STRUCTURE OF COUPLED AND UNCOUPLED CELL JUNCTIONS

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ABSTRACT

Cells of *Chironomus* salivary glands and Malpighian tubules have junctions of the "septate" kind. This is the only kind of junction discerned which is large enough to effect the existing degree of intercellular communication. The electron microscopic observations of the "septate" junction conform to a honeycomb structure, with 80-A-thick electron-opaque walls and 90-A-wide transparent cores, connecting the cellular surface membranes. A projection pattern of light and dark bands (the "*septa*") with a 150-A periodicity results when the electron beam is directed normal to any set of honeycomb walls. Treatment of the salivary gland cells with media, which interrupt cellular communication (without noticeable alteration of cellular adhesion) by reducing junctional membrane permeability or perijunctional insulation, produces no alterations in the junctional structure discernible in electron micrographs of glutaraldehyde-fixed cell material.

Two types of membrane junctions occur in communicating cell systems (13). In one type, the outer components of the cell surface membranes are so intimately adjoined that there is no discernible space between them (*occluding junctions*) (1–3, 5, 6, 8, 12, 21, 23–26; see also 7, 10, 20, 30); in another type, the membranes appear joined periodically by bridges (*septate junctions*) (31; see also 11, 32). In cell systems with occluding junctions, this junctional structure is the obvious, if not only, candidate for the function of cellular communication. Here sufficient insulation of the membrane junction from the extracellular fluid (*perijunctional insulation*)—a requisite for effective communication (13)—may be given by the close membrane apposition itself. The only condition is that parts of the apposing membranes (for instance, marginal parts) have the low ionic permeability characteristic of the nonjunctional surface membranes; and this is a condition likely to be satisfied. The other junctional structure which might be instrumental in communication for the same reasons, the desmosome plaque, is a less likely candidate; it occurs widely between nerve cells which do not communicate.

In cell systems with septate junctions, the situation is not so clear. The salivary gland of *Drosophila* is the only system of this kind in which junctional structure and permeability have been correlated. Here the septate junction is the most prominent structure and a likely site for communication (31). But the question remains outstanding, whether, in addition, a structure of the occluding type occurs in this junctional complex. In the present study, we look into two other communicating systems with septate junctions, the salivary glands and Malpighian tubules of *Chironomus*, and try to answer this question. We try also to analyze the configuration of the septate junction itself.

A further aim of this study is to examine the junctional structure when cellular communication is interrupted. Such interruption can be produced

by lowering the permeability of the membrane portions that serve as gateways for the cell-to-cell flow of ions (*junctional membranes*); or by rendering the elements of perijunctional insulation leaky. Effective lowering of junctional membrane permeability is produced experimentally by flooding the intracellular compartment (normally low in free Ca^{++}) with Ca ions; in contact with sufficient Ca^{++} ($> 10^{-4}$ M) the junctional membranes become as impermeable as the nonjunctional membranes (13, 17). Effective leaks in perijunctional insulation are caused by application of chelator or anisotonic media to the outside of the cell system (15, 17). Communication between salivary gland cells of *Chironomus* is interruped here in these two ways, and the junctional structure is examined.

MATERIALS AND METHODS

Salivary glands and Malpighian tubules were isolated by dissection from third instar larvae of *Chironomus thummi*. Contact between tools and the salivary gland cells used for comparing junctions in coupled and uncoupled states was minimized for prevention of cell injury: For isolation of the glands, the larva head was cut off while the larva midpart was compressed; the gland was thus expelled into the experimental medium by the pressure of the body fluids without getting in contact with dissecting tools. For transfer from experimental medium to fixative, the glands (and tubules) were sucked into a smooth pipette.

The following media were used (concentrations in mM; the number in parenthesis refers to numeration of Table I).

Control medium: NaCl, 87; KCl, 2.7; $CaCl_2$, 1.3; tris buffer, 10; pH, 6.3.

Ca-free medium (*No. 5*)*:* control medium Ca-free.

Chelating media: control medium with a complex of EGTA, 2.5, and Ca, 2.5, giving a free Ca^{++} concentration of 0.1 (No. *1*); Ca-free medium with EGTA, 2.5 (No. *2*)

Hypertonic medium (No. *3*): control medium plus sucrose, to give osmolarity twice control.

Hypotonic medium (No. *4*): control medium with control $[Ca^{++}]$; all other ions reduced to give osmolarities one-half control.

The glands or tubules were left in the media for periods ranging from 2.5 to 10 min. Spot checks of the state of junctional communication between gland cells were made by measurements of attenuation of membrane voltage across one cell junction (16) in gland samples.

The preparations were fixed for 1 hr at room temperature in 6.25% glutaraldehyde (28) in Millonig's (19) sodium phosphate buffer, pH 7.5. They were then washed for at least 1 hr in buffer alone at 4°C, fixed for a further hour at room temperature in 1% osmium tetroxide in the same buffer, and embedded in Epon (18). Sections, 500–700-A thick, were mounted on grids without supporting film; they were first stained in 1% uranyl acetate in 50% ethanol, and then in lead citrate (29). The sections were stabilized by coating with a thin film of evaporated carbon before electron microscopic examination (Siemen's Elmiskop I).

The linear measurements (intermembrane distance, band spacings, etc.) given in the results are averaged values on the basis of many electron micrographs.

