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Intercellular Communication and Some Structural Aspects of Membrane Junctions in a Simple Cell System

BIRGIT ROSE

Cell Physics Laboratory, Department of Physiology,
Columbia University College of Physicians & Surgeons,
New York, New York 10032

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Summary. A *Chironomus* salivary gland consists of a chain of 30 giant cells (G-cells) and 4 to 6 flat cells (F-cells) spanning a lumen. The surface membranes of these cells are linked by two kinds of organized structures: the *septate junction*, taking up nearly the entire surface of cell contact, and the *gap junction*, occupying a small fraction of this surface. (These junctional structures are examined in the electron microscope after La infiltration.) All cells are joined to their immediate neighbors by septate junctions, the G- to G-cells, the F- to F-cells, and the F- to G-cells; the G-cells, at least, are also joined by gap junctions. All cells are also in communication with each other: small inorganic ions, fluorescein (mol. wt. 330) and Procion Yellow (mol. wt. ≈ 550) pass from one cell interior to the next.

The salivary gland cells of *Chironomus* have been the object of extensive studies of cell-to-cell communication (*coupling*) (Loewenstein, Socolar, Higashino, Kanno & Davidson, 1965; Loewenstein, Nakas & Socolar, 1967; Politoff, Socolar & Loewenstein, 1969; Rose & Loewenstein, 1971; Socolar & Politoff, 1971*a*, *b*; Oliveira-Castro & Loewenstein, 1971). Because of their large size and transparency, these cells are ideal for such studies; much of our present knowledge of the mechanisms controlling permeability of cell membrane junctions derives from work on these cells.

The present study is directed to the structure of the gland cell junction, particularly to the question of the site of coupling. Earlier electron microscopy had shown that the cell surface membranes are joined by an organized structure, the *septate junction* (Bullivant & Loewenstein, 1968). I investigate this structure further with the aid of a tracer of extracellular space and search for other types of junctional structures, such as the *gap junction* (Revel & Karnovsky, 1967), known to be present in several other communicating cell systems (Revel & Karnovsky, 1967; Payton, Bennett & Pappas, 1969; Brightman & Reese, 1969).

A further objective of the investigation is the distribution of coupling in the gland. With electrical and fluorescent-tracer techniques, I study the coupling between the different cells forming the organ, and look into the distribution of junctional structures between the cells. The *Chironomus* salivary gland, an organ composed of only a small number of cells, is particularly suited for such work.

Materials and Methods

Preparation

Salivary glands of midfourth instar larvae of *Chironomus thummi* were used. At this stage, the gland cells are particularly large. The animal's head was torn off, and the attached salivary gland pair was pulled out of the body cavity, together with part of the digestive system. This allows one to isolate the glands, without touching them, by simply severing the gland ducts close to the head. The glands were immobilized in the center of a small petri dish by laying very fine, angled steel wires across the duct or on the edge of a cell. The bathing solution (control medium) had the following composition: NaCl, 28 mM; KCl, 2 mM; $CaCl_2$, 5 mM; Mg-succinate, 7 mM; Na_2-fumarate, 28 mM; TES[1], 5 mM; and glutamine, 80 mM; titrated to pH 7.4 with NaOH, 4 to 5 mM

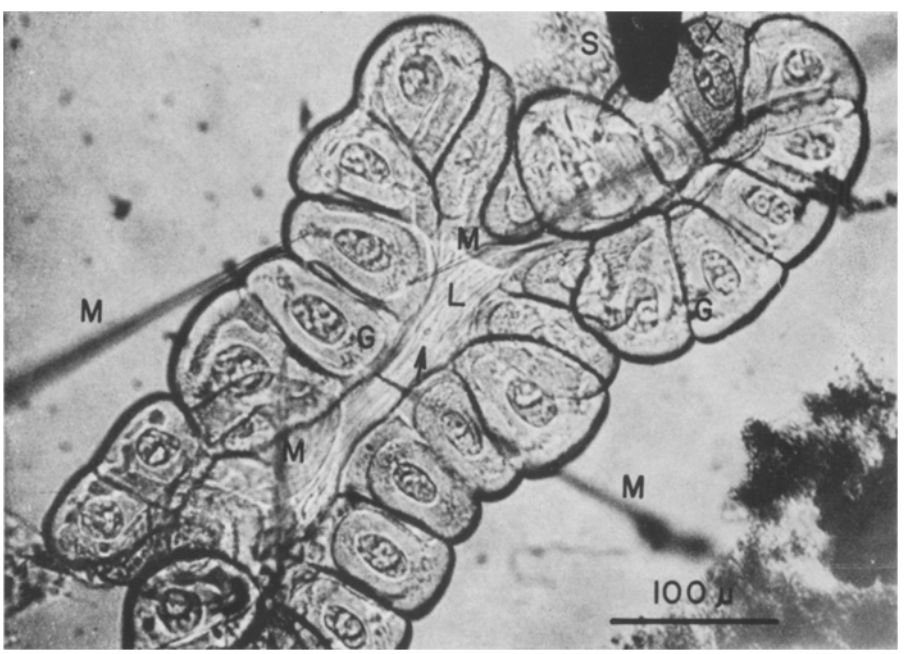

Fig. 1. Photomicrograph of *Chironomus* salivary gland (at end of a 2-hr-long experiment). *G* giant border cells (G-cells); *L* lumen; *M* microelectrodes in intracellular positions; *X* damaged cell; *S* end of steel wire used to immobilize gland. The arrow points to the nucleus of a flat cell (F-cell)

1 TES: N-tris(hydroxymethyl)-methyl-2-amino-ethane sulfonic acid.

(Politoff *et al.*, 1969). Integrity of the cells was ascertained before each experiment; damaged cells are readily recognized by the swelling of their nucleus and loss of transparency. (Moreover, membrane impedance and potential are low.) The glands were photographed routinely before and after each experiment (e.g., Fig. 1).

Electrical Arrangement

Micropipettes with less than 0.2-μ tip diameter served for intracellular electrical measurements. The micropipettes were filled with 3 M KCl, to which 1 to 2 mM K_2-citrate was added to reduce resistance. Such electrodes had resistances of 15 to 30 MΩ and $\leqq 5$ mV tip potential. To measure coupling between cells, inward current pulses (2 to 4×10^{-8} amp; 100 to 200 msec duration) were passed from an intracellular electrode M_i to the grounded medium. Two further electrodes, one in the same cell (M_I), the other in a different cell (M_X), measured the voltage changes V_I and V_X caused by the current pulse (Fig. 8 *inset*). Voltages and current were displayed on an oscilloscope.

