

# CALCIUM BINDING TO INTESTINAL MEMBRANES

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## ABSTRACT

Flame photometry reveals that glutaraldehyde and buffer solutions in routine use for electron microscopy contain varying amounts of calcium. The presence of electron-opaque deposits adjacent to membranes in a variety of tissues can be correlated with the presence of calcium in the fixative. In insect intestine (midgut), deposits occur adjacent to apical and lateral plasma membranes. The deposits are particularly evident in tissues fixed in glutaraldehyde without postosmication. They are also observed in osmicated tissue if calcium is added to wash and osmium solutions. Deposits are absent when calcium-free fixatives are used, but are present when traces of  $\text{CaCl}_2$  (as low as  $5 \times 10^{-5} \text{ M}$ ) are added. The deposits occur at regular intervals along junctional membranes, providing images strikingly similar to those obtained by other workers who have used pyroantimonate in an effort to localize sodium. Other divalent cations ( $\text{Mg}^{++}$ ,  $\text{Sr}^{++}$ ,  $\text{Ba}^{++}$ ,  $\text{Mn}^{++}$ ,  $\text{Fe}^{++}$ ) appear to substitute for calcium, while sodium, potassium, lanthanum, and mercury do not. After postfixing with osmium with calcium added, the deposits can be resolved as patches along the inner leaflet of apical and lateral plasma membranes. The dense regions may thus localize membrane constituents that bind calcium. The results are discussed in relation to the role of calcium in control of cell-to-cell communication, intestinal calcium uptake, and the pyroantimonate technique for ion localization.

## INTRODUCTION

When glutaraldehyde was introduced as a fixative for electron microscopy (18) it was apparent that aldehydes used alone would not provide adequate contrast. Osmium postfixation was introduced and is now in common use. Interest in the possibility that the osmium might remove or mask tissue components with intrinsic electron-scattering power led us to study unstained sections of tissues fixed in glutaraldehyde but not postfixated in osmium. These preparations present some problems to the microscopist because of their very low contrast. However, a striking feature observed in intestinal epithelia from various animals (rats, toads, and insects) was the presence of dense deposits adjacent to the plasma membranes. The deposits were present when the fixative was buffered with either phosphate, cacodylate, or collidine, and

were also present when unbuffered glutaraldehyde was used. The deposits were apparently removed or masked by postfixation with osmium, explaining why they have not been observed by other investigators. Here we describe the origin of these dense deposits, based on photometric analysis of fixatives, and on study of insect intestines fixed in various ways.

The first step was to determine if the deposits adjacent to the membranes were a component of the living tissue or if they were the result of tissue binding of some electron-opaque substance present in the fixative. Flame photometry revealed that calcium is present as a contaminant in glutaraldehyde from some sources as well as in most buffers. A fixative virtually free of calcium could be prepared from distilled glutaraldehyde and *s*-collidine

buffer. The dense deposits were absent when this fixative was used. Addition of calcium at concentrations of 0.05 mM/liter or less to the fixative resulted in formation of the deposits at the same sites as before. The deposits are thus the result of addition of calcium to the fixative.

The question that arises is whether or not the calcium deposition is related to the distribution of calcium with the living tissue. The deposits occur at calcium concentrations that are within the physiological range for the activity of calcium within cells. The deposition occurs mainly along apical and lateral plasma membranes in insect intestine. This distribution may reflect the location of calcium-binding sites, since it is well-known that cell membranes have a high affinity for calcium, and since the localization we observe correlates with physiological data. Previous studies of Oliveira-Castro and Loewenstein (14) predict that calcium should bind to the inner or cytoplasmic surface of the junctional membranes, since elevated intracellular calcium concentrations inhibit cell-to-cell coupling. In addition, calcium uptake in various intestinal epithelia is thought to be mediated by a calcium-binding protein (20, 21, 29-31) that is located along the apical brush border (27). We therefore discuss our findings with regard to two aspects of epithelial physiology, cell-to-cell communication and calcium uptake.

## MATERIALS AND METHODS

### *Tissues*

Adult male cockroaches (*Periplaneta americana*) were maintained on water and oatmeal. Animals were anesthetized with CO<sub>2</sub> and their midgut caeca (anterior diverticula of the midgut or ventriculus) were quickly dissected out and placed in fixative.

### *Analyses*

Fixatives and buffers from various sources were analyzed with an Eppendorf flame photometer. Fixatives were also analyzed for monomer and polymer content with a Bausch & Lomb 505 recording spectrophotometer.

### *Chemicals*

Sources of glutaraldehyde were: Fisher Scientific Company, Pittsburgh, Pa.; TAAB Laboratories, Emmer Green, Reading, England; Polysciences, Inc., Warrington, Pa.; and Ladd Research Industries, Inc., Burlington, Vt. Sources of buffers were: potas-

sium phosphate, Fisher Scientific Company; *s*-collidine, Fisher Scientific Company and Polysciences, Inc.; cacodylate, K & K Laboratories, Inc., Plainview, N.Y. Sources of ions were: sodium chloride, Fisher S-271; calcium chloride, Fisher C-79; and ethylenediaminetetraacetate (EDTA), Eastman P5416, Eastman Organic Chemicals Div., Eastman Kodak Co., Rochester, N.Y.).

### *Fixatives*

The following low calcium fixative was chosen on the basis of flame photometric analysis (see Results): 2.7% glutaraldehyde (Polysciences 216) in 0.08 M *s*-collidine buffer (Polysciences 346), with 5% sucrose added, pH = 7.2. In some cases the following salts were added to this fixative: 5 mM/liter calcium chloride, magnesium chloride, or lanthanum chloride, either singly or in combination, or 1 mM/liter EDTA. Lower concentrations of calcium (down to  $5 \times 10^{-6}$  M) were also tested. Tissues were fixed overnight in the cold. Those tissues that were not postfixed in osmium were dehydrated rapidly and embedded.

### *Postfixation*

Some tissues were washed in *s*-collidine buffer plus sucrose and postfixed in collidine-buffered osmium tetroxide. In some cases 5 mM/liter calcium chloride was added to both wash and osmium solutions.

### *Staining*

Some of the tissues fixed in glutaraldehyde and postfixed in osmium in the presence of calcium were stained in block in 0.5% uranyl acetate plus 0.5% calcium chloride for 2 hr at room temperature or overnight at 60°C and then dehydrated in ethanol.

### *Dehydration and Embedding*

Tissues were dehydrated rapidly in ethanol. In some cases the ethanol solutions were saturated with calcium chloride, but this did not appear to affect the results. Tissues were embedded in Spurr resin (25).

### *Microscopy*

Sections cut on a Huxley microtome were viewed either without staining (gold sections) or after staining (silver sections) with either ethanolic uranyl acetate (20 min) or alkaline lead citrate (5 min) or both. Micrographs were taken at magnifications up to 40,000 with a Hitachi HS-8, HU-11A, or HU-11E microscope. Approximately 42 caeca were sectioned and examined during this study.