TABLE I

Media*	Time of action‡
	min
Uncoupling media	
1. Chelating, 10^{-4} M $[Ca^{++}]$	2.5; 3; 10
2. Chelating, Ca-free	10
3. Hypertonic, 10^{-3} M $[Ca^{++}]$	2.5; 5; 10
Other media	
4. Hypotonic	4
5. Ca-free	10
Control medium	5; 10

* See "Materials and Methods" for composition.
‡ Time during which the preparation was in contact with the medium.

RESULTS

Coupled Junctions

The cells of the salivary glands and of the Malpighian tubules of *Chironomus* have septate junctions. In sections normal to the cell interface (hereafter transverse sections), the septa appear as regularly spaced 70-A-wide electron-opaque bridges separated by 80-A-wide light spaces, as described previously in salivary gland cells of *Drosophila* (31) and other cell systems (11, 32; see also 4, 22). The bridges extend from one cell membrane surface to another across the entire 170-A intercellular gap. They are not seen in the light space of the "unit membrane"; they do not seem to cross the membranes. In sections more oblique to the membrane, the bridges show up as longer parallel lines with width and spacing of the same order as in transverse section. This is so in both the salivary glands and Malpighian tubules (Figs. 1–5). In the glands, oblique sections, particularly sections tangential to the cell interface, show, in addition, a honeycomb pattern with a center-to-center spacing on the order of 170 A; the

hexagonal opaque honeycomb walls have widths of the same order as the bridges in transverse section (Fig. 5).

The septate junction extends from the lumen about halfway to the cell base in both cell types. Fig. 3 gives an illustration for the case of the tubule. No septa have been seen in the basal portion of the cell junction. It is difficult to follow this portion in electron micrographs because of its many and complex invaginations. However, if septa were present, one would expect to see them more readily in this portion, where each section gives views at many angles to the junctional plane, than in the relatively straight luminal half of the junction. At the luminal portion, the septa can be seen almost up to the lumen (Figs. 2, 4, 5); we never find intervening desmosome plaques or occluding membrane junctions of the kind seen in other kinds of communicating junctions in epithelia under similar conditions of fixation (5, 6). The septate junction seems to be the only bridging element here.

Absence of a particular junctional structure can only be proven, of course, by serial sections or by total views of the junctional regions involving all cell faces. This was not feasible. But we have examined several hundreds of random sections through junctions, and none showed desmosome plaques or occluding junctions. Thus, if such junctional structures were present at all, the junctional area occupied by them would, in all likelihood, be too small to be of functional significance in cellular communication. With junctional membrane conductivities of the order of 50 mho/cm^2, the highest conductivities for which there is evidence (cf. 13), the observed junctional conductance in the gland cells (2×10^{-5} mho, reference 16 *a*) would require a junctional area of 40 μ^2. So large an area of occluding junction would certainly have been detected in our electron micrographs of random sections. Even a junction one-tenth that area (which would have to be made of a membrane material with a conductivity only one order lower than that of cytoplasm) would still be readily detectable.

Uncoupled Junctions

Table I gives a summary of the media used for uncoupling and of the time they were allowed to act before fixation (salivary glands). Media *1* and *3* cause complete junctional uncoupling: the junctional membranes become 1–3 orders of magnitude less ion-permeable than normal, as shown by electrical measurements (15, 17); in effect, communication between cells is shut off. Medium *2* causes interruption of communication too; but it does so primarily by rendering the perijunctional insulation or the nonjunctional membranes leaky, not by changing the permeability of the junctional membranes (see references 13, 15 for mechanisms).

Of these three media, media *1* and *2* produced no alterations in junctional structure that could be discerned in electron micrographs. The septa were present, retaining their general aspect, dimensions, and spacing (Figs. 6 and 7). The septa seemed often less distinct than their counterparts in control medium. But this may well be caused by sampling errors; we have no means for weighing the significance of this difference in appearance.

Preparations treated with medium *3* had swollen and ruptured mitochondria and other signs of damage; they provided, therefore, no useful material for studying structural correlates of junctional uncoupling. Medium *4* caused no visible changes in junctional structure (Fig. 8). This medium is not a good uncoupling agent (17); the results are nonetheless included here, because they may be of interest from the purely structural point of view.

DISCUSSION

Coupled State

The conspicuous and apparently only coupling elements between the cells are the septa. A possible picture of the coupling structure, as it emerges from the electron micrographs of salivary glands, is an hexagonal arrangement built of 170-A-long honeycomb "cells" with electron-opaque walls (*t*) and transparent interiors (*a*) abutting at right angles on the surface membranes (Fig. 9 *C*). It is easily seen that in sections transverse to the membrane and thicker than two honeycomb "cells," the "cells" will transform, through overlap of electron opacities, into a pattern of alternating dark (*D*) and light (*L*) bands (the *septa*) with a periodicity equal to $(t + a) \cos 30°$, if the electron beam is normal to the plane faces of the honeycomb cells (Fig. 9 *A*). (In sections oblique to the membranes, the bands become longer.) The bands will merge into a relatively uniform opacity, when the beam is rotated 30° becoming parallel to a set of the plane faces (Fig. 9 *B*).

