

OPAQUE DEPOSITS ON GAP JUNCTION MEMBRANES AFTER GLUTARALDEHYDE-CALCIUM FIXATION

W. J. LARSEN

From the Department of Physiology and Biophysics, University of Miami School of Medicine, Miami, Florida 33152. The author's present address is the Department of Anatomy, University of Iowa, Iowa City, Iowa 52242.

ABSTRACT

When cloned hybrid cells (A/B_m-5) were grown to confluence and fixed in glutaraldehyde-calcium, electron-opaque deposits were observed on the cytoplasmic faces of plasma membrane. Deposits were most abundant at gap junctions. Deposits were often precisely paired, cell-to-cell, across the gap junctional membranes, and these paired deposits were frequently equivalent in size. This relationship was most often observed on long profiles of gap junctions, in contrast to the asymmetric distribution of larger deposits commonly found on short junctional profiles. Deposits were present with or without heavy metal staining but did not appear when calcium was omitted from the fixative. Fixation at room temperature yielded more and larger deposits than fixation at 0°C. The significance of these observations is discussed with regard to the possible binding of calcium at fixed membrane sites or the precipitation of calcium by anions produced by enzymes located at the gap junction.

Glutaraldehyde-calcium fixation results in the appearance of opaque deposits located most frequently on the cytoplasmic faces of plasma membranes (34). They have been observed in intestine (34, 35), squid axon (21, 33, 36), muscle (34, 42), and a slime mold.¹ They have also been observed within synaptic vesicles (8, 21, 42). The deposits are discrete spheres, hemispheres, or ellipsoids measuring about 5–80 nm along their major axis. X-ray microprobe analysis has shown that the deposits in squid axon contain calcium and phosphorus (21, 33).

When a hybrid cell line (A/B_m-5) was grown to confluence and fixed in glutaraldehyde-calcium, opaque deposits were abundant on gap junction membranes and were frequently paired with deposits in the adjacent cell. This relationship was

rarely observed on short profiles of gap junctions, where a few relatively large deposits were usually associated with only one side of the junction.

Studies of deposit formation on other membranes after glutaraldehyde-calcium fixation suggest that they may localize calcium-binding sites (34, 35, 42) or calcium-binding proteins (21). It has also been proposed that calcium deposited on squid giant axon neuralemma may localize an ATPase by precipitating with phosphate produced by the hydrolysis of endogenous ATP (33).

Although the mechanism of deposit formation on gap junctions is not clear at present, their possible significance is discussed with regard to studies implicating calcium in the control of the cell-to-cell movement of ions (27, 32, 44) and to studies on the roles of cyclic AMP (c-AMP) and cell contact in the regulation of cell division in tissue culture cells (2, 5, 13, 20, 37).

¹ Cutler, L. S. 1974. Personal communication.

MATERIALS AND METHODS

Cells

Cloned cell hybrids, resulting from the fusion of a rat hepatoma cell and rat liver fibroblast (4), were grown to confluence on Dulbecco's Modified Eagle's Medium supplemented with 10% fetal calf serum. These hybrid cells were used shortly after hybridization and while still exhibiting density-dependent growth regulation.² This particular cell line was considered most suitable for study since large numbers of gap junction transections are found in thin sections.

Electron Microscopy

Cells were fixed in their culture dishes either at room temperature or at 0°C. The fixative contained 2.5% glutaraldehyde (Ladd Research Industries, Inc., Burlington, Vt.), 0.05 M cacodylate, 2% sucrose, and 0.005–0.1 M CaCl₂ at pH 7.4. CaCl₂ (0.05 M) was added to all wash solutions, osmium, and alcohols in the dehydration series up to 100% EtOH. Cells were fixed in glutaraldehyde and osmium for 30 min. In one experiment, cells were stained in block with 0.5% uranyl acetate for 30 min. After dehydration, the cells were washed with several small volumes of Araldite (Ciba Products Co., Summit, N. J.) before embedment. Calcium was omitted from the glutaraldehyde in control experiments. Gold sections were viewed with and without staining in a Philips 300 electron microscope (Philips Electronic Instruments, Mt. Vernon, N.Y.).

Some cultures were stained with lanthanum (43), and others were frozen and fractured with a Balzer's freeze-fracture apparatus (BA360M; Balzer's High Vacuum Corp., Santa Ana, Calif.). Platinum carbon replicas were then prepared and examined in the electron microscope. Gap junctional areas were measured by tracing their perimeters with a compensating polar planimeter (Keuffel and Esser Co., Morristown, N. J.).

Silver Staining

Cells fixed in the presence or absence of calcium were stained with the von Kossa technique (23). After being stained with 5% silver nitrate in the dark, the cells were washed several times with distilled water and placed on a white piece of paper directly under a 60 W bulb for 5 min to 1 h. They were then dehydrated and embedded in Araldite.