TABLE I  
Ion Content of Fixatives and Buffers

	Ca <sup>++</sup>	Na <sup>+</sup>	K <sup>+</sup>
	meq/liter	meq/liter	meq/liter
Glutaraldehyde			
Fisher, 50%	0.11	0.322	0.017
TAAB, 24.8%	4.36	4.73	0.027
Polysciences, 8%	0	0.07	0.0056
Ladd, 10%	0	0.028	0
K-phosphate buffer, 0.1 M	1.1	0.306	—
s-Collidine buffer, 0.2 M	0.065	18.35	0
Na-cacodylate buffer, 0.1 M	0.391	—	0.033

## RESULTS

### *Ion Content of Fixatives and Buffers*

Table I summarizes the results of flame photometric analysis of the calcium, sodium, and potassium content of fixatives and buffers tested. Other ions may be present, but we have not assayed them. Calcium is present in glutaraldehyde from two sources and is undetectable in the other two tested (the sensitivity limit of our technique is 0.001 mm/liter). The calcium-free samples correspond to those containing the least polymer, as determined by spectrophotometry (Fig. 1). The calcium-free brands of glutaraldehyde are distilled by the manufacturers and supplied sealed in glass ampules. All buffers tested contained at least some calcium, s-collidine having the least. A fixative virtually free of calcium can be prepared from s-collidine buffer and either of the pure grades of glutaraldehyde. This fixative contained 0.03 mm/liter sodium, 0.006 mm/liter potassium, but calcium was undetectable.

### *Structure of the Midgut Caeca*

Various aspects of the structure of the columnar cells lining the insect midgut have been described elsewhere (1-3, 9, 12, 13, 23, 26). In the cockroach there are eight caeca, which are diverticula of the anterior portion of the midgut. Because of their anterior location, the caeca absorb much of the water and solutes flowing posteriorly from the crop (28). The caeca (Fig. 2) are composed of tall columnar cells similar to those of the remainder of

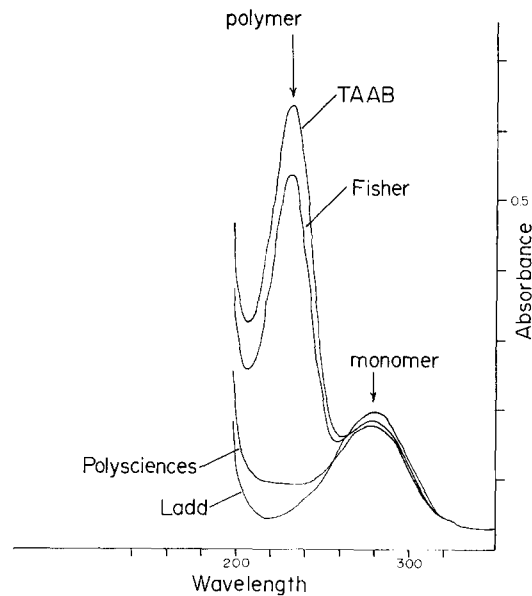


FIGURE 1 Absorption spectra of glutaraldehyde solutions from various manufacturers. All were diluted to 0.25% before analysis. Cross-linking properties responsible for fixation are due to the monomer. (Wavelengths in nanometers)

the midgut. Their over-all structure as observed at low magnification is similar to that of the columnar cells of vertebrate small intestine, except that the caecal epithelium lacks goblet cells.

There is a thin connective tissue layer between the basal surface and the blood space, or hemocoel. Embedded in this layer are both longitudinal and circular muscles, as well as tracheae. The structure of insect visceral muscles has been described elsewhere (22, 24).

### *Specializations of the Cell Surface*

The cells have extensive basal infolds (3), and the apical surface is folded into long narrow microvilli of uniform dimensions and spacing. Lateral plasma membranes of adjacent cells are closely apposed over large areas near the apical surfaces, while wider intercellular spaces are present basally. Both gap and septate junctions have been observed in the apical region, and deserve additional comment as well as further study. The most common image is that of a narrow intercellular cleft filled with a finely granular material of moderate density (Fig. 3). Septa have not been observed in junctional regions closest to the lumen, although