With the beam normal to the plane faces (Fig. 9 *A*), the banding pattern consists of regions of maximal and uniform opacity (d_1) flanked by

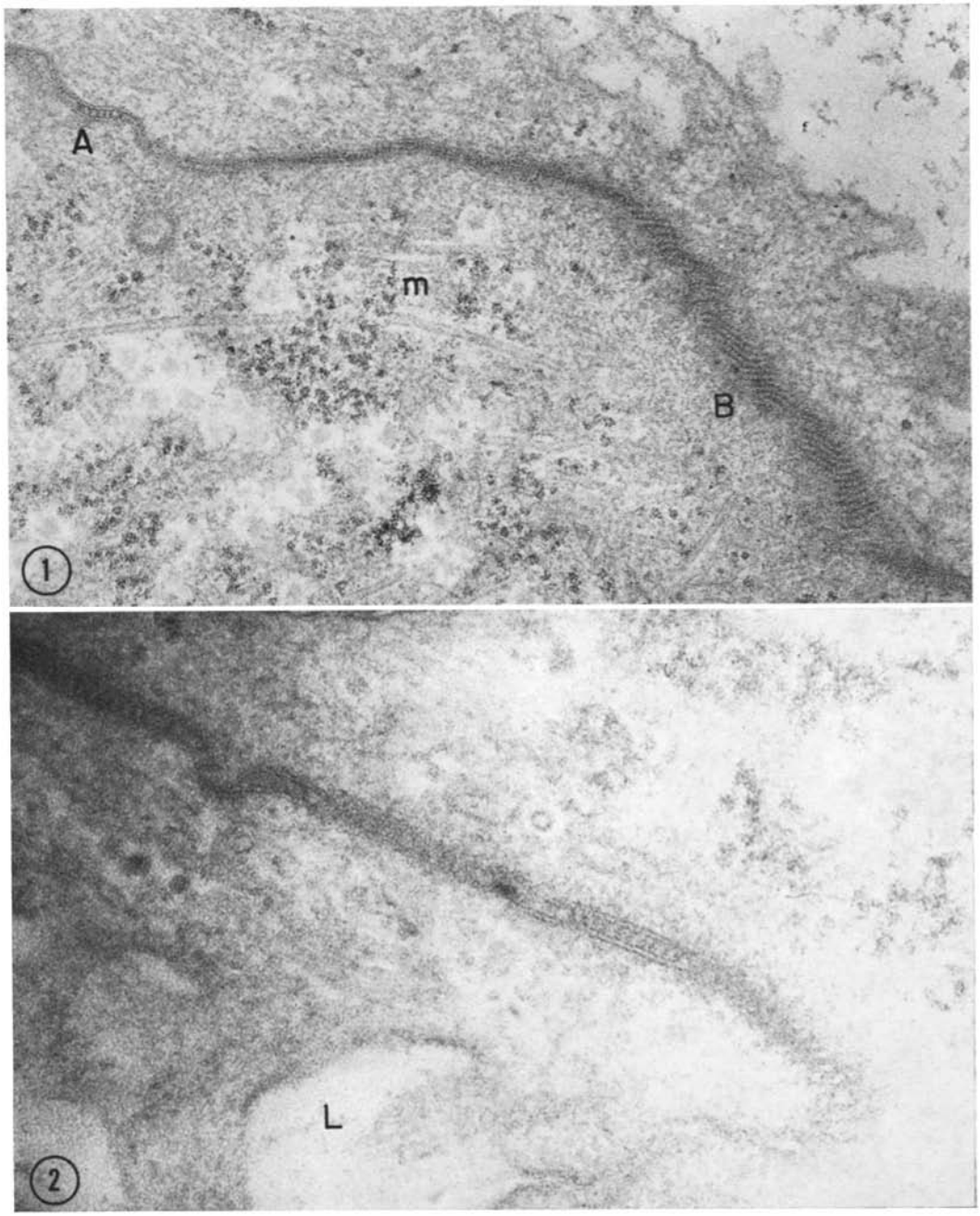

FIGURE 1 Electron micrographs of portions of two cells and their junctions near the lumen. *Chironomus* Malpighian tubule. Two aspects of the junction are seen: *A*, Section normal to cell interfaces (*transverse section*) shows the two cell surface membranes connected by "septa"; *B*, More oblique section in which the "septa" appear as parallel opaque lines. Many microtubules (*m*) are seen in the cytoplasm. × 60,000.

FIGURE 2 View at higher magnification of junctional region similar to that in Fig. 1. *Chironomus* Malpighian tubule. Junctional portion adjacent to lumen (*L*) is seen. There appears to be no occluding junction. Septate junction is again seen in both transverse and oblique section. × 120,000.