Injection of Fluorescein and Procion

Disodiumfluorescein was injected hydraulically or electrically. For the hydraulic injection, micropipettes of about 1-μ tip diameter were used. These pipettes were first filled with distilled water which was then exchanged for 10 mM fluorescein in 150 mM KCl. For electrical injection, pipettes of $\leqq 0.5$-μ tip diameter were filled in the same way with 10 mM fluorescein. Another fluorescent dye, Procion Yellow M4RS[2] (Kravitz, Stretton, Alvarez & Furshpan, 1968), was injected electrically only (pipettes filled with 4% Procion). The fluorescein or Procion anions were driven out of the pipette with rectangular electrical pulses (2 to 4×10^{-8} amp; 100 to 200 msec duration; 2.3 to 6.3/sec; pipette tip negative). Fluorescence was excited by blue light (Leitz filter BG 12) in the case of fluorescein and by ultraviolet in the case of Procion, and observed in the microscope dark field through a yellow filter (Leitz OG 1). Coupling at the same time was monitored continuously during the injection. In the case of electrical injection, the current carried by the fluorescein or Procion anions served for the measurement of coupling. In the case of hydraulic injection, the fluorescein-filled pipette was added to the three measuring electrodes.

Electron Microscopy

For electron microscopy, the isolated glands were fixed either immediately or after exposure to one of the following solutions: (1) *control medium* (3 hr) — see *Preparation*; (2) *Li medium* (3 hr) — Li^+ substitutes for Na^+ of control medium; (3) *Ca, Mg-free Na medium* (3 hr) — NaCl, 38 mM; KCl, 2 mM; Na_2-succinate, 7 mM; Na_2-fumarate, 28 mM; TES, 7 mM; glutamine, 80 mM; (4) *Ca, Mg-free Li medium* (3 hr) — Li substitutes for Na of Ca, Mg-free Na-medium; or (5) *DNP medium* (25 min) — 10^{-4} M 2,4-dinitrophenol (DNP) dissolved in control medium. All media were at pH 7.4 and room temperature. Fixation followed in 3% glutaraldehyde and 1% OsO_4 in Millonig's phosphate buffer (Millonig, 1961) each for 1 hr at 0 to 4 °C. The tissue was then dehydrated in a graded alcohol series and embedded in Durcupan ACM (Fluka AG, Switzerland). Thin sections were made on a Sorvall MT2 ultramicrotome. They were collected on uncoated copper grids, stained with uranyl acetate and lead citrate (Reynolds, 1963), and viewed in a Siemens Elmiskop 1. La infiltration was done according to Revel and Karnovsky's recipe (1967), but using sodium cacodylate buffer (200 mM, pH 7.3) and adding La to all aqueous solutions of fixation and dehydration at 0 to 4 °C.

2 Obtained from Imperial Chemical Ind. Organics Inc. (Providence, R.I.).

Results and Discussion

Membrane Junctions

Septate Junction. Fig. 2 shows a diagram of the *Chironomus* salivary gland constructed on the basis of serial sections. The gland consists of about 30 giant border cells (G-cells) 0.1 to 0.15 mm in diameter, arranged typically in single file around a lumen; and 2 sheets of 2 to 4 flat cells (F-cells) spanning the lumen on either side of the gland (Figs. 1 & 2).

All cells are connected by septate junctions: G- to G-cells, F- to F-cells, and F- to G-cells (*see*, e.g., Figs. 3 & 4). (*See* also Kloetzel & Laufer, 1969.[3]) In sections normal to the plane of membrane contact, the junctions appear as 300 to 350 Å thick structures, consisting of the 70 to 90 Å thick membranes of the two cells, separated by a gap of 170 Å. The gap is bridged by 60 to 80 Å thick septae at spacings of 80 to 100 Å (Figs. 3 & 4). Sections in the plane of the junction show a polygonal structure, the "cores" of the polygons being electron transparent with a center-to-center spacing of about 180 Å, and the polygonal "walls" being relatively electron opaque. The general appearance and dimensions of the junction are consistent with previous descriptions (Wiener, Spiro & Loewenstein, 1964; Locke, 1965; Bullivant & Loewenstein, 1968; *see* also Wood, 1959).

In glands impregnated with colloidal La, the electron-opaque La is seen only in the gap between the two membranes, everywhere along the septate junction, but not in the cytoplasm or in the inner structure of the membranes.

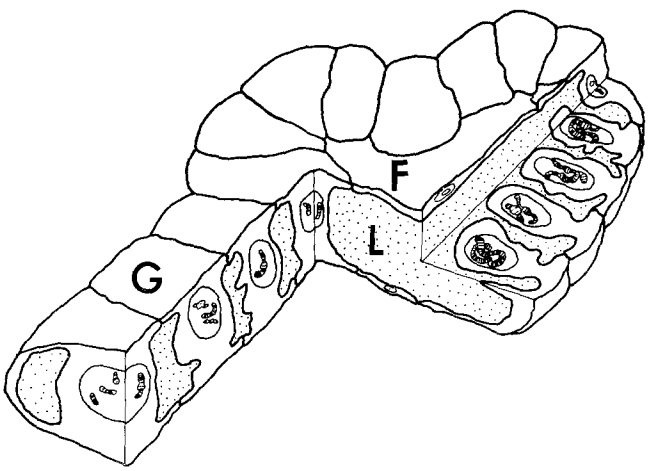

Fig. 2. Schematic drawing of the gland, based on serial sections (not to scale). *G* G-cell; *F* F-cell; *L* lumen. Note lumen interdigitating the G-cells