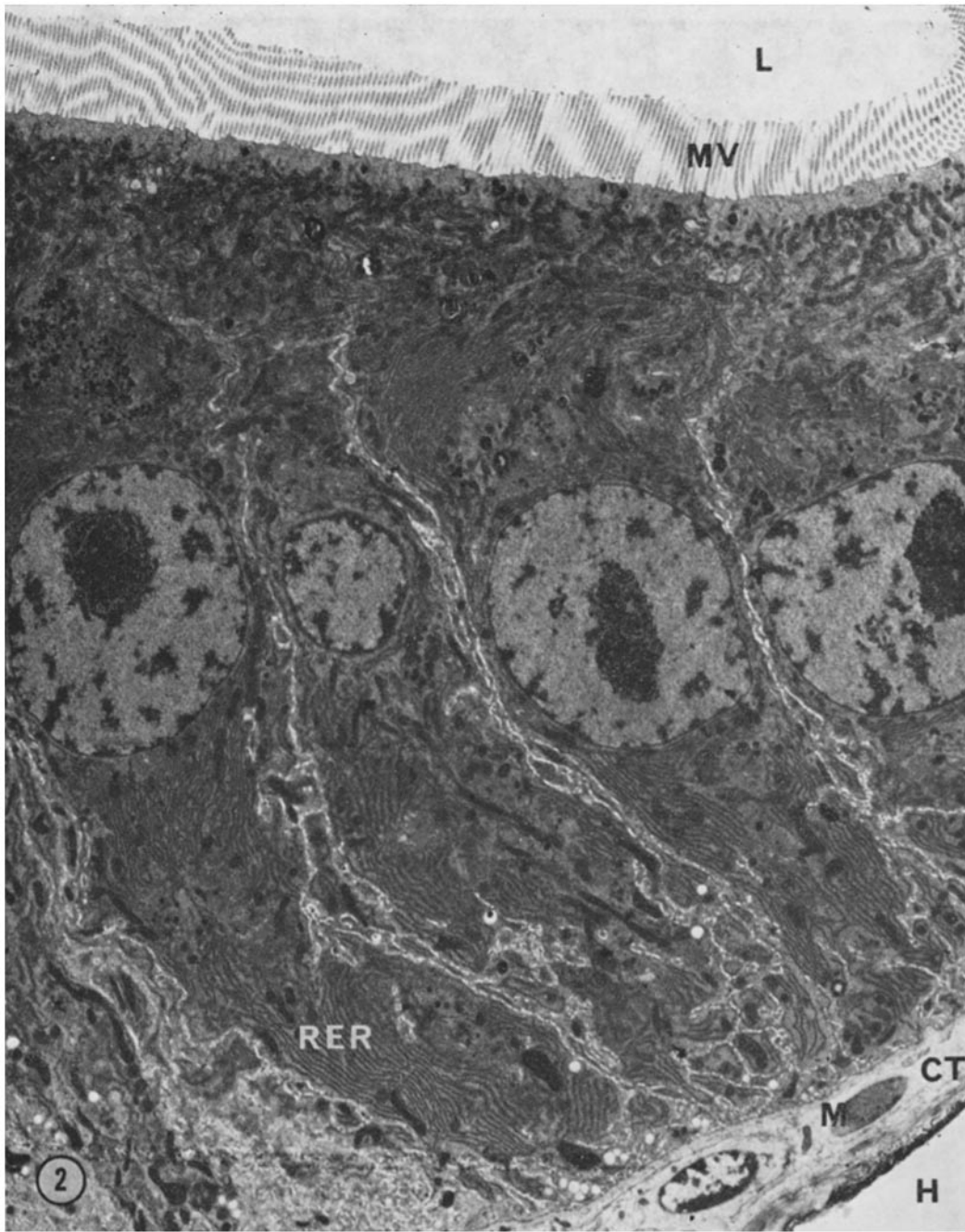
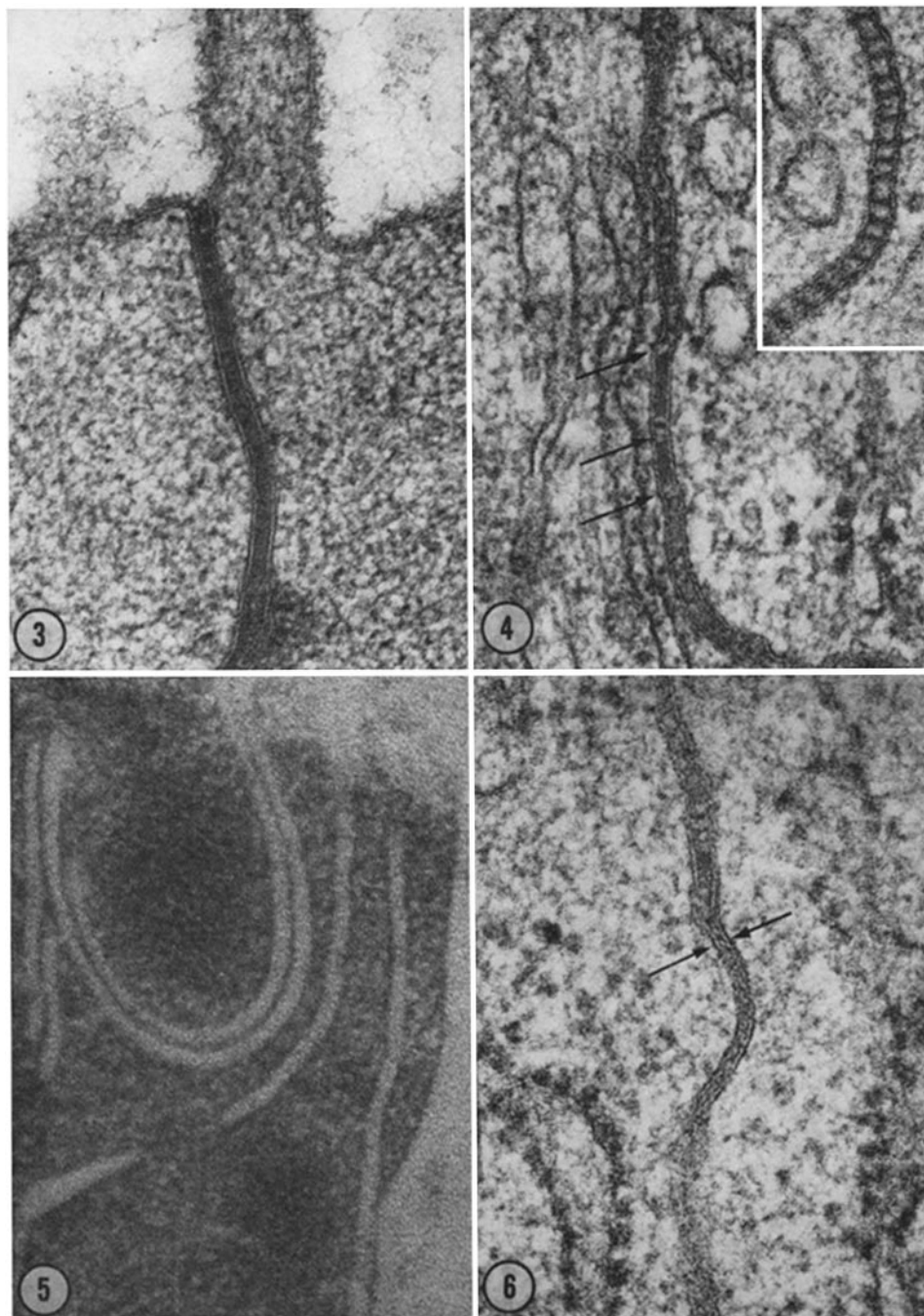


FIGURE 2 Low magnification survey of cockroach midgut caeca, conventional preparation. Microvilli (*MV*) line the lumen (*L*). The cells contain abundant rough endoplasmic reticulum (*RER*) and mitochondria, the latter most abundant near the apical surface. A thin layer of connective tissue (*CT*) with muscles (*M*) embedded in it faces hemocoel (*H*).  $\times 3350$ .



**FIGURE 3** Junctions near the apical cell border. Intercellular space contains a dense granular material. Section is perpendicular to cell surface as judged by plane in which microvillus has been sectioned.  $\times 90,000$ .

**FIGURE 4** Junction distal to region shown in Fig. 3. Gaps occur in dense intercellular material (arrows), and may be atypical septa.  $\times 86,000$ . *Inset*, more typical septate junction in Malpighian tubule. Here septa are dense lines between less dense extracellular substance.  $\times 89,000$ .

**FIGURE 5** Face view of lanthanum-treated junctional surface. Septa are clear gaps in dense intercellular material. Spacing between septa varies.  $\times 165,000$ .

**FIGURE 6** The gap junction (arrows).  $\times 110,000$ .

sections of various obliquities have been examined. Fig. 3 is an example of a section nearly perpendicular to the apical surface, as noted by the fact that the microvilli are seen in longitudinal profile. In more distal portions of the junctions, the dense matrix appears to have occasional interruptions or discontinuities, i.e., clear gaps occur in the dense intercellular substance (Fig. 4). Fixation in the presence of ionic lanthanum enhances the contrast of the intercellular matrix, and face views of the junctions in tissues treated in this manner confirm the presence of the gaps in the matrix (Fig. 5). Such preparations show as well that the gaps continue for some distance parallel to the cell surface, i.e., that they are channels extending through the intercellular matrix. The spacing between these "septa" is variable. Where the septa are far apart, the intervening junction closely resembles the "continuous junction" described by Noirot and Noirot-Timotheé (12).

Junctions similar to those described here have been observed in the hepatic caeca of *Daphnia* (10) and have been termed "septate" junctions, although it seems that such clear septa differ from the conventional sort (e.g. 8, 16) which consist of dense lines between regions of less opacity. We have not observed any dense septa in our material, although they are abundant in Malpighian tubules prepared by identical methods. An image of the junction of the Malpighian tubule is included as an inset to Fig. 4 in order to illustrate the fundamental difference between the two junctional types. Gap junctions are encountered frequently, and one is illustrated in Fig. 6.

#### *Effects of Calcium*

Fig. 7 shows the apical surface of a caecal cell fixed in collidine-buffered glutaraldehyde plus 5 mM/liter calcium chloride. The tissue was not osmicated and the section was not stained. Electron-opaque deposits occur along both apical and lateral membranes. The deposits are clearly present in such preparations, but are not observed if the tissue is osmicated without calcium. Deposits are also found in cells of the midgut proper, which resemble caecal cells in other details. That the deposits are due to the presence of calcium in the fixative is shown by comparison with Fig. 8, in which sodium was added rather than calcium. Here there is very little contrast, but it is possible to observe faint images of the microvilli and apical plasma membrane, both of which lack the deposits.