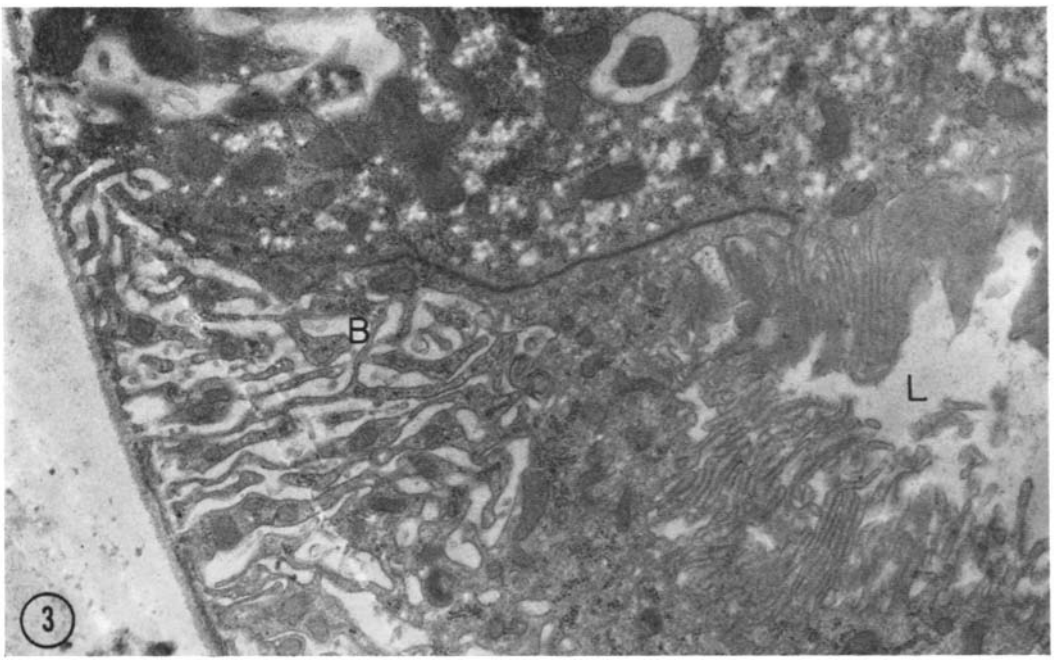

FIGURE 3 Low-power micrograph of two Malpighian tubular cells showing junction extending from lumen (L) to the infolded basal region (B). × 15,000.

regions with progressively diminishing opacity (d_2) merging into regions of minimum and uniform opacity (l). There is little contrast between the d_2 edges and l. The electron microscopic image of the banding will be blurred towards the d_2 edges. The point from which the more opaque regions are seen as dark bands is thus uncertain; it will be located somewhere between the l- and d_1-borders of d_2. On the assumption that this point lies at the middle of d_2, the interior width (a) of the honeycomb "cells" is $2/3\ (1 + d_2)\ \cot 30° \approx 90$ A; the wall thickness (t) is $(d_1 + d_2)/\cos 30° \approx 80$ A, (center-to-center periodicity, about 170 A). This fits the electron microscopic observations within experimental error (e.g., Fig. 5).

With the beam parallel to a set of the plane faces (Fig. 9 B), light bands (l, periodicity $(a + t)/2$) reduce to regions less than 10 A wide, $(a - t)/2$, below electron microscopic resolution. This fits the observations in which cross-sections through the junctional region appear rather uniformly electron opaque (e.g., Fig. 5).

The honeycomb picture may be elaborated further. If the honeycomb system is the coupling device, it must contain the elements of perijunctional insulation and the water channels essential for free cell-to-cell diffusion (13). A simple possibility that suggests itself here is that of a honeycomb system in which the walls have low ion permeability (say, of as low an order as the nonjunctional cell surface membrane) and the honeycomb cores have high permeability (see reference 16 for a discussion of possible molecular arrangements).

A coupling system of this sort places two interesting conditions on the relationship between coupling apparatus and membrane, and on the membrane itself: (i) the fitting between honeycomb wall and membrane must be leak-proof, and (ii) the membrane portions to which the walls are connected must be of low permeability. These are merely the requirements for adequate perijunctional insulation. The first condition would seem almost certainly to be fulfilled; the septa are clearly contiguous to, if not continuous with, the membrane surface (Fig. 2). The second condition would make the membrane a mosaic in which permeable and impermeable pieces follow the outlines of the honeycomb core and walls, respectively (Fig. 9 C). This is of much interest in relation to the possible molecular mechanisms that bring about the permeability differentiation at these membranes (14, 15).