3 This work was in progress when Kloetzel and Laufer's report appeared.

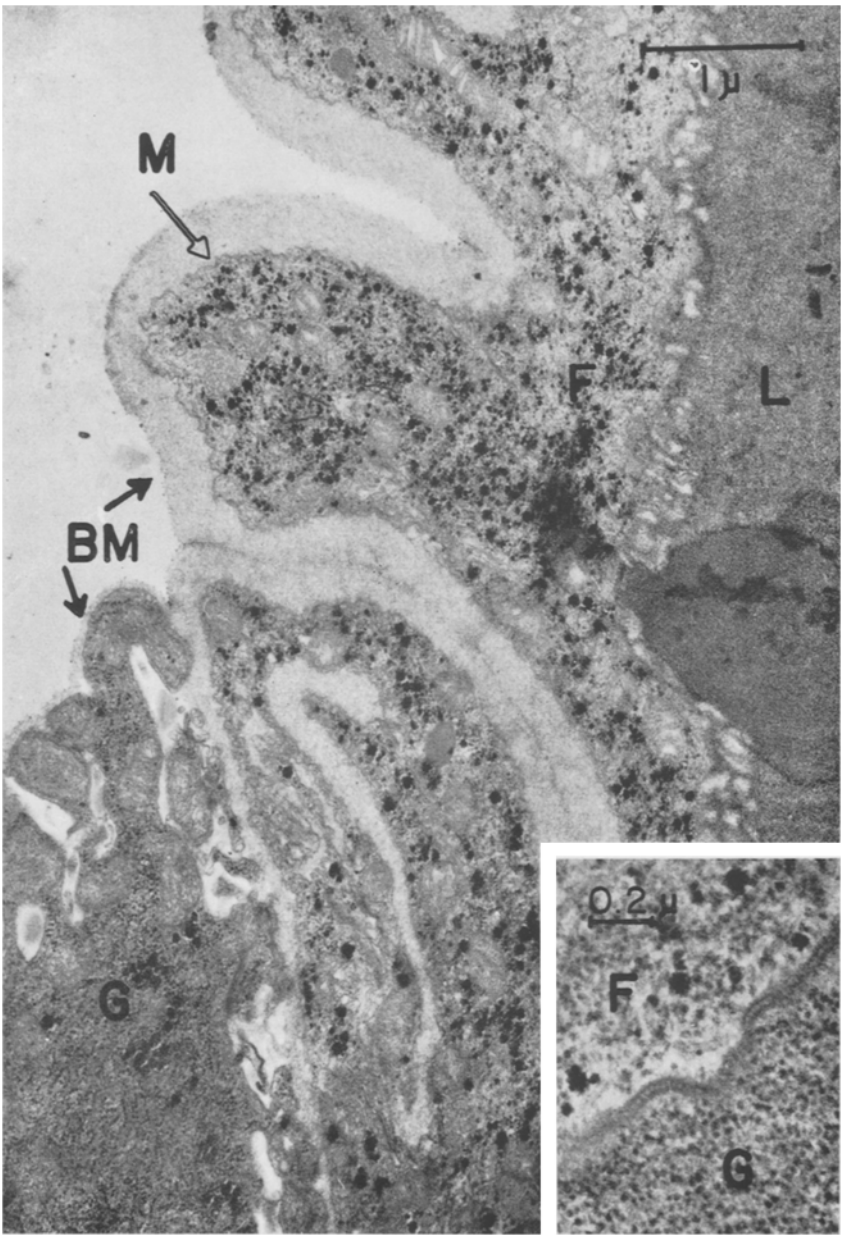

Fig. 3. Electron micrograph of *Chironomus* salivary gland cells. *G* G-cell; *F* F-cell; *L* lumen. Solid arrows indicate basement membrane (*BM*); open arrow indicates plasma membrane (*M*). (Note the difference in thickness of basement membranes of the two cell types: 3,300 Å vs. 430 Å.) *Inset*: Septate junction between F- and G-cell. (Gland exposed to control medium.)

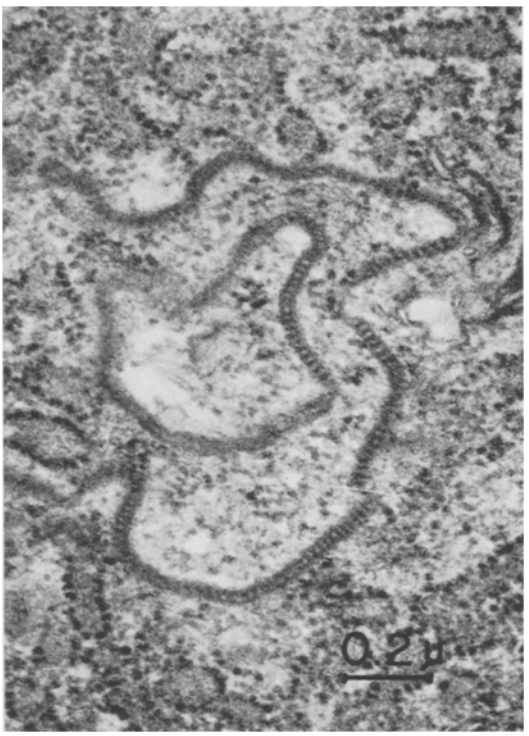

Fig. 4. Septate junction between two G-cells. (Control medium)

La accentuates the septation of the junction; the space between the two membranes is now a 200 Å wide electron-opaque line interrupted periodically at distances of 80 to 150 Å by relatively electron-transparent 40 to 60 Å thick bands (Figs. 5 & 6 *inset*). Sections in the junctional plane reveal again the polygonal pattern of the junctional complex; the "cores" are now strongly electron opaque (La), with an average diameter of 160 Å and a center-to-center spacing of 200 to 220 Å (Figs. 5b & 6), and the polygonal "walls" are relatively transparent.

Because it offers a highly organized form of continuity between cells, the septate junction is a candidate for cell-to-cell communication. As a coupling device, the junction would have to incorporate the intercellular ion pathway as well as its perijunctional insulation from the exterior, required by the results of electrical measurements (Loewenstein, 1966). From earlier electron-microscopical work, a honeycomb appeared as a plausible structure for this junction; a simple possibility was a coupling model in which the honeycomb cores constitute the ion path, and the honeycomb walls the insulation (Bullivant & Loewenstein, 1968). The

