THE CELL JUNCTION IN A LAMELLIBRANCH
GILL CILIATED EPITHELIUM

Localization of Pyroantimonate Precipitate

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ABSTRACT

The junctional complex in the gill epithelium of the freshwater mussel (Elliptio complanatus) consists of an intermediary junction followed by a 2–3 µ long septate junction. Homologous and heterologous cell pairs are connected by this junction. After fixation with 1% OsO₄ containing 1% potassium pyroantimonate, electron microscopy of the gill reveals deposits of electron-opaque precipitate, specifically and consistently localized along cellular membranes. In both junctional and nonjunctional membrane regions, the precipitate usefully outlines the convolutions without obliterating the 150 Å intercellular space, which suggests the rarity or absence of either vertebrate-type gap or tight junctions along the entire cell border. The precipitate appears on the cytoplasmic side of the limiting unit membranes of frontal (F), laterofrontal (LF), intermediate (I), lateral (L), and postlateral (PL) cells. The membrane surfaces of certain vesicles of the smooth endoplasmic reticulum, of multivesicular bodies, and of mitochondrial cristae contain precipitate, as does the nucleus. In other portions of the cell, precipitate is largely absent. The amount of over-all deposition is variable and depends on the treatment of the tissue prior to fixation. Deposition is usually enhanced by pretreatment with 40 mm NaCl as opposed to 40 mm KCl, which suggests that the precipitate is in part sodium pyroantimonate. Treatment with 0.2 mm ouabain does not enhance deposition. Regional differentiation of cell membranes with respect to their ability to precipitate pyroantimonate is found in at least three instances: (a) between the ciliary membranes and other portions of the cell membrane: the precipitate terminates abruptly at the ciliary base, (b) between the LF and I cell borders: the precipitate is asymmetric, favoring the LF side of the junction, and (c) between the septate junctional membrane and adjacent membrane: the precipitate occurs periodically throughout the septate junction region with the periodicity corresponding to the spacing of the septa. This suggests that different regions of the cell membrane may have differing ion permeability properties and, in particular, that the septa may be the regions of high ion permeability in the septate junction.

INTRODUCTION

This paper will describe the junctional complex of a classic invertebrate cell system, the lamellibranch gill epithelium, that exhibits coordinated activity of the tissue across cell boundaries, notably illustrated by the metachronal wave traversing the rows of ciliated cells.

In the past 10 years, cell biologists have become increasingly concerned with the morphology and
the functional significance of the junctional specializations of membranes of epithelial cells, both in vertebrates (Farquhar and Palade, 1963, 1965; Revel and Karnovsky, 1967; Brightman and Reese, 1969) and in invertebrates (Wood, 1959; Locke, 1965; Gouranton, 1967; Bullivant and Loewenstein, 1968; Stuart and Satir, 1968; Danilova et al., 1969). The junctional specializations of vertebrates include (a) the gap junction, thought to be a region of low membrane resistance to the passage of ions and to function in coupling cells electrically in certain situations, and (b) the tight junction, that forms a barrier to the free passage of material through the intercellular space. It is not now definitely established whether the tight junction also serves a coupling function. The unit membranes of a tight junction are fused at their junctional surfaces so that the intercellular space is completely obliterated; a small 20 A gap persists between the unit membranes in the gap junction.

The main morphological specialization of the junctional surface of epithelial cells of most invertebrates that have been carefully studied (Table I) corresponds neither to a true tight junction nor to a gap junction but is rather a septate junction or septate desmosome (Wood, 1959). Bullivant and Loewenstein (1968) claim that the septate junction is the only membrane element of any extent in Drosophila salivary gland cells that could act to couple the cells electrically (Loewenstein and Kanno, 1964; Wiener et al., 1964). Accordingly, the septate junction may have both occluding and coupling properties so as to substitute in these cells for both tight and gap junctions. One important criticism of this conclusion arises because the cell border is difficult to trace from one end of the cell to another, and small but functionally significant regions of tight or gap junction might be missed. Furthermore, between certain insect cells there are in addition to septate junctions what seem to be tight or perhaps gap junctions (Stuart and Satir, 1968) so that the possibility that these junctions occur in a given invertebrate epithelium is a real one.

Junctional differentiations of the cell membranes of the gill epithelial cells have not been examined in detail previously. Preliminary reports on the presence of septate junctions between the cells and their possible role in cell coupling have appeared elsewhere (Satir, 1961; Gilula and Satir, 1969, 1970).