An alternative interpretation of the junctional

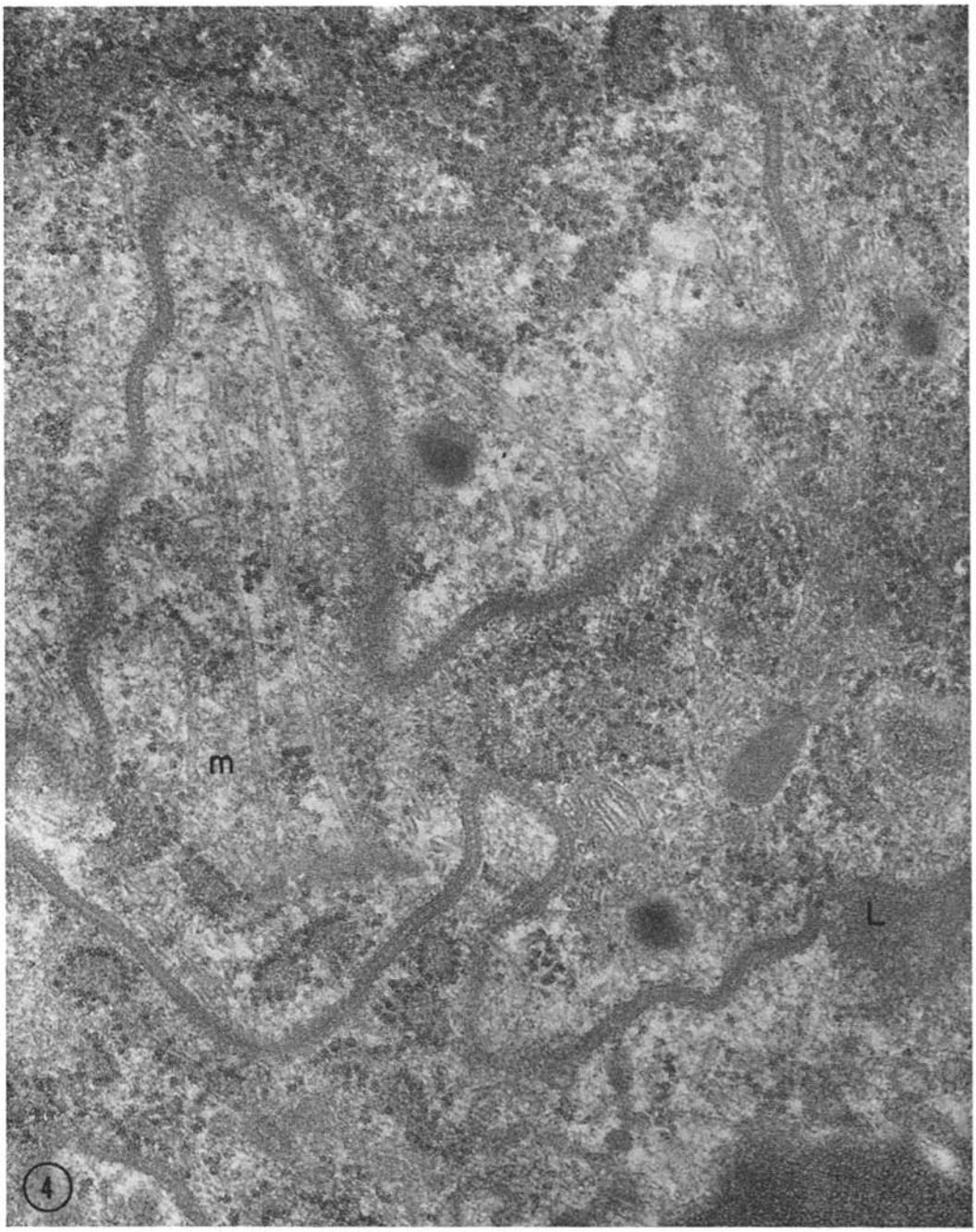

FIGURE 4 Junction between two cells in the region adjacent to the lumen (*L*). *Chironomus* salivary gland. Transverse sections showing "septa," transverse sections wherein the intermembrane space appears rather uniformly opaque showing no "septa," and oblique sections are seen in different parts of the junction. There are many microtubules (*m*) in the cytoplasm. × 52,000.