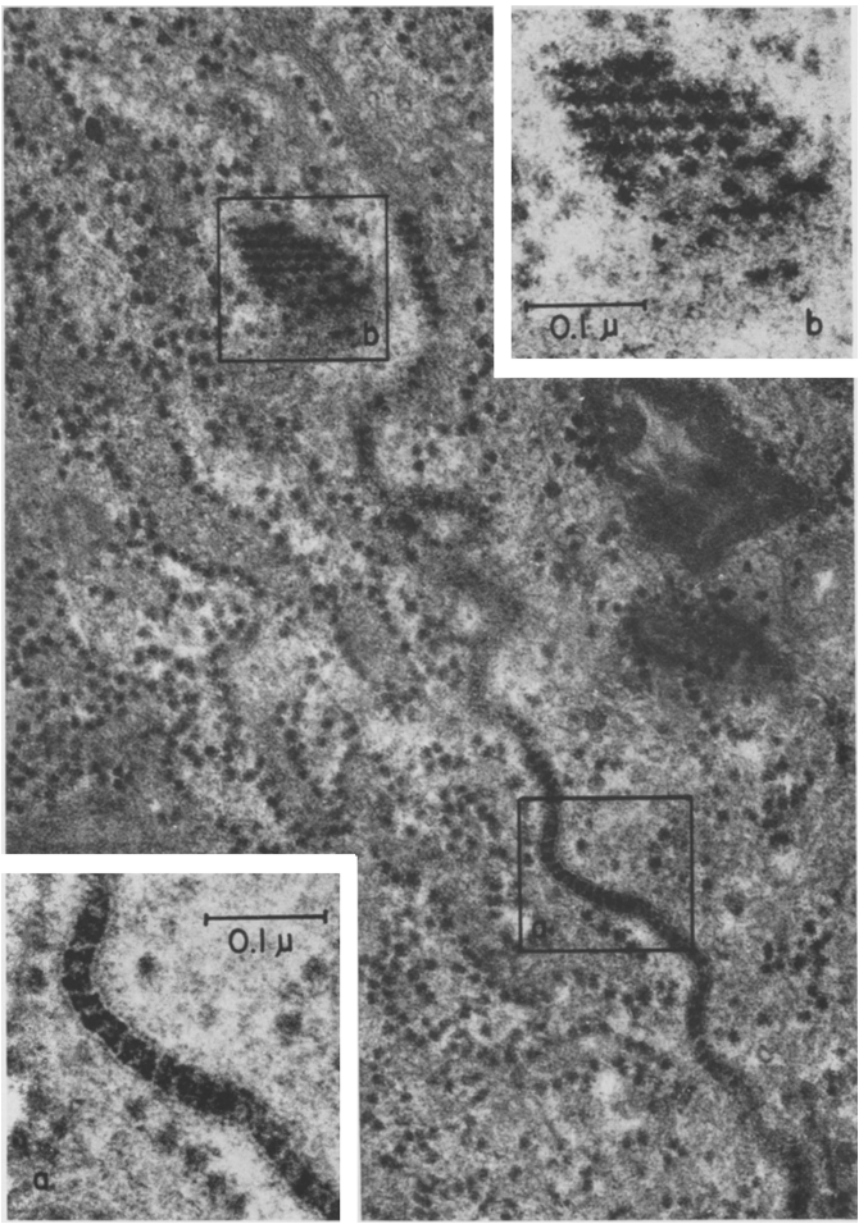

Fig. 5. La-impregnated septate junction between G-cells. a, section normal to plane of junction; b, section in plane of junction. (Control medium)

present results throw new light on this structure. If La is truly a tracer for extracellular space, as has been proposed (Revel & Karnovsky, 1967), then the "honeycomb cores", where La is shown to be located, must be open

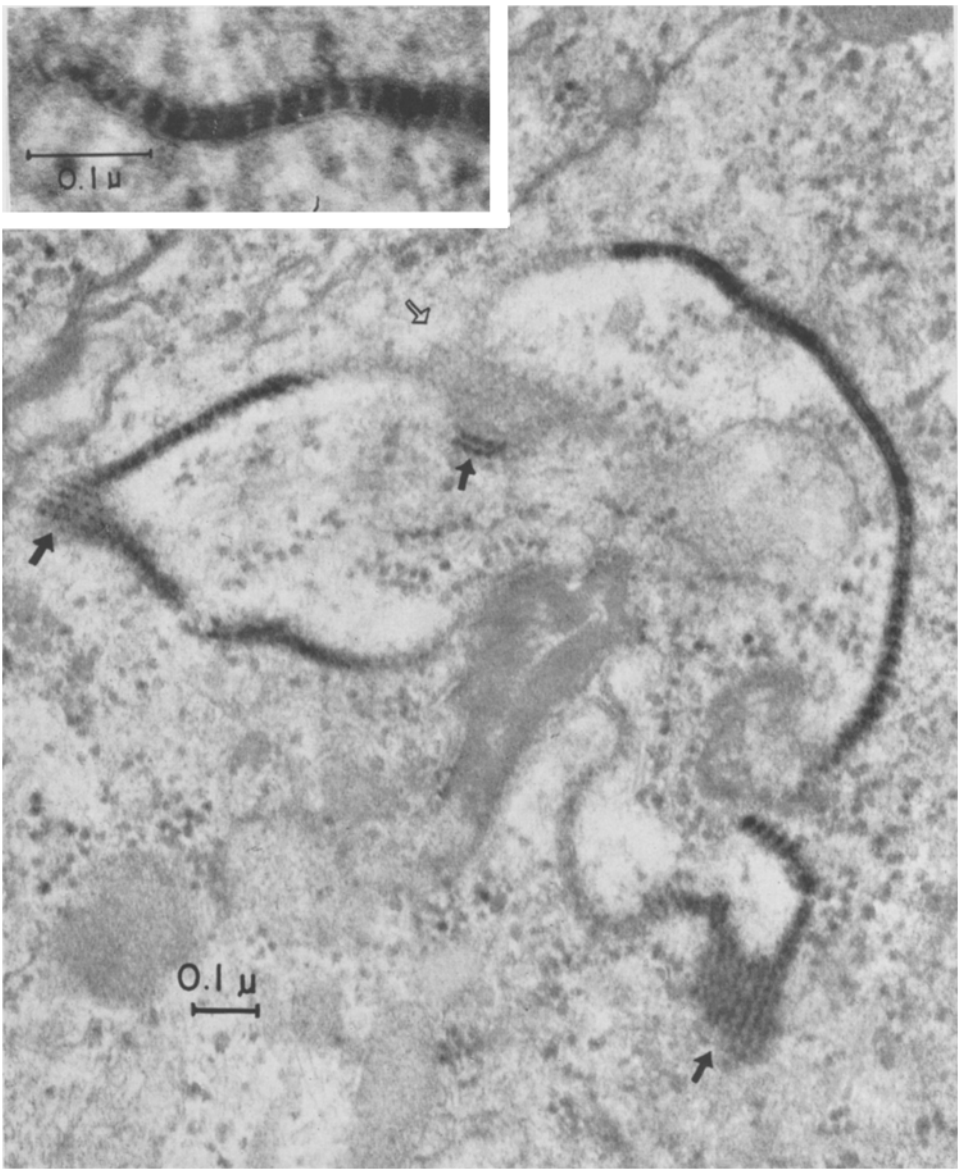

Fig. 6. Septate junction (G-cells), partially impregnated with La. The arrows point to regions in the plane of the junction. Solid arrows point to La-impregnated junctional region; open arrow, equivalent region not impregnated. *Inset*: A La-impregnated junctional cross section at higher power (G-cells). Note that outer membrane leaflets appear discontinuous at some septae, whereas the inner ones are continuous. (Ca, Mg-free Li-medium)

to the exterior, and the septae themselves would have to contain all the coupling elements. On the basis of their electron-microscopical results on the septate junction of mussel gill epithelium, Gilula, Branton and Satir (1970)[4] propose an interesting model in which the septae are zigzag-pleated sheets subserving the coupling. In the interpretation of the present results, everything hinges on whether $La(OH)_3$ is a reliable tracer for extracellular space (i.e., space in communication with the external medium). Work is now in progress to determine if the "cores" are indeed in communication with the exterior.