In 1962, Komnick proposed that potassium pyroantimonate (K\textsubscript{5}Sb\textsubscript{3}O\textsubscript{14}H\textsubscript{2}O) could be utilized for intracellular localization of sodium ion (Na\textsuperscript{+}) at the electron microscope level. Apparently, under proper conditions, sodium pyroantimonate precipitates as a fine, electron-opaque particle, although at the present time some controversy exists as to whether the technique really reflects the localization of all intracellular sodium. Kaye et al. (1965) showed that, in rabbit corneal cells, the pyroantimonate precipitate appears on the cytoplasmic side of unit membranes, and that the amount of precipitate increases after pretreatment of the preparation with ouabain. Utilizing the Komnick and Kaye procedure, we can demonstrate regional differentiation of the apical and junctional cell membranes of the gill cells with respect to the occurrence of the precipitate.

**MATERIALS AND METHODS**

Freshwater mussels have been obtained from Carolina Biological Supply Company, Gladstone, Oregon. As reported previously (Satir, 1965) the specimens used are mainly *Elliptio complanatus*, but occasional animals are from other genera (e.g. *Anodonta*). No
| Phylum         | Genus                | Tissue                        | Junction type* | Reference                  |
|---------------|----------------------|-------------------------------|----------------|----------------------------|
| Coelenterata  | Chlorohydra;         | epidermis, gastrodermis       | sj             | Wood, 1959, 1961           |
|               | Pelmatohydra         |                               |                |                            |
|               | Cordylophora         | ectoderm                      | sj             | Overton, 1963              |
| Rotifera      | Asplanchna           | hypodermis-coronal cell junction | sj          | Koehler, 1965              |
| Platyhelminthes| Dugesia              | auricular epithelum (receptor cells) | sj          | MacRae, 1967               |
| Annelida      | Hirudo               | visual cells                  | sj             | Lasansky and Fuortes, 1969 |
|               | Lumbricus            | epidermis                     | i, sj, d       | Coggeshall, 1966           |
| Mollusca      | Elliptio             | gill epithelium               | i, sj          | Satir, 1961; this study    |
|               | Buccinum; Neptunea   | osphradium                    | i, sj          | Welsch and Storch, 1969    |
| Arthropoda    | Artemia              | maxillary gland epithelium    | i, sj          | Tyson, 1969                |
|               | Procambarus          | gut epithelium                | sj             | Komuro and Yamamoto, 1968  |
| Insecta       | Calpodes             | epidermis                     | i, sj, tj      | Locke, 1965                |
|               | Chironomus           | salivary gland                | sj             | Bovillant and Loewenstein, 1968 |
|               | Zoatermopisis        | sternal gland;                | i, sj          | Stuart and Satir, 1968     |
|               |                      | campaniform sensillum         | sj, tj, d      | Smith et al., 1969         |
| Echinodermata | Tripneustes          | blastula                      | sj             | Bolinsky, 1959             |
| Chordata      | Mesocricetus         | nervous system supporting cells | sj             | Bargmann et al., 1962      |

* sj, septate junction; i, intermediary junction (zonula adherens); tj, tight junction; d, desmosome (macula adherens).
obvious species specific differences have been discovered in the characteristics of the cell membrane that concern us in this report.

Pretreatment

Mussels were stored in a laboratory aquarium in deionized water prior to study. The gills are excised from the mussels and stripped (Satir, 1963) in deionized water. For this study, pieces of gill tissue have been activated with 0.04 M NaCl or KCl or with these salts and 0.2 mm ouabain, or with 1% K$_2$Sb$_2$(OH)$_4.4$H$_2$O (Fisher Scientific Co., Pittsburgh, Pa.) (0.04 M K$^+$) for a minimum of 20 min in the activating solution before fixation. Some pieces are fixed in water and are inactive. Adjacent pieces from one gill are used for comparative studies. The gills are examined microscopically prior to fixation, and estimates of metachronism have been made. The metachronism of the L cilia is generally preserved at fixation when osmium tetroxide is used as the initial fixative (Satir, 1963).