Fig. 9 shows that the deposits extend along the

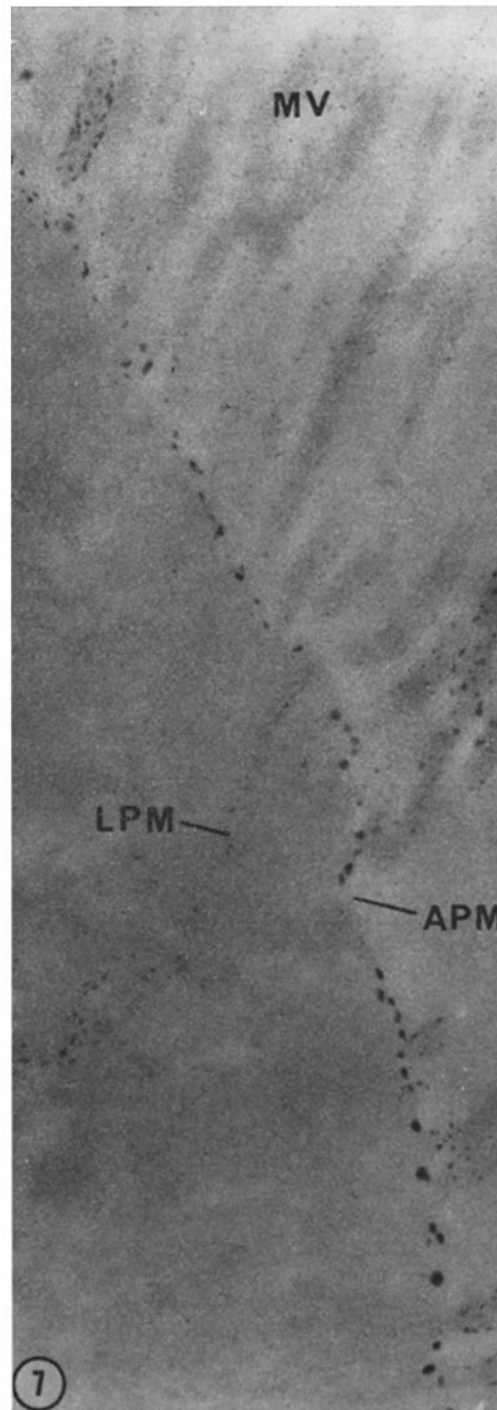


FIGURE 7 Unstained section of apical surface of a caecal cell fixed in glutaraldehyde plus 5 mM/liter calcium chloride. Unosmicated tissue. Electron-opaque deposits occur within microvilli (MV) and along apical (APM) and lateral (LPM) membranes.  $\times 56,000$ .

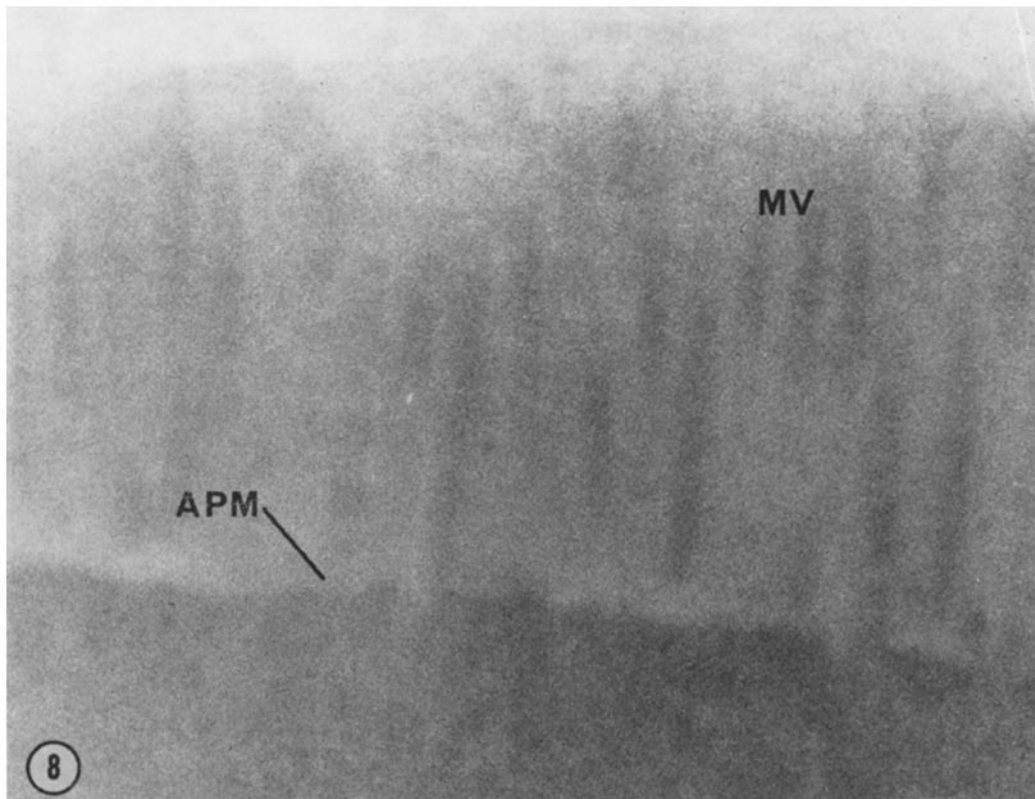


FIGURE 8 Unstained section of apical surface of a caecal cell fixed in glutaraldehyde plus 5 mM/liter sodium chloride; tissue unosmicated. Apical plasma membrane (*APM*) and microvilli (*MV*) can be resolved, but both are free of deposits.  $\times 48,000$ .