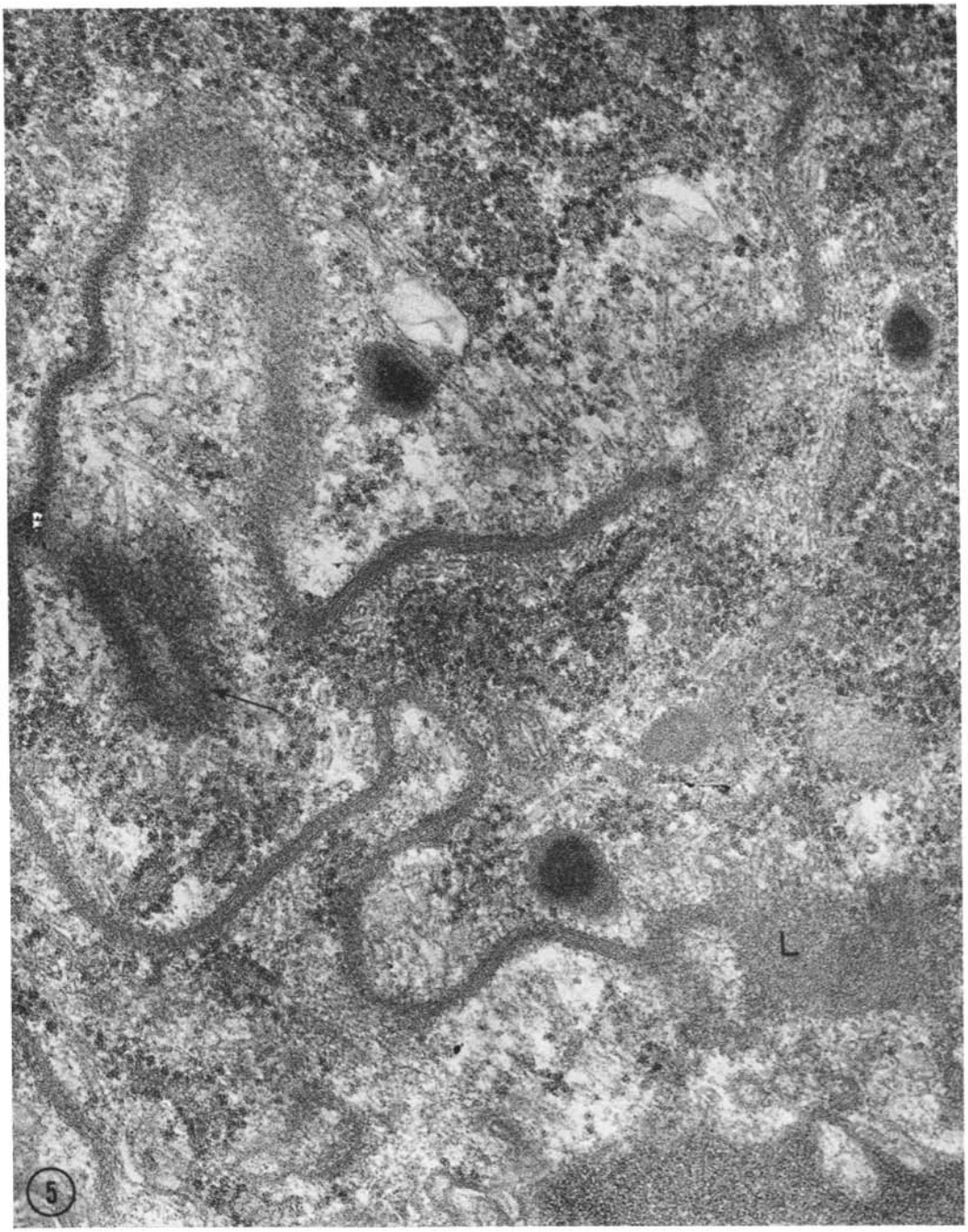

FIGURE 5 Micrograph of serial section parallel to that in Fig. 4, but not immediately adjacent to it. The appearance of the junction, as its angle changes with respect to the section, can be compared in the two figures. In particular, there is an oblique view of part of the membrane junction (arrow) not seen in Fig. 4. The "septa" in face view (sections tangential to cell interfaces) have a hexagonal appearance. There appears to be no occluding junction between the septate part of the junction and the lumen (*L*). × 52,000.

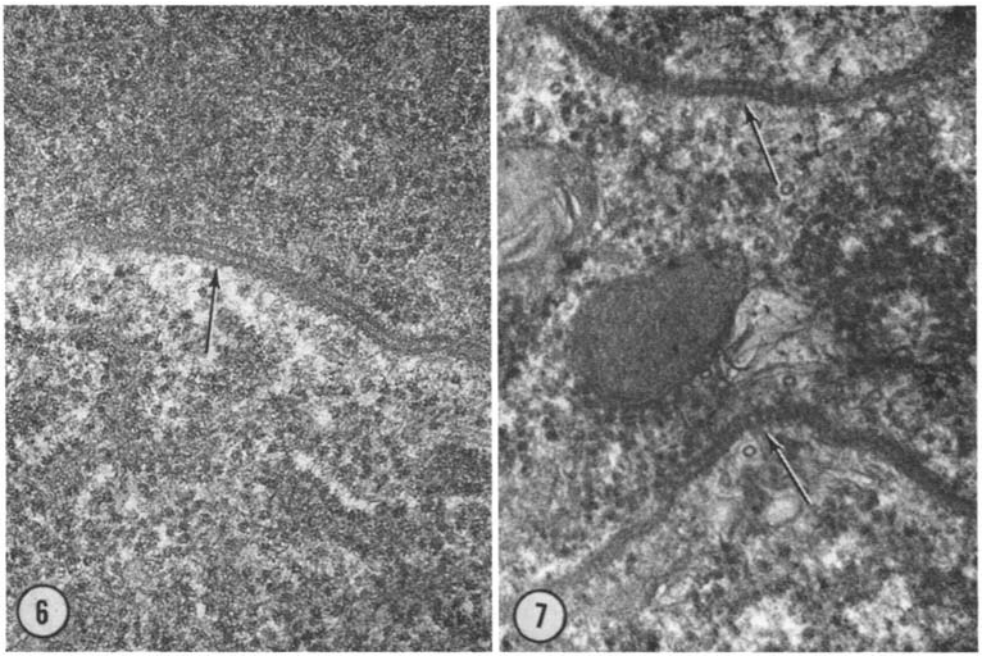

FIGURE 6 Junction of *Chironomus* salivary gland cells treated for 10 min with an uncoupling medium containing a chelator-calcium complex giving a free Ca^{++} concentration of 10^{-4} M. (Medium No. *1*; see Methods and Table I). Septate structure is present (arrow). × 60,000.

FIGURE 7 Junction of *Chironomus* salivary gland cells treated for 10 min with a chelating uncoupling medium, calcium-free (Medium No. *2*). Septate structure is present (arrow). × 60,000.

structure is that the septa are strips, 70 A thick, placed every 150 A at right angles to the membranes. This fits most observations too, particularly those on Malpighian tubules. But it would be hard to account, then, for the hexagonal arrays seen in electron micrographs of salivary glands. No optical effect can transform parallel lines into hexagons; and the hexagonal arrays are seen only at the level of the septate junction, not at the level of the nonjunctional membranes (including the basal portion of the cell junction).

One may look now for structural similarities between membrane junctions of the septate kind here and those of the occluding kind found in other cell systems. Functionally, the two junctions seem to be, at least in some cases, analogous, as far as cell-to-cell flow of small ions is concerned (cf. 13, 21, 23 *a*). Structurally, there are two obvious analogies: tangential views of membranes at occluding junctions show hexagonal arrays, and transverse views show, in some cases, a banding pattern (1, 24, 26, 27). It was, in fact, an occluding type of junction in which hexagonal and banding patterns were first described by Robertson (26). The dimensions of the arrays, however, are very different: the center-to-center distance of the hexagonal arrays in the septate junction is more than twice that of the occluding junctions; and the distance between the junctional membrane surfaces is many times greater.