Electron Microscopy

Preparation Procedures

Tissue has been fixed in (a) 1% OsO$_4$, buffered in standard buffers, or unbuffered, or (b) unbuffered or collagen-buffered 1% OsO$_4$ containing 1% potassium pyroantimonate for 1 hr, or (c) 2.5% or 5% glutaraldehyde in 0.1 M cacodylate buffer pH 7.2 for 2-4 hr followed by either (a) or (b). All fixations were done at room temperature. In many localization experiments during dehydration, all aqueous solutions contained 4 or 40 mm NaCl and 4 mm versene. Material was embedded in Epon or Araldite and sectioned into solutions also containing sodium-versene. Thin sections were generally gold-silver to gray in color. Sections were examined with a Siemens 1A or 101 Elmoskop unstained or stained with either (a) saturated uranyl acetate in 50% ethanol for up to 90 min or 7.5% aqueous uranyl magnesium acetate for 17-20 min, or (b) uranyl salts followed by lead citrate for 1.5-5 min.

RESULTS

Differentiation of the Epithelial Cell Membranes

Previous studies of the fine structure of gill epithelium, e.g. of Mya (Fawcett and Porter, 1954), Anodonta (Gibbons, 1961), and Elliptio (Satir, 1961, 1965), have been primarily concerned with gill cilia. These reports also contain a general morphological description of the columnar portion of the gill epithelium that will be useful for orientation here. For example, in Elliptio (Satir, 1961, 1963; see Fig. 1) on one lamellar face there are several rows of columnar frontal cells (F) that possess short cilia, terminated by a single row of laterofrontal cells (LF) with their sail-shaped cirri, a single row of nonciliated intermediate cells (I), and four rows of lateral cells (L) with long cilia that beat abfrontally to drive water into the water tubes. These are the cilia that best exhibit metachronism. Finally, there are rows of nonciliated postlateral epithelial cells (PL) that progressively become squamous. Certain lamellibranch gills, e.g. Anodonta, possess a single row of prolaterofrontal cells between F and LF rows (Gibbons, 1961). The epithelium rests on a fibrous lamina. It is apparently innervated by branches of unmyelinated nerves that pass through the underlying connective tissue to the cell bases. Nests of small branches of unmyelinated nerve have been observed under the F, L, and PL cells but synapses of the vertebrate type have not yet been found. This appearance is in accord with the description of lamellibranch nerves by other workers (Gupta et al., 1969).

The cells with which we are principally concerned all possess a brush border. The microvilli are about 1 μ long. They must be reasonably permanent differentiations of the cell cortex because their spacing and number is rather precise. The cell cortex is well defined. The microvilli contain microfilaments which join a terminal web or filamentous belt around the cortex (Fig. 3). In the cells which possess cilia, the cilia are spaced in rows separated by rows of microvilli. At their bases, the cells in general show no particular membrane specializations. Their membranes are not greatly infolded, nor are they thickened when in apposition to the basement lamina.

CELL JUNCTION AND LATERAL SURFACE:
The complex cell junctions of vertebrate epithelia (Farquhar and Palade, 1963; Brightman and Reese, 1969) cannot be found between gill cells. Belts of tight junction are never seen. Desmosomes or gap junctions are not observed and, if present at all, membrane specializations of the vertebrate type must be rare. Typically, at the point where the microvillus border turns down to become the lateral edge of the cell, the intercellular space (gap) is about 225 Å, somewhat wider than normal, and the cytoplasmic surfaces of the unit membranes are backed by dense material and appear thick-
ened (Figs. 4 A, 7, 9, 11 A). This region, 0.2–0.5 μ in length, is comparable to the intermediary junction (zonula adherens) of vertebrate epithelia, and this term will be adopted for the regions. The intermediary junction encircles the cell completely. In glutaraldehyde-fixed material, the dense region abutting the cytoplasmic sides of the junction contains microtubules, as well as the microfilaments of the terminal web (Fig. 3) and amorphous material. The terminal web lies in the cytoplasm closely apposed to the intermediary junction and runs parallel to it. The microtubules, which also

The last portion of the figure legend of electron micrographs (Figs. 2–11) contains in order the following information: activating solution, fixative, embedment, stain, and magnification (see Methods). Figs. 2–4 are standard images without pyroantimonate. Figs. 5–11 show pyroantimonate precipitate.

**FIGURE 2** Transverse section of gill epithelium in L cell region. Arrow marks multivesicular bodies in L cell cytoplasm. The lateral border of the cells shown is comprised of three regions: (a) a 0.2–0.5 μ long intermediary junction (i) (zonula adherens), (b) a 2–3 μ long septate junction (sj), and (c) a nonjunctional region (x) that extends to the base of the cell. The junction joins both homologous (L-L) and heterologous (L-1; L-PL) pairs of cells. 0.04 M KCl, 5% glutaraldehyde, then 1% OsO₄ (collidine buffer). Epon. 7.5% aqueous uranyl, then lead citrate. × 11,000.
run parallel to the junction surface, are not present in material fixed in osmium tetroxide alone.