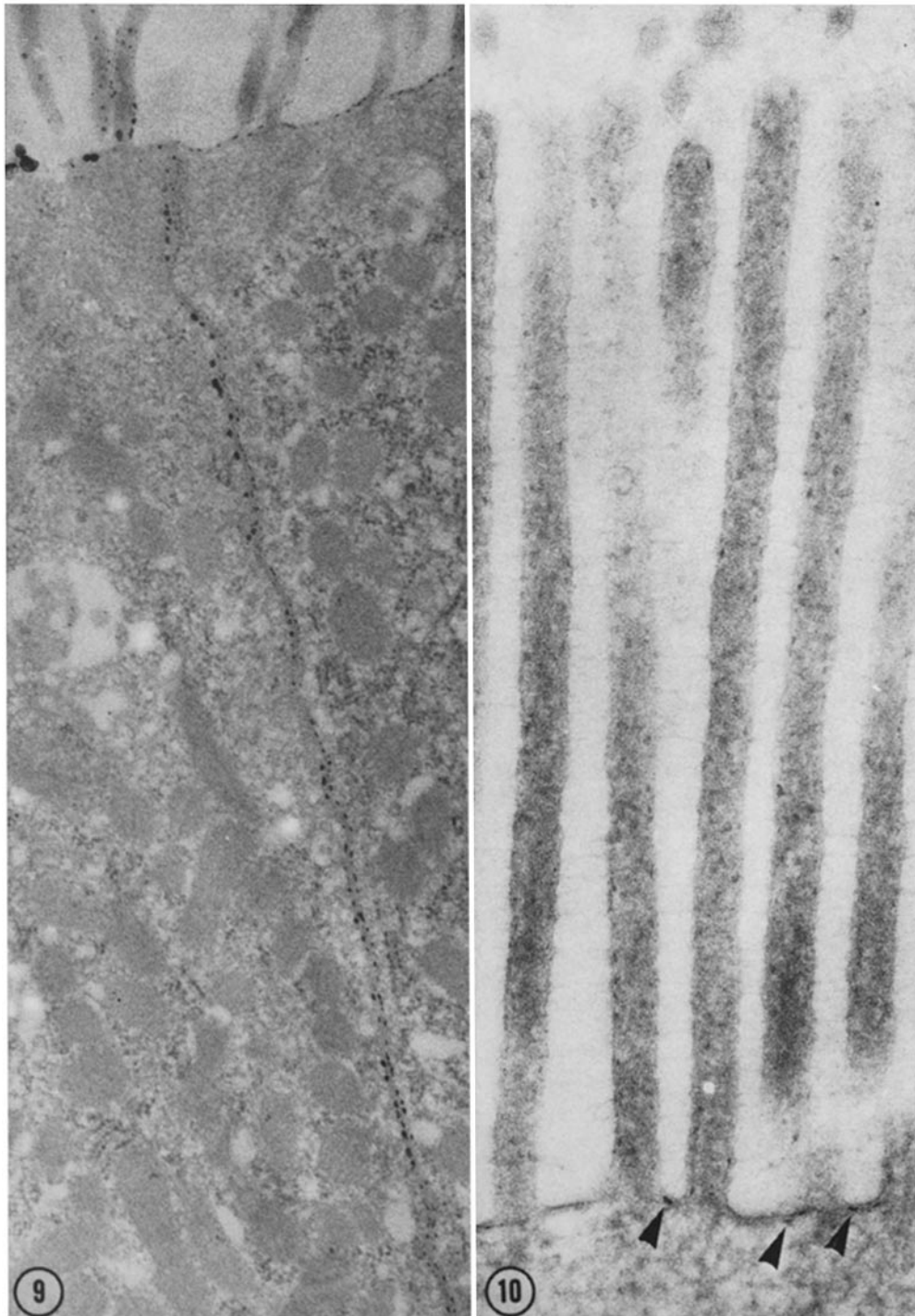
lateral plasma membrane. This section was stained with uranyl acetate, so it is possible to discern some of the other features of the cells, although contrast is still lower than normal because the tissue was not osmicated. The deposits are most abundant adjacent to the apical surface and along the lateral membranes where the junctions are located. They are observed infrequently along the remainder of the lateral plasma membrane and basal surface. These images are quite similar to those obtained by Satir and Gilula (19) in the ciliated epithelium of lamellibranch gills fixed in the presence of potassium pyroantimonate. Their study showed pyroantimonate precipitate along all parts of the plasma membrane, but particularly in register with the septa of the septate junctions. Our preparative method does not provide clear images of the septa, but the deposits resulting from addition of calcium to the fixative do occur at intervals along the junctional membranes (Fig. 9). An effort

has been made to determine how much calcium is required to produce the deposits. Identical results are obtained with 50, 5, and 0.5 mM/liter concentrations. At 0.05 mM/liter the deposits are less abundant and at 0.005 mM/liter they are very difficult to find, although occasional faint dense patches have been observed.

#### *Effects of Other Ions*

Other ions have been tested to determine if they duplicate the calcium effect. Magnesium, strontium, barium, manganese, and iron all produce dense deposits along apical and lateral membranes when added to the fixative at 5 mM/liter concentrations. Lanthanum does not produce the deposits when added to the fixative, but may not have access to the cell interior. Mercury also gives negative results, although it does enter the cell, as evidenced by its ability to stain the ribosomes





**FIGURE 9** Caecal cell fixed in glutaraldehyde plus 5 mM/liter calcium chloride; unosmicated; section lightly stained with uranyl acetate. Electron-opaque deposits along apical and lateral membranes appear to be larger in cell to left.  $\times 49,000$ .

**FIGURE 10** Caecal cell fixed in glutaraldehyde plus 5 mM/liter each of calcium chloride, magnesium chloride, and lanthanum chloride. Although tissue is unosmicated and the section only lightly stained with uranyl acetate, apical membrane can be resolved as a double layer, with electron-opaque deposits (arrowheads) occurring as patches along the inner leaflet of the membrane.  $\times 83,000$ .



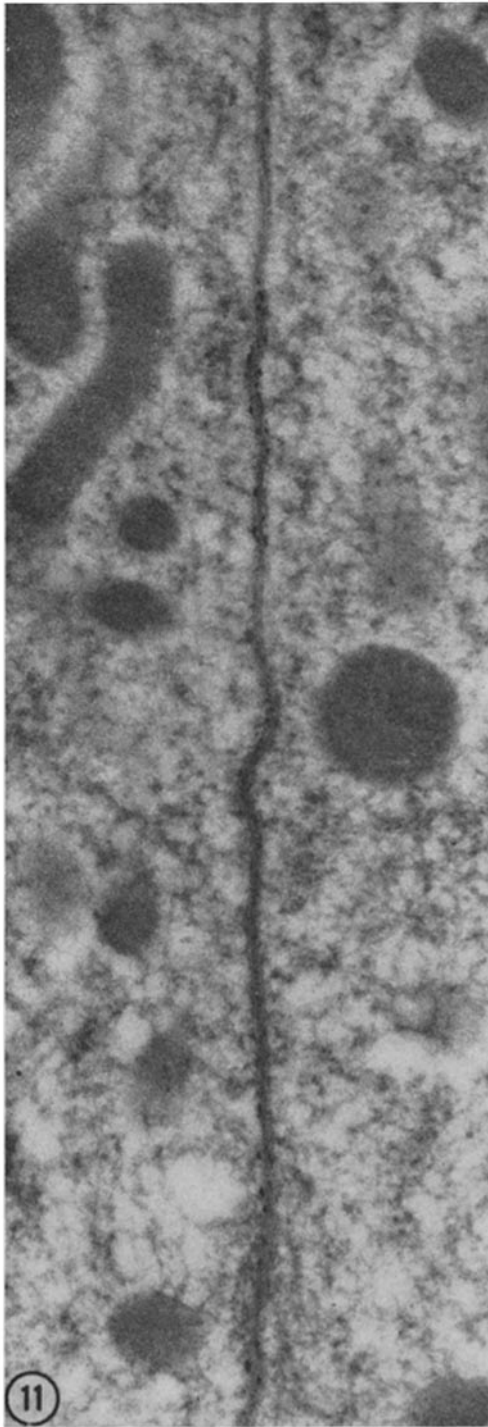


FIGURE 11 Caecal cell prepared as in Fig. 10. Dense deposits occur as patches along the cytoplasmic side of the lateral membrane.  $\times 37,000$ .

(unpublished results). As will be discussed below, however, caution is required when interpreting the positive results because of possible calcium contamination in the reagents we used and because very low calcium concentrations are adequate to produce deposits.

#### *Combination of Ions*

Simultaneous addition of calcium, magnesium, and lanthanum to the fixative results in images such as those illustrated in Figs. 10 and 11. The membranes can be resolved into two layers, with electron-opaque material occurring as patches along the membranes. These preparations suggest that the dense material is attached to the inner or cytoplasmic leaflets of the membranes, and this interpretation is supported by results obtained when the specimens are postosmicated in the presence of calcium ions.