Gap Junction. Another type of organized membrane junction occurs toward the basal part of the cells. This type of junctional complex is about 130 to 140 Å thick in cross section. It was found over short distances only, often not exceeding 1 μ. In this type of junction, the membranes of the two adjacent cells are separated by a (La-impregnated) gap of 40 Å. Here and there the gap is interrupted periodically by narrow bands at 90 Å spacings (Fig. 7). The general appearance of this membrane junction and its dimensions are those of Revel and Karnovsky's (1967) "gap junction".

The occurrence of the gap junction in this tissue raises again the question of the structural site of cell coupling. Which of the junctions mediates coupling – the extensive septate junction, the much smaller gap junction, or both? Many coupled cell types lack septate junctions: certain nerve cells (Payton *et al.*, 1969; *see* also Robertson, 1963; Pappas & Bennett, 1966; Brightman & Reese, 1969), heart cells (Revel & Karnovsky, 1967; McNutt & Weinstein, 1970), liver cells (Revel & Karnovsky, 1967; Benedetti & Emmelot, 1968), thyroid and skin cells (Farquhar & Palade, 1963, 1965). Since the first three tissues, and probably also the last two, have gap junctions, the gap junction appears as a likely site of coupling. But there are also coupled cells, such as the mussel gill cells, which appear to have only septate junctions (Satir & Gilula, 1970; Gilula *et al.*, 1970).

The aforementioned cells with only gap junctions have a lower degree of coupling than the salivary gland cells: the coupling coefficient (*see* next section for a definition) in the nerve cells is 0.1 to 0.3 (Furshpan & Potter, 1959; Watanabe & Grundfest, 1961; Bennett, 1966), in heart muscle about 0.4 (Weidmann, 1952; Barr, Dewey & Berger, 1968), in thyroid 0.2 to 0.3 (Jamakosmanovic & Loewenstein, 1968), in skin 0.4 (Penn & Loewenstein, 1967), and in liver, 0.8 (Penn, 1966), as against a coefficent of nearly 1 in salivary gland (Loewenstein & Kanno, 1964; Politoff *et al.*, 1969; Rose &

4 The present work was done in 1969–70, before I had knowledge of the work of Gilula *et al.* (1970).

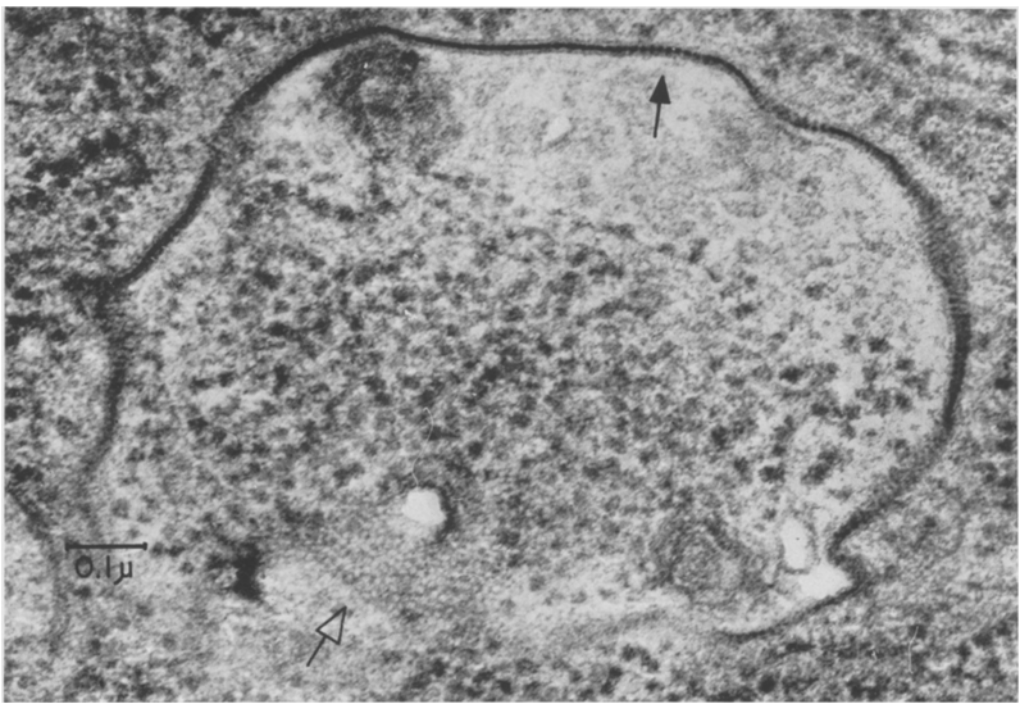

Fig. 7. La-impregnated gap junction. Solid arrow points to banding in a cross-sectional region; open arrow points to section in the plane of the junction, revealing polygonal structure (Ca, Mg-free Li-medium)

Loewenstein, 1971). The question now is can the gap junction alone mediate the high degree of coupling in the salivary gland cells, or, stated more meaningfully, is the area of gap junction sufficient to account for the observed junctional conductance? The junctional resistance can be calculated from measurements of voltage attenuation in a cell chain, such as those illustrated in Fig. 9. The resistance of the cytoplasm-junctional membrane complex, r_j, is given by:

$$r_j = \frac{V_I}{i_I} \frac{s}{\lambda} \left(\tanh \frac{a}{\lambda} + \tanh \frac{b}{\lambda} \right) \tag{1}$$

(Loewenstein *et al.*, 1967), where i_I (4×10^{-8} amp) is the current passed between the interior of cell 1 and the medium; V_I (24 mV) the voltage in cell 1; s (90 μ) the mean cell length; b (1,123 μ) the distance along the cell chain from the center of cell 1 to the end of the chain (cell 16); a (100 μ) the corresponding distance along the chain in the opposite direction; and λ (1,368 μ) the space constant (data of experiment of Fig. 9). If we select an

origin anywhere between the current source and the end of the chain, then the potential at any other point X on the chain on the same side of the current source is given by:

$$V_X = V_0 \frac{\cosh \frac{D-x}{\lambda}}{\cosh \frac{D}{\lambda}} \tag{2}$$

(Loewenstein & Kanno, 1964) where D is the distance between the origin and the end of the chain; x the distance between X and the origin; and V_0 the voltage at the origin. When $x = D$, the expression simplifies to:

$$V_{x=D} = \frac{V_0}{\cosh \frac{D}{\lambda}}, \quad \text{and} \quad \cosh \frac{D}{\lambda} = \frac{V_0}{V_{x=D}}$$

λ was thus obtained from the voltage measurements in cells 4, 5, 8, or 10 (V_0), their respective distances (D) from the end of the chain (cell 16), and the voltage in cell 16 ($V_{x=D}$). The Table gives the λ values so obtained; mean $\lambda = 1{,}368$ μ. Thence the junctional resistance $r_j = 5.4 \times 10^4$ Ω [Eq. (1)]; this includes the resistance of the cytoplasm which is of the same order of magnitude. The resistance of a gap junction of 20 μ^2 area in an electrotonic nerve synapse is $\approx 5 \times 10^6$ Ω (Asada, Pappas & Bennett, 1967; Pappas Asada & Bennett, 1967). Hence, to account for r_j, the area of such a gap junction in the salivary gland would have to be 2,000 μ^2 (i.e., nearly half as large as the entire area of cell contact). This is certainly not the case. One must conclude, therefore, that either a major part of the coupling in salivary gland cells is provided by a structure other than the gap junction or that the junctional membrane in the gap junction here is structurally very different from that in the nerve synapse, with a specific resistance several orders of magnitude smaller.