Immediately below the intermediary junction lies the septate junction (Figs. 2-4, 6, 7, 9-11). All cells in the epithelium, homologous (Figs. 2, 7, 9) and heterologous (Figs. 2 and 10) cell pairs, are joined by these junctions. In L cells, the septate junction is 2-3 µ long under normal conditions (Fig. 2). A transverse section through the junction reveals as many as 82 septa in this junctional length. The septate junction also forms a girdle around the cell. In face view, in glutaraldehyde-fixed material, the septa form arrays suggestive of close-packing of hexagonal subunits (Fig. 3). In material fixed in osmium tetroxide alone, the arrays are stacks of pleated sheets, and hexagons cannot be readily visualized. Even after glutaraldehyde fixation, the sides of the hexagons formed by the pleated sheets are more pronounced. Over long distances the septal sheets are not entirely straight and the cell border is apparently "fingerprint-like." In transverse sections, in positively stained material, the width of a septum measures about 110 Å (Figs. 4, 11). Usually, the intercellular space in this region is 150 Å, approximately the same as the space of the nonjunctional regions elsewhere along the L cell border. In 40 mM KCl or NaCl, the septate junction may open and large intercellular spaces sometimes develop. Smooth-surfaced cisternae of the endoplasmic reticulum often run parallel to the junction (Figs. 3, 4, 11). These form a reticular network (Fig. 3) apposed to the cytoplasmic sides of the junction, and separated from it by about 100 Å (Figs. 4, 11). Connections are sometimes seen between the cell and the reticular membranes at the level of a septum in well preserved regions (Fig. 4 B). In addition to the reticulum, multivesicular bodies are often found in the L and LF cell cytoplasm in the region of the junction (Figs. 2, 3, 7, 10).

The septa stop abruptly and the remaining cell borders run parallel to one another without intercellular specializations to the basal lamina. We have been able to follow the border for long distances (~10 µ) from cell apex to base in many cells and have never observed an obliteration or substantial diminution of the intercellular space that could not be accounted for by membrane tilt. The course of the cell borders is especially apparent after pyroantimonate treatment (Fig. 11).

In stained material, at high magnification, trilaminar profiles of the unit membrane are visible along all cell borders (Figs. 4, 10, 11). In osmium tetroxide-fixed, uranyl-stained material, the cytoplasmic and junctional dense lamellae of the unit membrane are equally thick (25-30 Å, Fig. 4 A) in the septate junction region. Some slight thickening of the cytoplasmic lamella is observed with glutaraldehyde fixation and lead staining (Fig. 4 B). No critical differences in appearance or thickness of the unit membrane is observed anywhere on the cell in these preparations.

**Pyroantimonate Localization**

**Effect of EM Preparative Procedures:** Although localization of pyroantimonate precipitate in the gill epithelium is highly specific in experiments with different gills, quantitatively different amounts of precipitate form and only similarly fixed portions of the same gill should be compared quantitatively. Material fixed in osmium-pyroantimonate alone is preferable to material prefixed with glutaraldehyde. In the latter fixation where precipitate is discernible, it generally follows the localization pattern of osmium-pyroantimonate alone, except that precipitate sometimes also appears on the extracellular side of the cell membrane.

No consistent differences in amount of precipitate are found in Epon vs. Araldite embedments. Sodium-versene solutions, added either during
dehydration or in the boat when the material is sectioned, alter neither the place of deposit nor the gross amount of precipitate. Staining with uranyl or uranyl and lead solutions increases the contrast of the section without altering the localization.

Fig. 5 shows an unstained section of the gill epithelium pretreated with 40 mm KCl and fixed in osmium-pyroantimonate. The fine black pyroantimonate precipitate is readily visualized in the cells. Stained specimens are shown in Figs. 6-11. Localization is summarized in Fig. 1 and in Table II.

**Description:** The precipitate appears on the cell borders of all cells examined. The apical, basal, and lateral cell borders, including the regions of intermediary (Figs. 9, 11) and septate junction (e.g., Figs. 7, 11), contain precipitate. The microvillous membrane is characteristically as dense with precipitate as the adjacent cell membrane, but the ciliary membrane usually contains little or no pyroantimonate precipitate. In Figs. 5, 9, and 10, note the abrupt termination of precipitate at the point of fusion of cell and ciliary membranes and the lack of precipitate in the ciliary shafts.