#### *Effects of Osmication*

When tissues are fixed in glutaraldehyde and postfixed in osmium, the dense deposits are not observed, in either unstained or stained sections. This explains why the deposits have not been observed previously. When calcium is added to the glutaraldehyde, wash, and osmium solutions, however, dense patches are observed along the membranes. Figs. 12 and 13 illustrate these dense regions in close association with both apical and lateral membranes. These dense regions are different in form from the larger granular deposits obtained with glutaraldehyde (plus calcium) fixation alone. As this will be discussed below, we are uncertain of the significance of this finding. The clearest images of the dense material as well as of the membranes are obtained with postosmicated (plus calcium) tissues that have been stained in block with uranyl acetate (Fig. 14). Here the dense regions are clearly resolved as a part of the cytoplasmic leaflets of the membranes.

#### *Observations on Muscle*

The muscles located within the connective tissue of the midgut have a well-developed transverse T system with clear openings between the T tubules and the outer surface of the muscle (Fig. 15). When the specimens are postosmicated and stained in block with uranyl acetate with calcium added to all solutions, dense deposits occur along the sarcolemma, particularly along the T tubules (Fig.

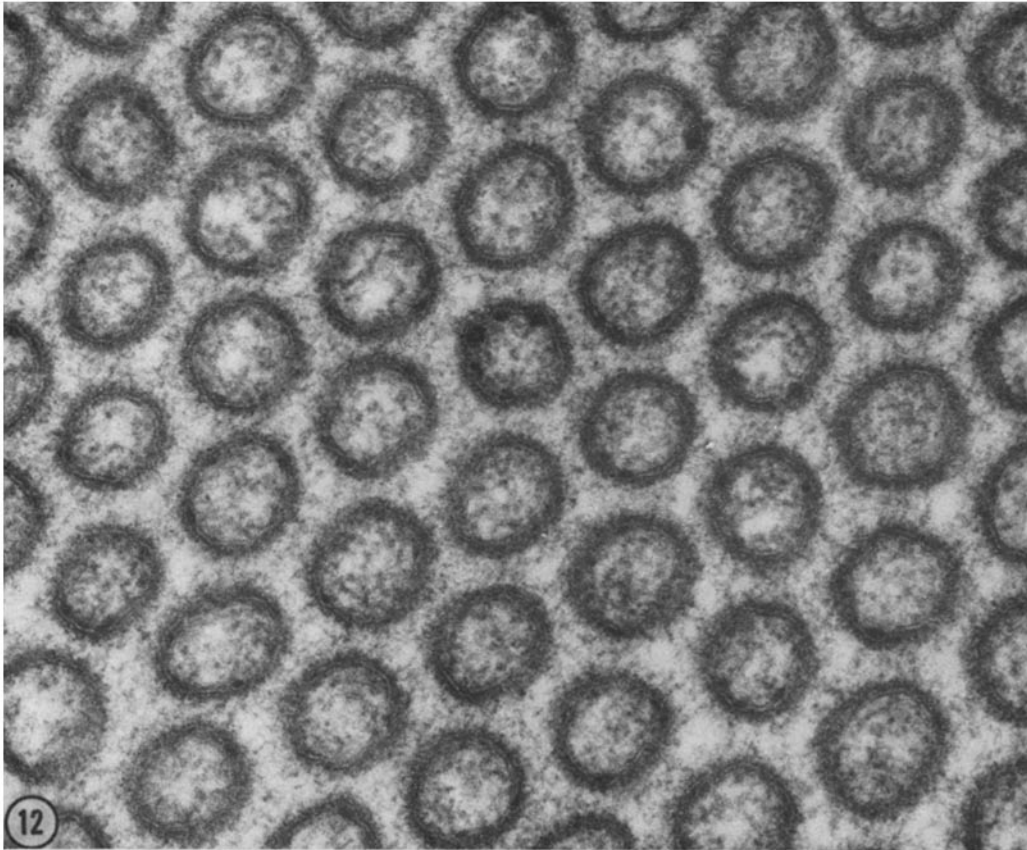


FIGURE 12 Caecal microvilli. Tissue fixed with glutaraldehyde and osmicated with 5 mM/liter calcium chloride present in all solutions. Section stained with uranyl acetate and lead citrate. Electron-opaque deposits are associated with inner leaflet of plasma membrane.  $\times 125,000$ .

16). These dense deposits are also present in unstained sections of glutaraldehyde (plus calcium)-fixed tissue that has not been postosmicated or stained in block with uranyl acetate.

## DISCUSSION

### *Calcium in Fixatives*

Calcium is probably present to a variable degree in fixatives in common use at the present time. When purified glutaraldehyde is used to prepare the fixative, calcium may still be present in the final fixative due to impurities in the buffer. Histochemists should be aware of this variable, since calcium inhibits some enzymes and could thus affect the amount of reaction product formed. It is also clear that ions present in fixatives could

interfere with histochemical reactions such as the pyroantimonate technique for sodium localization.

### *Nature of Calcium Binding*

We do not yet know the chemical composition of the dense structures observed adjacent to the membranes. The electron-scattering power of calcium (atomic weight = 40) is much less than that of osmium (atomic weight = 190). For this reason a significant part of the density might be due to elements originally present in the tissue, and the effect of calcium might be to stabilize components of the cell. In any case, the images are suggestive that the calcium-binding component is closely associated with the plasma membrane, if not a component of the membrane itself.

After postosmication (plus calcium) the dense

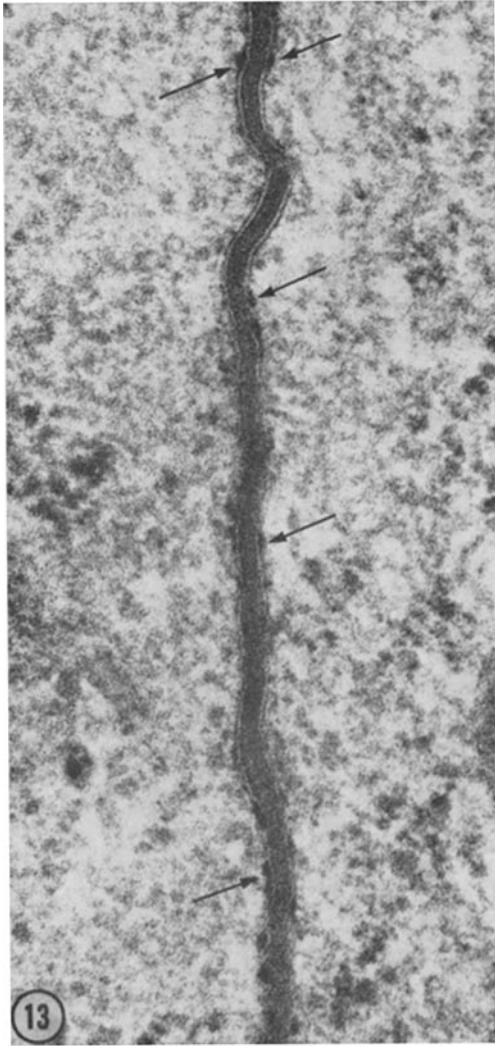


FIGURE 13 Caecal tissue prepared as in Fig. 12. Dense deposits appear associated with inner leaflet of lateral plasma membranes (arrows).  $\times 90,000$ .

deposits appear to be closely adherent to the membranes, while glutaraldehyde (plus calcium) alone yields a more granular deposit. We are uncertain of the significance of this observation, but can offer one suggestion. Osmium postfixation seems to stabilize the membranes, as the membranes are poorly resolved when osmication is omitted. Perhaps glutaraldehyde (plus calcium) fixation leaves the material to which calcium is bound susceptible to extraction during dehydration, and the extracted material accumulates in clumps adjacent to the membranes.