Table. *Data*[a] *for calculation of space constant* (λ)

Origin cell no.	V_0 (mV)	$V_{x=D}$ (mV)	L (μ)	λ (μ)
4	23	18.7	878	1,320
	22.1	18.7		1,475
5	21.8	18.7	806	1,418
8	21	18.7	616	1,257
10	19.7	18.7	452	1,369

[a] From the experiment illustrated in Fig. 9.

No change in the structure of the gap junction and septate junction was discernible in the electron microscope when, prior to fixation, the glands were exposed to one of the following media known to depress junctional membrane permeability: DNP medium (Politoff *et al.*, 1969); Li medium; Ca, Mg-free Na medium; or Ca, Mg-free Li medium (Rose & Loewenstein, 1969, 1971). (For composition of the media, *see Methods.*) A similar negative result was obtained by Bullivant and Loewenstein (1968) on the septate junctions of glands treated with other agents depressing junctional permeability.

Topology of Coupling

The G-cells are known to be coupled with respect to their content of small ions (Loewenstein *et al.*, 1965). This can be shown by injecting a pulse of current into a G-cell (I), and measuring the resulting voltage changes (V) in this and an adjacent G-cell (II). The ratio V_{II}/V_I (*coupling coefficient*) is a convenient index for the degree of coupling between cells (Loewenstein & Kanno, 1964). Intact G-cells possess a coupling coefficient of nearly 1; evidently a large portion of the injected current flows from one cell interior to the next through membrane regions of low resistance (*junctional membranes*).

The preceding results on gland morphology showed that G-cells are joined to each other as well as to F-cells. The question now arises whether coupling is directly via-G-cell junctions, or via junctions with G-cells, or both. It is difficult to make coupling measurements in F-cells, because, aside from being thin, these cells have a very thick basement membrane (*see* Fig. 3). However, it is easy to interrupt the G-cell chain and to get an answer by measuring coupling in G-cells across the interruption. Fig. 8 illustrates an experiment wherein the membranes of three separate cell groups were slit open (hatched). This is known to cause a drastic fall in permeability of the junctions of the damaged cells in media containing divalent cations (Loewenstein *et al.*, 1967). Coupling between cells was then tested the following way: a current-passing electrode M_i and a voltage-recording electrode M_1 were inserted into a G-cell (1), and left there for the entire experiment. In random sequence, a third, roving electrode M_X measured the voltage changes V_X (resulting from the current passed into cell 1, i_I) in other G-cells of the gland. Simultaneously the voltage changes in cell 1 were recorded (V_1). The corresponding V_X/V_1 values are plotted in Fig. 8, in the order of the position of cells X on the G-cell chain, with respect to cell 1. Interruption of coupling along the G-cell chain was complete; the interiors of the slit cells were completely shunted to the exterior as shown by measurements of input resistance (not shown on graph), and some of their immediate neighbors (cells -4, 13), damaged during the slitting procedure, showed no coupling with cell 1 ($V_X/V_1 \approx 0$). Nonetheless,

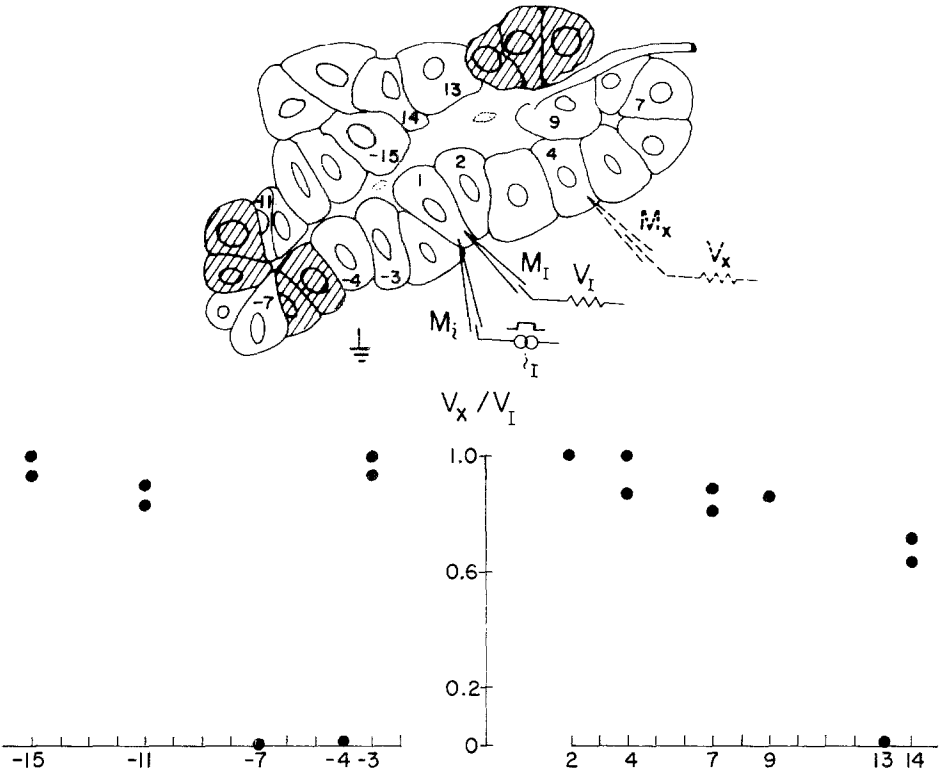

Fig. 8. Coupling pathways in the gland. Coupling via F-cells. *Top*: Tracing of gland photomicrograph. The G-cell chain was interrupted at three separate regions by slitting open several G-cells (hatched). Cells −4, 13 were damaged in the process. M_i fixed electrode passing current pulses (4×10^{-8} amp; 200 msec duration; inward) from cell 1 to grounded medium; M_I fixed electrode measuring voltage changes V_I in the same cell; M_X roving electrode measuring voltage changes V_X in other G-cells. *Bottom*: V_X/V_I values plotted according to the corresponding cells' position on the G-cell chain, with respect to cell 1. Two measurements (not successive) were made in each numbered cell; identical values are plotted as single points

G-cells on either side of the interruption were in good ionic communication (cells 14, −15 with cell 1, Fig. 8). The lumen is excluded here as a significant intercellular coupling path because no significant coupling between lumen and G-cells is found when electrodes are inserted into the lumen. A junctional coupling path therefore must exist between the F- and G-cells.