The ground cytoplasm is almost empty, as is the nucleus, except for the nucleolus, which contains a very fine precipitate (Fig. 8); the nuclear membrane is clear. Precipitate appears on certain cytoplasmic vesicles of the smooth endoplasmic reticulum, particularly in the I cell, but is not present on elements of the granular endoplasmic reticulum nor in the cisternal membranes of the endoplasmic reticulum apposed to the septate junction (Fig. 11). The prominent multivesicular bodies of the L and LF cells are surrounded by membranes with precipitate (Figs. 7, 8, 10) but the precipitate is not found on the vesicular membranes themselves. Mitochondria contain a small amount of fine precipitate that apparently adheres to the surface of the cristae (Fig. 8). Except for a few random massive clumps the extracellular space above the gill filament contains no precipitate. In the fibrous lamina below the epithelium the pyroantimonate precipitate is characteristically particulate. The particles are roughly spherical and range up to about 15 m in diameter. They are rarely clumped together. This morphology is distinctive and nowhere in the cells does the precipitate have this form. The cell borders of the small nerve processes and connective tissue cells are outlined with precipitate.

**Membrane Localization of Precipitate:** In stained specimens, the localization of the precipitate to the cytoplasmic side of the unit membrane is evident (Figs. 7, 9, 11). This agrees with the localization reported by Kaye et al. (1965). The cytoplasmic dense lamella of both intracellular and cell membranes is thickened by about 50 A or more wherever the precipitate occurs. The precipitate is found either as an electron-opaque amorphous addition to the membrane or as a very fine particulate. Regional differentiation of the membrane with respect to its ability to precipitate pyroantimonate is found in at least three locations: (a) Between the ciliary membrane and other portions of the cell membrane, as noted above. The precipitate terminates abruptly at the ciliary base (Figs. 5, 9, 10). (b) Between the cell borders of the LF and I cell. The precipitate is asymmetric, favoring the LF side of the junction (Fig. 10). (c) Between the septate junctional membrane and the adjacent membranes.

Careful examination of the character of the precipitate in the region of the septate junction (Figs. 7, 9, 11) often reveals an apparent periodicity of precipitation along the cytoplasmic sides of the unit membranes that corresponds to the spacing of the septa at the junctional sides of the membranes. The adjacent membrane regions of intermediary junction and L cell border (Fig. 11) do not show this periodicity, nor do the microvillous or basal portions of the cell membranes. In these

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**FIGURES 4 A, B** Transverse sections of the septate junction (sj) region. Note close apposition of cisternae of the smooth endoplasmic reticulum to either side of the septate junction (asterisks). Connections between the junctional membrane and the cisternal membrane are sometimes seen opposite the septa (arrows). A. Junction between LF cells. Note equal thickness of cytoplasmic and junctional dense lamellae of the unit membrane in osmium-fixed material without lead stain. 0.04 M KCl. 1% OsO4 (Veronal-acetate buffer). Epon. Saturated uranyl acetate in 50% ethanol × 198,000. B. Junction between L cells. 0.04 M KCl. 5% glutaraldehyde, then 1% OsO4 (collidine buffer). Epon. Aqueous uranyl, then lead. × 188,000.
regions the precipitate usually consists of coarser granules or is more glasslike than in the septate junction region. In oblique or face views of the septate junction (Fig. 6) the precipitate occurs in lines that appear to correspond to the septal corrugations as seen in osmium-fixed material.

**EFFECT OF PRETREATMENT:** None of the pretreatments of the gill with water, NaCl, KCl, NaCl-ouabain, or KCl-ouabain change the sites of precipitate localization. Pretreatment of the material with 40 mM NaCl instead of 40 mM KCl usually increases the amount of pyroantimonate precipitate found in the sections (Figs. 7 vs. 8 and Table II). For example, mitochondria and ciliary membranes contain almost no precipitate in KCl-treated material (Fig. 7) while a comparable section (Fig. 8) of the same material pretreated with NaCl shows precipitate.