Uncoupled State

Electron micrographs of gland cells treated with media Nos. *1* and *2* of Table I were of acceptable quality for a comparison of the junctional structures in uncoupled and normally coupled cells. Medium No. *1* is known to cause uncoupling in two steps. First, the diffusion barrier insulating the interior of the communicating cell system from the extracellular fluid is rendered permeable to Ca^{++}, presumably chiefly at the perijunctional insulating element; and then, extracellular Ca^{++} entering the interior causes the permeability of the junctional

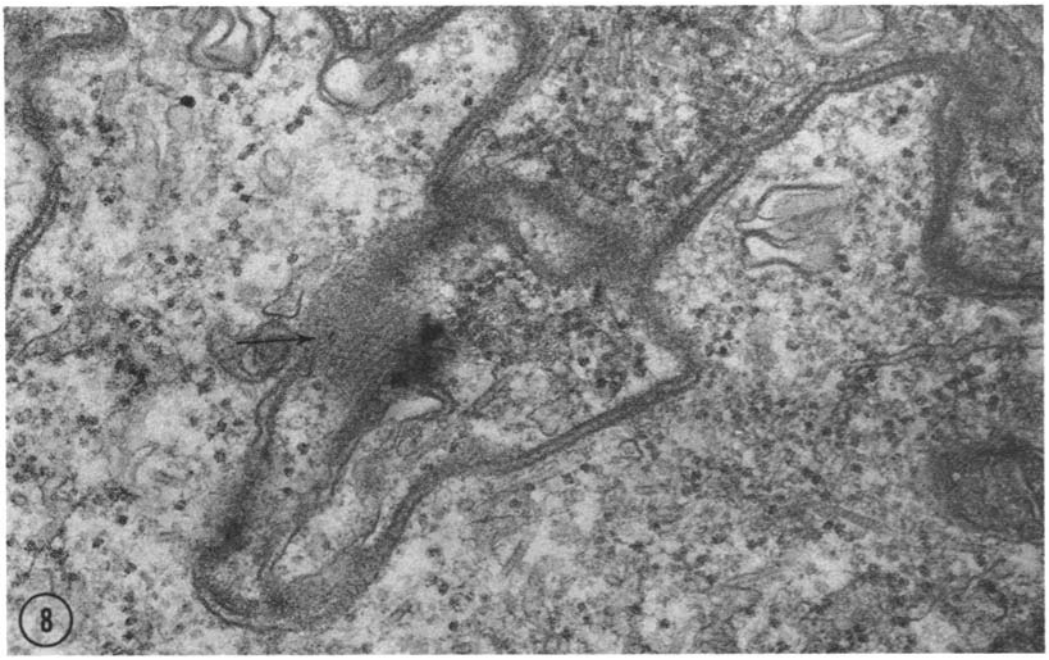

FIGURE 8 Junction of *Chironomus* salivary gland cells treated with a hypotonic medium for 4 min (Medium No. 4). Septate structure is present and has the characteristic appearances in transverse, oblique, and frontal sections (arrow). × 60,000.

membrane to fall (14, 15, 17). With medium No. 2, the second step is missing.

The obvious elements, then, on which a search of structural correlates of uncoupling should be focused are the septa and the cell membrane portions connected with the septa. We were guided further in this search by information on the magnitude of the permeability changes at each step. The conductance of the perijunctional insulating elements increases with decreasing free extracellular Ca^{++} concentration (14, 15). But, as is well known, cell adhesion also decreases with decreasing extracellular Ca^{++}. Thus, it is first of all necessary to distinguish between the processes of adhesion and perijunctional insulation (or, at least, total surface insulation), particularly since there are good reasons for suspecting a close relation between the structural elements of cell adhesion and insulation (13). We chose, therefore, the highest possible free Ca^{++} concentration (10^{-4}) which would satisfy the conditions of the two uncoupling steps. At this concentration there are no detectable alterations in cell adhesion.[1] The permeability change in the perijunctional insulating elements is relatively small at this concentration. The over-all input conductance of the cell system changes sometimes by no more than a few per cent (see, for example Fig. 12 of reference 17). But it is always sufficient to set off the second uncoupling step.

The permeability change associated with the second step is large. The permeability state changes here so radically that the junctional system, norm-

[1] 10^{-4} M Ca^{++} leaves a safety margin of several orders of magnitude for cell adhesion. In this respect, *Chironomus* salivary gland cells show a clear advantage over other cell systems, such as mouse liver or toad urinary bladder, used in our trial runs in search for an appropriate cell material.

No experiments were made with stronger levels of chelation. If Ca^{++} sequestering is intensified, cells come apart; and cells thus separated emerge each with complete surface membrane equipment, and survive, as is plain from tissue culture work. It is thus clear *a priori*, that with progressive intensification of Ca^{++} sequestering, a state will be reached at which the septate junction will split, as do other junctions (e.g., reference 9). But the structural aspects of such a state may be quite irrelevant to the processes of cell communication; in fact, for the reasons discussed above, they are likely to provide misleading information.