The existence of such a path could be demonstrated also by direct measurements in a few experiments in which electrode impalement of F-cells was successful. However, the coupling coefficient V_F/V_G was then at most 0.7 – much lower than expected from the aforegoing results. Possibly the coupling had been reduced as a consequence of F-cell impalement.

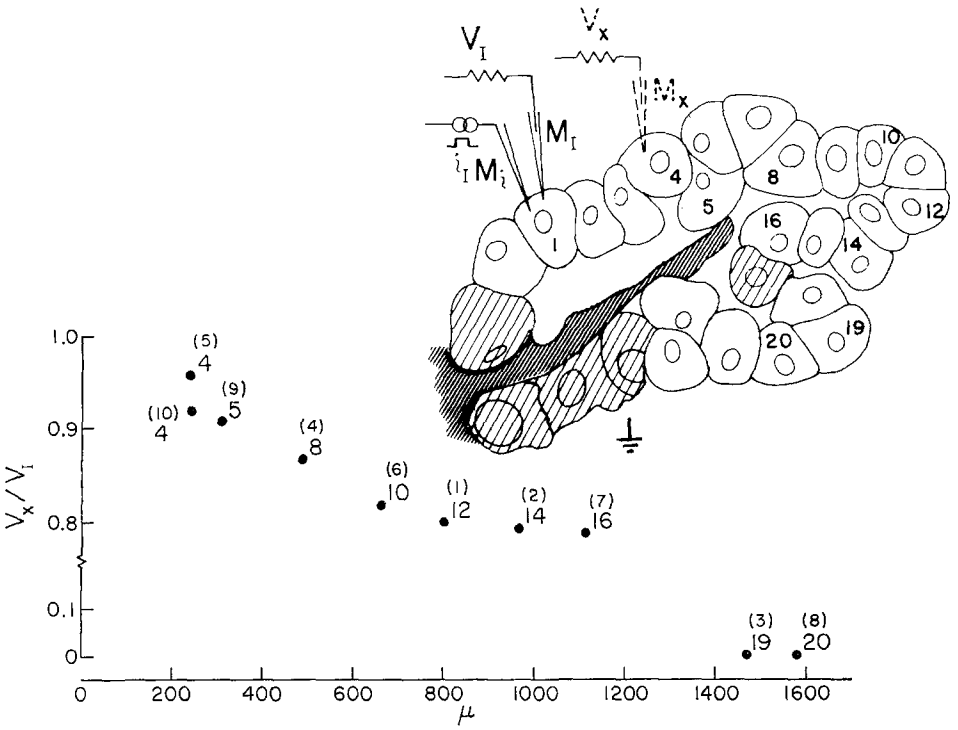

Fig. 9. Direct G-cell coupling. *Top*: Tracing of gland photomicrograph. The F-cells were destroyed by a longitudinal cut through the lumen (densely hatched), thereby eliminating all cross connections between G-cells. (Hatched G-cells were damaged during the process.) Electrical arrangement and measuring procedure as in Fig. 8. *Bottom*: V_X/V_I vs. the distance $M_X - M_i$, measured along the G-cell chain. Numbers above dots identify cells (*see* tracing); numbers in parentheses give the sequence of the measurements

G-cells are also coupled directly to each other. This can be shown by eliminating the F-cell paths: if a gland is cut longitudinally through the lumen so as to destroy the F-cells, the G-cells remain well coupled[5] (Fig. 9). The corresponding V_X/V_I values now decrease with increasing distance between the recording electrode M_X and the current source M_i (measured along the G-cell chain), as expected in an unbranched coupled cell chain.

In earlier measurements of voltage attenuation on whole glands, V was also found to decrease with the $M_i - M_X$ distance along the G-cell chain (and the calculated junctional membrane resistance values were of the same order as the present ones) (Loewenstein *et al.*, 1965, 1967). The F-cells, then not known, were probably unwittingly uncoupled and the measurements were thus also effectively in a single G-cell chain: for immobilization, the gland was compressed along the lumen by a wisp of glass in these earlier experiments.

5 There is no leakage via the open F-cells under these conditions; the F–G junction seals in the presence of Ca^{++} (Oliveira-Castro & Loewenstein, 1971).

Fluorescent Tracer Studies

In the previous measurements of cell coupling, the current was carried by the small cytoplasmic ions (e.g., K^+, Cl^-). The junctional membranes of *Chironomus* salivary gland cells are permeable also to larger molecules. I showed this for the anions fluorescein (mol. wt. 330) and Procion Yellow (mol. wt. 550), both of which can easily be traced in the cell interiors owing to their fluorescence.

Fluorescein has already been used successfully for probing junctional membrane permeability in several other tissues (Loewenstein & Kanno, 1964; Pappas & Bennett, 1966; Bennett, Dunham & Pappas, 1967; Furshpan & Potter, 1968). This ion is excited maximally with ultraviolet but is also excited with blue light. Fluorescein diffuses well in cytoplasm where it can easily be detected at a very low concentration because of its high fluorescence yield. I introduced fluorescein with hydraulic or electric pressure from a micropipette into a G-cell and observed the fluorescence excited by blue light (the cells exhibit strong autofluorescence in ultraviolet). Typically, fluorescein spreads throughout the cell and beyond the cell's boundaries into neighboring cells without visible leakage to the external medium (or to the lumen) (Fig. 10).

The spread of fluorescein along the G-cell chain is clearly visible but no fluorescence in F-cells was detected at the fluorescein concentration used – probably because of the thinness of these cells. However, there is certainty that fluorescein passes also from F- to G-cells; when an F-cell is injected, fluorescein is seen in the adjacent G-cells.