Metachronism in the L cilia still develops when the gill is pretreated with 40 mM NaCl or KCl containing 0.2 mM ouabain. No clearcut change in the amount of pyroantimonate precipitate could be detected in these specimens. Metachronism also develops when the gill is activated with 1% potassium pyroantimonate instead of 40 mM KCl. Pyroantimonate alone does not poison the cells nor prevent the transmission of the metachronal wave. Lower concentrations of sodium azide or sodium potassium cyanide inhibit metachronism completely (Satir, 1961). Effects of ouabain or pyroantimonate on the frequency of metachronism have not been closely studied here.
FIGURE 6 Junctional localization of precipitate. The precipitate occurs in rows, reinforcing the oblique striations that are the pleated sheets (septa) seen in oblique view (at lines). 0.04 M KCI. 1% unbuffered OsO₄-pyroantimonate. Saturated uranyl acetate in 50% ethanol containing versene, then lead. X 120,000.

DISCUSSION

In the gill epithelium of freshwater mussels the junctional complex girdling the cell border has two components: (a) an intermediary junction that seems to be the attachment point of the terminal web, and is therefore probably a shape-determining region that perhaps strengthens the outer edge of the epithelium by increasing the rigidity of the brush border, and (b) the septate junction.

The septate junction, or septate desmosome as originally described by Wood (1959), is the main junctional element of many invertebrate epithelial cells (Table I) particularly in the annelid-arthropod line as opposed to the tight and gap junctions of vertebrate epithelia (Farquhar and Palade, 1963; Revel and Karnovsky, 1967; Brightman and Reese, 1969). The only report known to us of a possible septate junction in vertebrates (Barros and Franklin, 1968) describes the junction formed between sperm and egg at fertilization. Septate junctions seem to be present in echinoderm epithelia but are not present in buccal epithelium in Amphioxus. In invertebrates, gap junctions are found at some electrical synapses in the nervous system (Payton et al., 1969). Regions of occlusion of intercellular space (tight junctions) are found together with septate junctions in the campaniform sensilla of termites (Stuart and Satir, 1968) and elsewhere. Previously, the septate junction has been assigned functions associated with tight junctions (occlusion), gap junctions (cell coupling), and desmosomes (intercellular adhesion).

There are three principal morphological differences between septate and gap junctions: (a) the intercellular space in gap junctions is reduced to 20 Å, while in septate junctions there is no reduction (intercellular space = 150 Å), (b) the size of the hexagonal subunits is greater in septate than in gap junctions, and (c) the septate junction is a complete girdling belt ("zonula") while the gap junction is apparently only a spot ("macula") or a series of spots or bands on the membrane. It has not yet been possible to demonstrate convincingly the absence of short segments of true tight or gap junctions along an entire invertebrate epithelial cell border.

In our material it is possible to explain the appearance of the few instances of intercellular obliteration that are present along the junctional surfaces of the cells from apex to base in any single sections (Fig. 11) by section tilt without assuming the presence of either tight or gap junctions. We can-

| Subcellular localization | 0.04 M NaCl | 0.04 M KCl |
|--------------------------|-------------|------------|
| nucleoplasm              | ++          | ++         |
| nm                       | +           | +          |
| nl                       | ++          | +          |
| ground cytoplasm         | +           | +          |
| cm                       | +           | +          |
| mv                       | +           | +          |
| sj                       | +           | +          |
| c                        | +           | +          |
| s-er                     | +           | +          |
| m                        | +           | +          |

* ++++, very heavy; ++, heavy; +, readily detectable; ±, light or absent; −, absent. Compare Figs. 7 and 8.
not rule out the complete absence of points or short segments of tight junction, but these would have to be rare and certainly could not encircle the cells. If, as postulated by Loewenstein and Kanno (1964) for Drosophila salivary gland, the septate junctions here were to function in cell communication, all cells in the gill epithelium—like and unlike in a three-dimensional array—could be coupled to one another even across gill filaments, since every cell border possesses a belt of septa. We have some preliminary records indicating that electrical coupling occurs in the gill over long distances (Gilula and Satir, 1969).

This study demonstrates specific and consistent sites of localization of pyroantimonate, particularly on the cell membranes of mussel gill epithelial cells. The precipitate outlines the cell border so that the membrane can be traced around the cell without difficulty. The pyroantimonate precipitate always occurs selectively along the cytoplasmic side of the unit membrane both along the cell border and intracellularly, but different classes of intracellular membranes and even specific regions along a cell border behave differently with respect to the pyroantimonate reaction. Of particular interest are the following findings: (a) the ciliary membrane can be distinguished from the contiguous cell membrane with this reaction, (b) the asymmetry of the junction between LF and I cells with respect to the precipitate, and (c) the apparent periodicity of reaction product along the septate junction.

