest is the situation observed in lanthanum-impregnated sections where the twin line appearance of the gastrodermal junction septa fuses into one thin line as the angle of section gets further away from a true tangential view. If solid septa spanned the entire 15-nm intermembrane space between cells, then they would be expected to appear as wide bars as the section is tilted about an axis along the lines of the septa. Instead the appearance, initially of a wide blurred line, but then of a single narrow line approximately the same thickness as one side of the twin septa, but with longer side projections, suggests that the septa may not fully span the 15-nm space between membranes.

The main purpose of this paper is to report the 2 new types of septate junction. A more precise understanding of the structure of the septa themselves depends on observations with the tilting stage of the electron microscope. Such experiments are at present in progress.

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