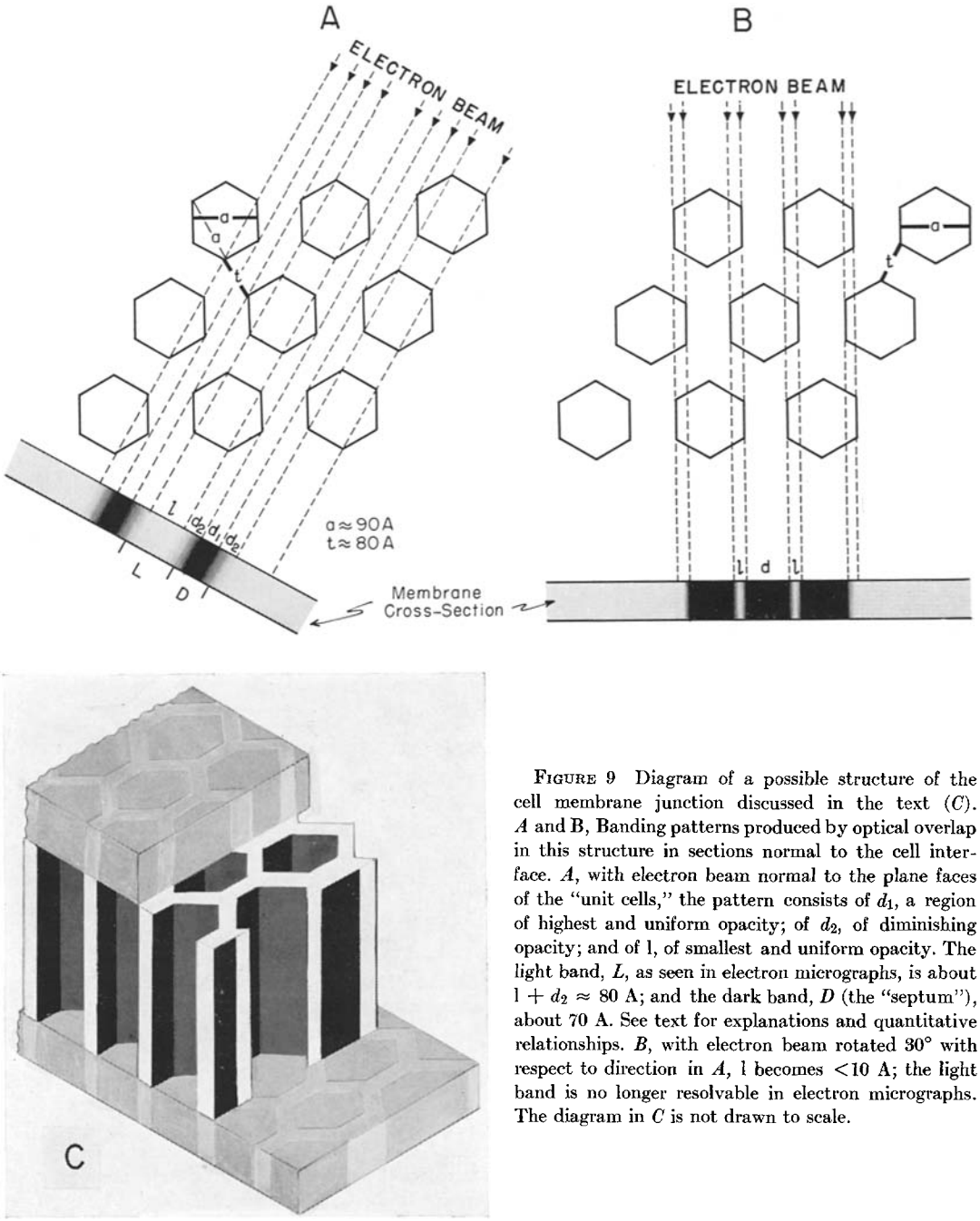

FIGURE 9 Diagram of a possible structure of the cell membrane junction discussed in the text (*C*). *A* and B, Banding patterns produced by optical overlap in this structure in sections normal to the cell interface. *A*, with electron beam normal to the plane faces of the "unit cells," the pattern consists of d_1, a region of highest and uniform opacity; of d_2, of diminishing opacity; and of l, of smallest and uniform opacity. The light band, *L*, as seen in electron micrographs, is about $l + d_2 \approx 80$ A; and the dark band, *D* (the "septum"), about 70 A. See text for explanations and quantitative relationships. *B*, with electron beam rotated 30° with respect to direction in *A*, l becomes <10 A; the light band is no longer resolvable in electron micrographs. The diagram in *C* is not drawn to scale.

ally freely permeable to all kinds of ions, is virtually sealed off upon completion of the second step (17). Yet there is no significant structural change discernible in electron micrographs of the septate junction in this step, nor in the preceding one. This would perhaps not be too surprising if we were dealing with membrane permeabilities to small ions only (K^+, Cl^-, etc.). In this respect, the information obtained from electron microscopy of membrane material fixed with presently available

techniques is evidently too limited. But it is disappointing that these limitations seem also to extend to permeabilities involving large ions and molecules with hydrodynamic diameters on the order of 50 A, or greater (13).

This touches on the question of how much functional information is provided by electron micrographs showing junctions with close membrane connections. Since good perijunctional insulation is a requisite for effective communication between cell interiors, all communicating cell systems are likely to have some form of close membrane junction, occluding, septate, or the like. But the reverse, of course, is not necessarily true. This means only that it is unsafe to rely on electron microscopic data alone to classify a membrane junction as communicating.

This work was supported by research grants from the National Institutes of Health and the National Science Foundation. We thank Dr. M. Nakas and Dr. F. Wald for assistance in some of the experiments on uncoupling, and Dr. S. J. Socolar, for helpful discussion.

Received for publication 25 October 1967.

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