Several workers (Ronkin and Buretz, 1960; Steinbach and Dunham, 1962) have suggested that the ciliary membrane may be physiologically different from other parts of the cell membrane, perhaps in ion permeability characteristics. The striking difference between cell membrane and ciliary membrane with respect to the ability to precipitate pyroantimonate might reflect different ion binding properties of these membranes.

The periodicity of precipitate in the septate junction region corresponds to that of the extracellular septa and appears to be a cytoplasmic indication of the septal site. The precipitate is usually more glasslike in regions adjacent to the septate junction (Fig. 11). We recognize that the longitudinal view of this precipitate localization may be misleading. However, the occurrence of rows of precipitate particles along the face view of the septate junction (Fig. 6) supports our interpretation. If the precipitate corresponds to the location of an ion carrier in life, the septa would appear to be likely channels for ions to pass from cell to cell. On this assumption, the asymmetry of deposition of precipitate on the septate junction separating LF and L cells may imply that ion or ion carrier distributions at opposite sides of this junction are unequal, which suggests an asymmetry of function such as must occur at a rectified electrical synapse.

Exactly what the pyroantimonate reaction localizes is still uncertain. Komnick (1962) originally proposed the reaction as a method for localizing intracellular sodium ion. A number of workers have utilized this technique to study sodium ion localization in vertebrate tissues such as avian salt gland (Komnick and Komnick, 1963) or mammalian cornea (Kaye et al., 1965), gall bladder (Kaye et al., 1966), muscle (Zadunaisky, 1966), cochlea (Vinnikov and Koichev, 1969), and kidney (Bulger, 1969). Several lines of evidence suggest that the greater part of the precipitate is sodium pyroantimonate: (a) The amount of precipitate increases on membranes on rabbit corneal cells when ouabain is used as an inhibitor of ion transport in this tissue, indicating bound sodium at a site of a high affinity sodium ion carrier (Kaye et al., 1965). (b) The amount of radio-

**Figures 7-8** Comparison of localization in KCl- vs. NaCl-activated cells.

**Figure 7** KCl-activated L cells. Precipitate occurs on cell membrane and multivesicular body membranes; note the precipitate in the septate junction region (arrowhead) and the relative lack of precipitate in mitochondria (m). 0.04 M KCl, 1% unbuffered OsO$_4$—pyroantimonate. Araldite. Saturated uranyl acetate in 50% ethanol containing versene, then lead. $\times 45,000$. Inset shows detail of localization on the septate junction. Lines mark correspondence of precipitate to septa. $\times 120,000$.

**Figure 8** Another portion of the same gill, NaCl-activated. The localization is similar to Fig. 7 but note the relative abundance of precipitate on the membranes and especially in mitochondria (m). Part of the nucleolus (nl) contains precipitate. 0.04 M NaCl. $\times 21,000$. 

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FIGURE 9 Membrane localization of precipitate in L cells. Precipitate occurs along the cytoplasmic surface of the intermediary (i) and septate junctions (sj) and along microvillous portions of the cell membrane. Note the abrupt termination of precipitate at the origin of the ciliary membrane (arrows). 0.04 M KCl, 1% OsO$_4$ (collidine buffer)—pyroantimonate. Epon. Aqueous uranyl, then lead. $\times$ 60,000.

active sodium remaining in washed muscle tissue after fixation with osmium-pyroantimonate is considerably greater than after fixation with osmium alone because of the formation of insoluble sodium pyroantimonate (Zadunaisky, 1966). (c) Electron diffraction (Hartmann, 1966) and electron microprobe analysis (Lane and Martin, 1969) of selected specimen areas containing precipitate
indicate that the precipitate is predominantly sodium pyroantimonate. However, Tice and Engel (1966) criticize the method because the distribution of all precipitate "may not accurately reflect the distribution of all intracellular sodium." Localization does appear to differ considerably in different tissues and does not always correspond to a priori expectations of sodium ion locale, formulated in part from biochemical or physiological studies.