### Calcium and Intercellular Communication

The distribution of the deposits adjacent to the junctional membrane in insect intestine is similar to the distribution of pyroantimonate precipitate in clam gill observed by Satir and Gilula (19). The phenomena are related, because it has been possible to duplicate the effects of pyroantimonate on clam gill simply by fixing that tissue in a glutaraldehyde fixative containing calcium (D. J. Crawford and J. L. Oschman, unpublished). In their studies Satir and Gilula added sodium and EDTA (Versene) to their dehydrating solutions and to the water bath in the microtome, presumably in an effort to rule out divalent cation binding. We suspect that much of the precipitate they observed was calcium antimonate that formed during fixation. Such precipitates probably would be insensitive to EDTA treatment at later stages of tissue processing.

Loewenstein and his colleagues have made extensive studies on cell-to-cell communication in the *Chironomus*, salivary gland epithelium in which cells are connected by septate and gap junctions. They found that cell-to-cell coupling in this tissue is markedly depressed by addition of calcium, magnesium, strontium, barium, and manganese to the cell interior via a hole in the nonjunctional membrane (14), and suggested that this was caused by binding of these divalent cations to the junctional membranes. Rose and Loewenstein (17) also found that cells became uncoupled when cellular calcium levels were raised either by substitution of extracellular sodium by lithium or by addition of dinitrophenol (both of these treatments interfere with the calcium extrusion mechanism). In both cases it was not possible to restore coupling by injection of chelators (EDTA or EGTA) into the cell interior even with high concentrations of chelator. This can be explained either by high affinity of the binding sites for calcium, or by poor diffusion of chelator through the cytoplasm to the binding sites.

To what extent do these physiological studies done on *Chironomus* salivary glands relate to the insect midgut? We have obtained some *Chironomus* salivary glands (through the courtesy of Dr. William J. Larsen of the University of Miami) and have found that they also have dense deposits adjacent to their junctional membranes when fixed in the presence of calcium. We find that the same cations (calcium, magnesium, barium, strontium, and manganese) that cause uncoupling in salivary

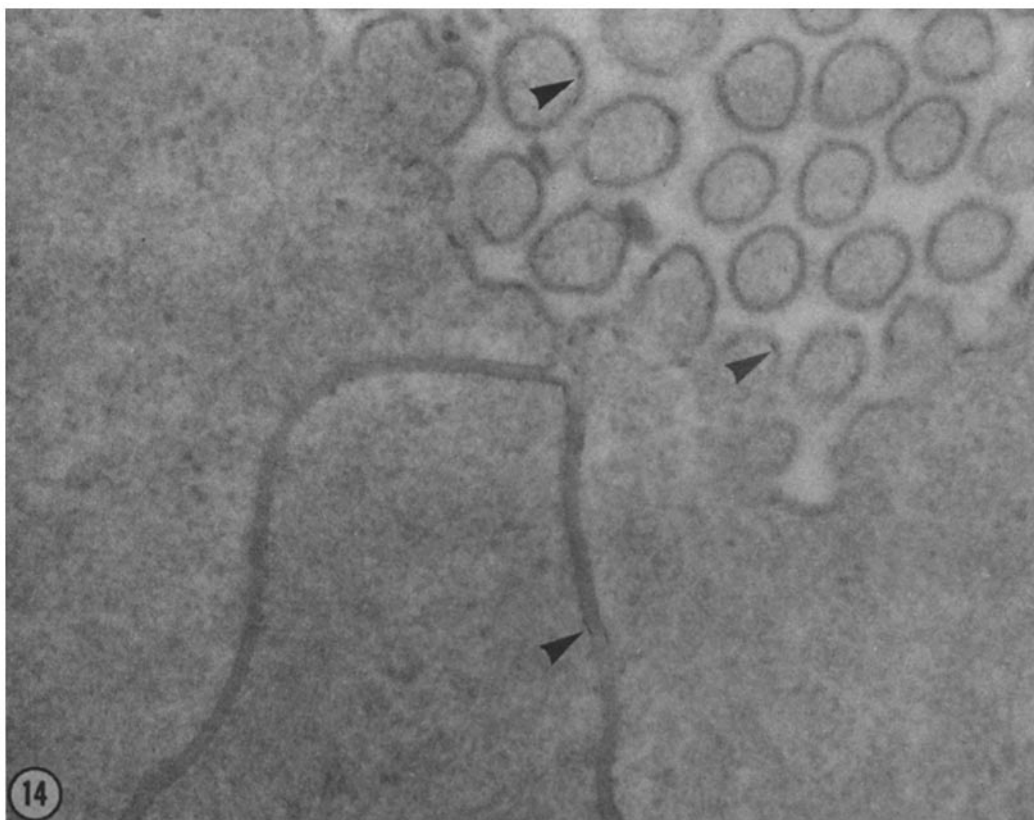


FIGURE 14 Caecal tissue fixed in glutaraldehyde, postfixed in osmium, and stained with uranyl acetate in block, all in presence of calcium ions. Section unstained. Dense deposits occur on both lateral and apical membranes (arrowheads). The deposits may localize a component of the membranes.  $\times 100,000$ .

glands (14) also form the dense deposits along the membranes of insect intestine.<sup>1</sup> The deposits are produced with concentrations of calcium in the

<sup>1</sup> It would be premature to conclude that these divalent cations duplicate the effect of calcium, as we have tried them at only one concentration, 5 mM/liter. There are difficulties in preparing these salts free from calcium contamination, and reagent grade chemicals contain significant amounts of calcium. From the impurity assays of the divalent cations we used and from flame photometry of our solutions, we can conclude that 5 mM/liter solutions of strontium, barium, and iron salts would contain enough calcium to produce the deposits were calcium the only ion with the ability to bind to the membranes. We are more confident about the results with the other divalent cations, as calcium was undetectable in the magnesium chloride and was very low in the manganese chloride solutions.

same range as that found by Oliveira-Castro and Loewenstein. In one experiment we fixed tissues in the presence of calcium and then washed with buffer + EDTA. This treatment removed the dense deposits. Since EDTA does not restore coupling in living salivary glands (see above), it is possible that fixation allows the EDTA to have access to the sites of calcium binding.