The spread of fluorescein is likely to be via junctions for several reasons. First, fluorescein is not taken up detectably at the concentrations existing in the medium during the experiments.[6] Moreover, cell-to-cell flow has been shown in *Drosophila* salivary gland for several dye molecules which are not lost by the cells to any significant extent (Kanno & Loewenstein, 1966; *see* also Schmidtmann, 1925; Potter, Furshpan & Lennox, 1966). Furthermore, in an electrical synapse, there is cell-to-cell spread of Procion Yellow, a fluorescent dye not taken up by the cells even at high concentrations in the medium (Payton *et al.*, 1969). I have injected Procion into *Chironomus* salivary gland cells, which also fail to take it up (at 3%

6 Fluorescein is taken up detectably by the cells only when it is present at visible concentrations in the bathing medium. The cells gradually lose the injected fluorescein (as is apparent from their gradual loss of fluorescence), but this is diluted into a volume 10^7 times that of a cell and, hence, never reaches visible concentration in the medium (or in the lumen).

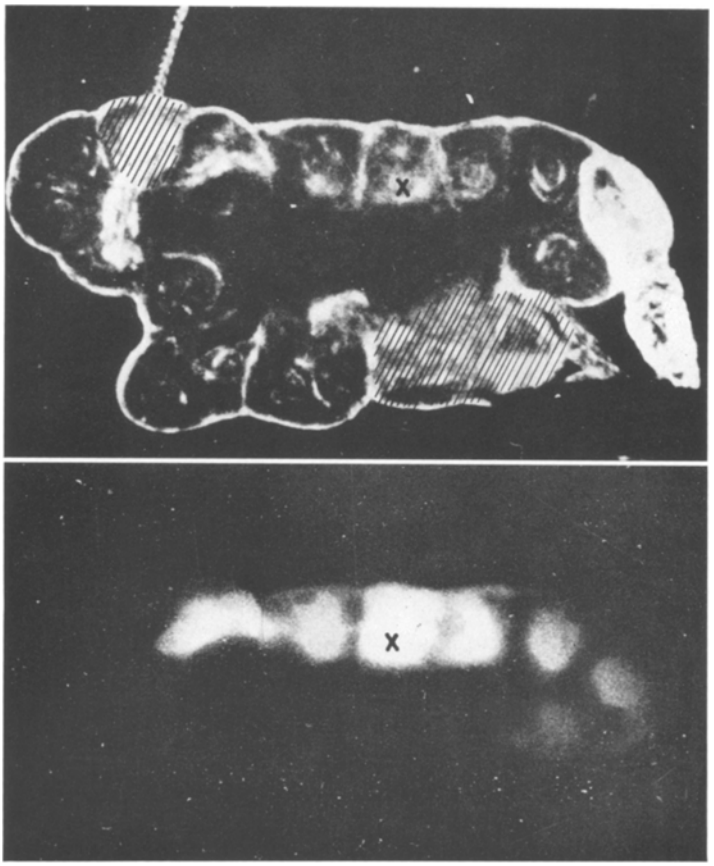

Fig. 10. Junctional passage of fluorescein. *Top*: Dark-field photomicrograph of gland. Fluorescein is injected into cell marked *X* (hydraulic injection). Cross-hatched cells were slit open. *Bottom*: Photomicrograph of fluorescence in the gland, 15 min after injection

in the medium), and obtained results similar to those with fluorescein (Fig. 11).

The possibility of nonjunctional fluorescein uptake here is unlikely for still different reasons. Cell-to-cell flow of fluorescein occurs only between coupled cells, as determined electrically, not between cells in which electrical coupling is interrupted. In the example of Fig. 10, electrically determined coupling was interrupted at two locations in the G-cell chain, at some distance from the injected cell, by slitting open several G-cells (hatched). Fluorescein is seen to have spread in both directions along the G-cell chain up to the borders with the damaged (not coupled) cells. Fluorescein is not detectable in the damaged cell region even after repeated injection, or after periods of observation longer than those of the experiment in Fig. 10.

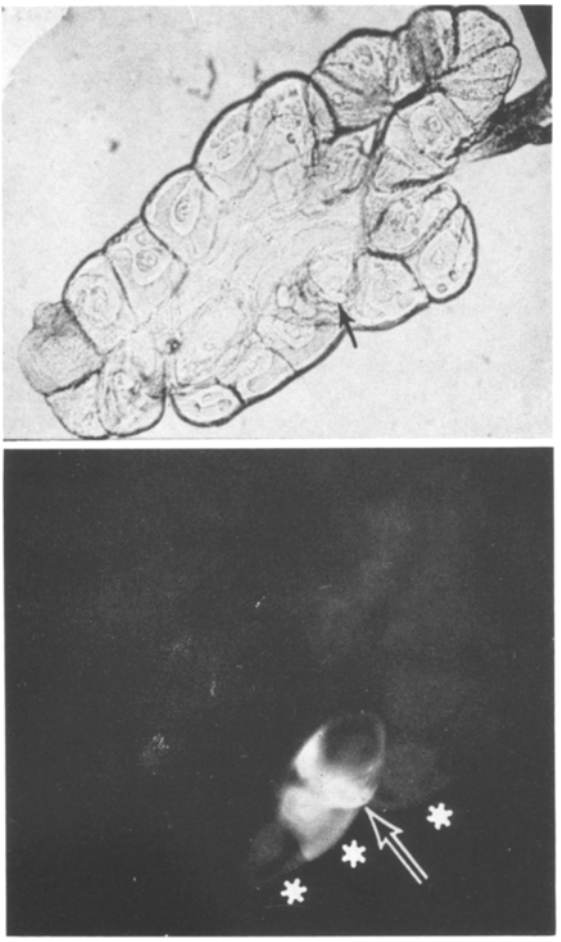

Fig. 11. Junctional passage of Procion Yellow. *Top*: Bright-field photomicrograph of gland; arrow marks cell injected with Procion (electrical injection). *Bottom*: Photomicrograph of fluorescence (UV-excitation) in the gland, 30 min after injection. The fluorescence of Procion is orange-yellow, whereas the cells' autofluorescence is yellow-green; the fluorescence of Procion is here distinguishable by its stronger intensity (cells marked*)

This is in line with the results of fluorescein studies in other situations of experimental interruption of coupling, such as when Ca^{++} is injected into cells (Kanno & Loewenstein, 1964; *see* also Oliveira-Castro & Loewenstein, 1971) or when Li^{+} substitutes for extracellular Na^{+} (Rose & Loewenstein, 1971). In all of these situations, cell-to-cell passage of fluorescein is blocked whenever the electrical conductance of junctional membranes is depressed. Thus it would seem that the large molecules take the junctional path, as do the small ones.

Note added in proof: It should be realized that the results of La-infiltration into the septate and gap junctions do not conflict with the concept *perijunctional insulation*, which is defined as a diffusion barrier between the cell system's interior and exterior and as an integral part of each *junctional unit* (Loewenstein, 1966). Each unit is thus a separate, insulated intercellular passageway; many such units make up a junction. This is compatible, of course, with extracellular spaces between the junctional units open to the exterior.

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