The results of this study are in accord with the conclusion that the precipitate is in part sodium pyroantimonate, particularly because the amount of precipitate can be increased by presoaking the tissue in sodium chloride (Table II). Carvalho et al. (1963) have presented evidence that supports the idea that there is a common cation binding site on cell membranes, and that Ca++, Mg++, H+, and K+ compete for this site. Pretreatment of the gill with NaCl rather than KCl could cause additional binding of sodium ion by such a cation exchange. If the pyroantimonate precipitate that we see reflects bound sodium ion, this would explain the increase in precipitate, after such treatment, on cell membranes, and the appearance of precipitate on ciliary and mitochrondrial membranes. We find no clear increase in the amount of precipitate after ouabain treatment, but this is perhaps not unexpected if ouabain does not significantly inhibit ion transport in this system at the concentration employed, a conclusion suggested by the fact that metachronism is still present in the gill after ouabain treatment. No information is presently available on the presence of sodium and potassium-activated ATPase in the gill epithelium. Sleigh (1968) has found that 10^-4 M ouabain affects frequency and velocity of ciliary beat in ctenophore comb plates.

Since pyroantimonate does not poison the gill in life, we conclude that the cell membrane is not normally permeable to pyroantimonate. Glutaraldehyde fixation may not alter the permeability of the membrane to this ion, or it may change the distribution or binding of sodium ion. In any case, initial fixation with glutaraldehyde prior to pyroantimonate localization eliminates most of the precipitate within the cell, may lead to spurious results, and should be avoided. Other workers have come to similar conclusions (Bulger, 1969). Osmium fixation apparently permits pyroantimonate to enter the cell and to react in a consistent and reproducible manner. Since mitochondrial cristae and nucleoli show precipitate, it is likely that the pyroantimonate can penetrate all cellular compartments. Whatever the exact chemical nature of the precipitate may be for any given organelle, it seems likely that the reaction has a consistent biomolecular basis because localization is specific and differential. In addition, the usefulness of the precipitate as a stain for following the convolutions of the cell membrane is readily apparent.

We acknowledge the technical help and assistance of V. Sylvester at the University of Chicago and especially of C. Clark at the University of California. Part of this work was assisted by staff and facilities of the Scientific Photography and Electron Microscope Laboratories on this campus.

This work was supported by grants from the USPHS (GM 13859) and the American Medical Association Education and Research Foundation. Mr. Gilula is a United States Public Health Service trainee under grant GM 1021.

A portion of the work was presented at the 3rd International Congress of Histochemistry and Cytochemistry and an abstract appears in the proceedings of the meetings (Satir, 1968).

Received for publication 22 January 1970, and in revised form 1 June 1970.

Note Added In Proof: While this paper was in press, a report appeared discussing the use of a 2% potassium pyroantimonate alone (without the addition of osmium tetroxide) as a "fixative" for certain tissues (Tandler et al. 1970. J. Cell Biol. 45:355). Intracellular precipitation of insoluble salts of Ca++, Mg++, and Na+ was observed. Also, a report of gap junctions in the clam and in Daphnia has appeared (cf. Goodenough and Revel. 1970. J. Cell Biol. 45:272).

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FIGURES 10 A, B. A. Membrane localization of precipitate at laterofrontal-intermediate (LF-I) cell border. Note heavy precipitate on LF side of junction, light precipitate on I side. Arrow shows ciliary membrane point of origin and abrupt end of heavy microvillus membrane precipitate. 0.04 M KCl, 1% OsO₄ (collidine buffer)—pyroantimonate. Epon. Aqueous uranyl, then lead. X 30,000. B. Enlargement of LF-I junction showing asymmetric precipitate localization. Note localization only on cytoplasmic dense lamella of unit membranes. X 80,000.
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Figures 11 A and B. Detail of border between L cells. B is a continuation of A. The same mitochondrion (m) is identified on both parts of the figure. Note the cisternae (asterisks) opposing each border in the septate junctional (\(x\)) and nonjunctional (\(x\)) regions. No tight or gap junctions are visible near the apical border and in the nonjunctional regions. When the intercellular space is obscured in places (arrows), this appears to be the result of membrane tilt. Pyroantimonate precipitate appears only on the cytoplasmic sides of the unit membranes and is sometimes glasslike (\(g\)) and sometimes particulate (\(p\)). 0.04 \(\times\) KCl, 1% OsO\(_4\) (collidine buffer)—pyroantimonate. Epon. Aqueous uranyl, then lead. \(\times 90,000\). In inset, arrows indicate examples where the precipitate occurs along the cytoplasmic dense lamella of the unit membranes at regions corresponding to the septa. \(\times 350,000\).