Our results confirm that the junctional membranes bind the divalent cations tested by Oliveira-Castro and Loewenstein (14) with the reservations mentioned above. Further study will determine if the deposits are more concentrated adjacent to particular types of junctions. There were premature reports that the septate junction is the only junctional element present in coupled invertebrate epithelia (6, 19), but septate and gap junctions have now been observed side-by-side in a variety

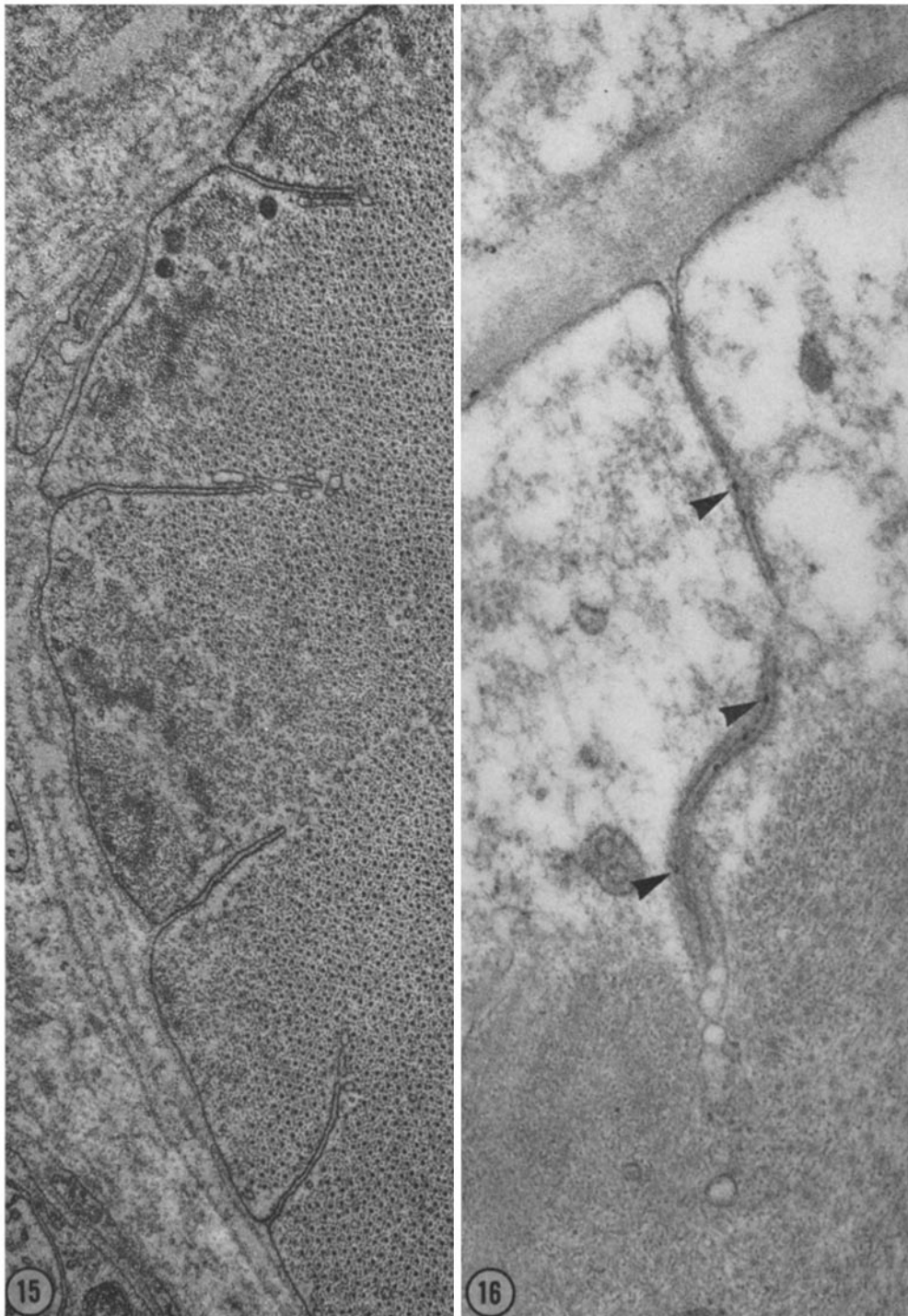


FIGURE 15 Longitudinal muscle of midgut caeca, conventional preparation. The T tubules clearly open to the surface and are associated with profiles of the sarcoplasmic reticulum.  $\times 32,000$ .

FIGURE 16 Longitudinal muscle of midgut caeca, fixed with glutaraldehyde and postfixed with osmium with 5 mM/liter calcium chloride present in all solutions. Section lightly stained with lead citrate. Dense deposits occur along sarcolemma and T-system membranes (arrowheads) although it is not clear which leaflet of the plasma membrane the deposits are associated with.  $\times 67,000$ .

of epithelia (4, 8, 10, 15, 16) including the midgut (present study). Although the septa have been suggested as the structural basis for cell-to-cell communication (6, 7, 16), it has not yet been firmly demonstrated that a particular junctional element is responsible for coupling.

### *Relation to Calcium Transport*

In insect intestine the binding sites in association with the apical membranes may be involved in calcium uptake from the lumen. A possible relationship between such binding sites and ion transport is suggested from a study on a vertebrate tissue, the corneal endothelium. Kaye et al. (11) fixed corneas in the presence of pyroantimonate, and observed precipitates similar to those observed in our study. As in insect intestine, these occurred along the cytoplasmic sides of the membranes. The precipitates were more abundant in ouabain-treated corneas. Ouabain decreases sodium transport into the corneal stroma, producing a thickening and clouding of the cornea. The interpretation was that ouabain inhibits the transport adenosine triphosphatase, preventing sodium from being extruded from the cell, so that it accumulates at the carrier site. Our results may complicate the interpretation of such results, since we can no longer be certain which ion is localized by the pyroantimonate (see also reference 5).

Intestinal membranes of vertebrates are able to accumulate calcium against concentration gradients. It would appear that this process occurs in two stages, uptake across the mucosal border, followed by sodium-dependent extrusion into the serosal fluid (21). A potentially important development in the study of this calcium uptake mechanism is the finding that a calcium-binding protein (CBP) can be isolated from intestines (20, 29–31). The amount of this protein in intestinal homogenates correlates with the rate of calcium absorption, which varies with age, pregnancy, calcium intake, and vitamin D<sub>3</sub> intake (20, 31). Further studies by Taylor and Wasserman (27) localized CBP to the brush border by means of immunofluorescence.

Little is known about calcium uptake in insect intestine. However, we have found that when the rat duodenum is fixed in the presence of calcium, images are obtained that are nearly identical to those of insect intestine (unpublished results). Similar deposits are found consistently along apical and lateral membranes in toad intestine (also

unpublished results). While the correspondence of these deposits with the sites of calcium transport could be a coincidence, it seems possible that our technique enables us to visualize the sites of calcium-binding material that function in calcium uptake *in vivo*.

### *Muscle*

Muscle cell membranes in the insect intestine also have dense deposits associated with them, particularly the T-system membranes. It is not clear on which side of the membrane the deposits lie, nor what their role might be in calcium metabolism in muscle. This preliminary finding is presented in order to illustrate that membranes of cells other than intestine also show the same sorts of deposits. We have also observed deposits on the inner surfaces of motor neurons embedded in the connective tissue sheath of the midgut.

### CONCLUSIONS

Here we describe the apparent binding of calcium to fixed intestinal membranes. Under appropriate conditions, addition of calcium to fixatives results in formation of electron-opaque deposits along lateral and apical membranes. Deposits are absent from other membranes within the cells, such as nuclear envelope, mitochondria, endoplasmic reticulum, and sarcoplasmic reticulum of muscle. Whether or not the distribution of the calcium-binding sites along lateral and apical membranes corresponds to that in living tissue is an open question, although this seems logical on the basis of what we know of calcium uptake mechanisms, the effects of calcium on cell-to-cell communication, and the distribution of pyroantimonate precipitates in other tissues. This is, however, circumstantial evidence, and the images we observe could be due to calcium binding to sites exposed by the fixative. Should these sites prove to be those actually involved in calcium binding *in vivo*, addition of calcium ions to fixatives could serve as a simple method for localizing such sites in other tissues.

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