RESULTS

Junctional structure in A/B_m-5 cells

Junctional structure in this cell line was investigated with three different techniques. In stained thin sections, closely apposed membranes of adjacent cells were characterized by intercellular densi-

ties with a periodicity of 8 nm (Fig. 1). In this respect these membrane transections are similar to profiles of gap junctions described in other fibroblasts in tissue culture (3, 40) and in rabbit granulosa cells (7). They differ from many other descriptions of gap junctions since no 2–3 nm gap is observed. The membranes in these areas of cell-to-cell apposition were stained more heavily than plasma membrane facing the culture medium and in addition could be differentiated by their characteristic straight or slowly curving profiles. After fixation in the presence of lanthanum (43), narrow electron-dense regions 3–4 nm wide were observed between adjoining cells. In areas where the apposed membranes were closest, the lanthanum-stained region was interrupted by unstained intercellular bridges with an 8-nm periodicity (Fig. 2). Finally, freeze-fracture replicas of these cell membranes revealed aggregates of intramembranous particles on fracture face A of approximately 8 nm diameter and with a minimum center-to-center spacing of 8 nm (Fig. 3). These structures are similar to gap junctions described in a large variety of vertebrate tissues (11, 14, 18, 24, 30). The average area of 63 such particle aggregates from confluent cultures was 3.3×10^6 nm² with a range of 3.5×10^4 – 28.4×10^6 nm². No other specialized intercellular structures were observed in this cell line using any of these techniques.

Calcium Deposition on Gap Junctions

Unstained thin sections of cells fixed at room temperature in glutaraldehyde containing 0.005–0.1 M CaCl₂ revealed aggregates of dense deposits preferentially associated with areas of close cell contact characterized by dense parallel membranes (Fig. 4). The diameter of these deposits ranged from about 4 to 100 nm. Even without osmication or heavy metal staining, parallel rows of deposits were frequently observed where cells adjoined one another (Fig. 5). After osmication, dense deposits were found in areas of cell apposition typified by straight or slowly curving profiles of dense parallel membranes (Fig. 6). After heavy metal staining, deposits were found preferentially associated with cell junctions characterized by the presence of intercellular densities with an 8-nm periodicity (Fig. 7).

To avoid possible artifacts introduced by the staining of sections with heavy metals, most of the junctional profiles studied in this investigation were not stained. It was considered necessary,

² Azarnia, R. 1974. Personal communication.

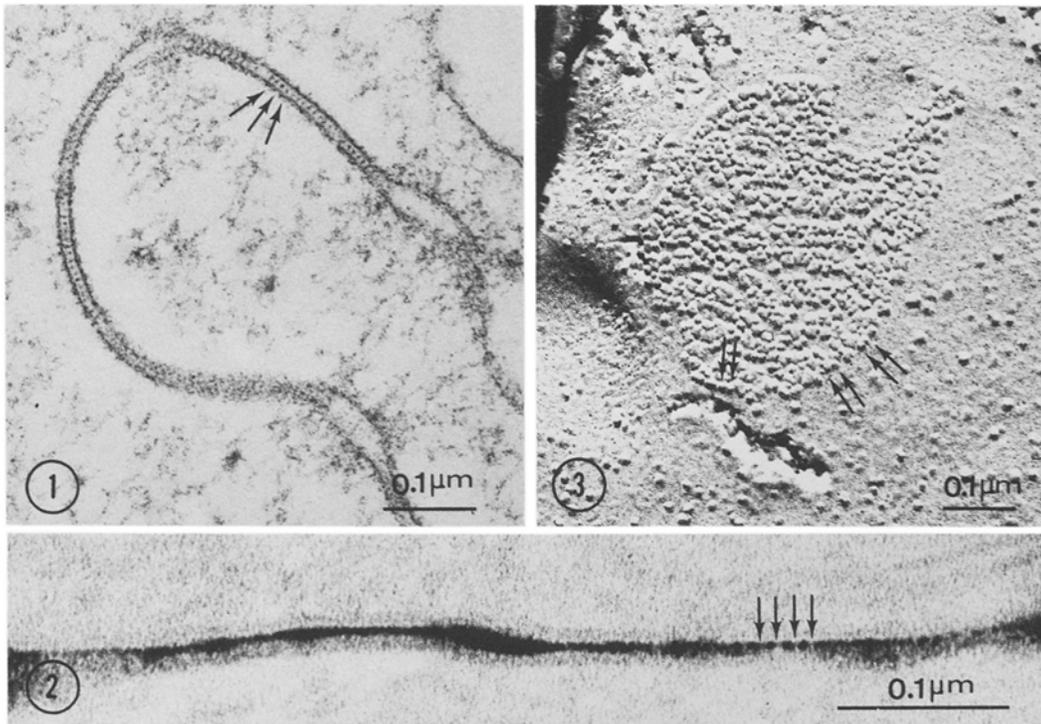


FIGURE 1 Stained section of gap junction in A/B_m-5 cells. Note periodic densities (arrows) between cells at gap junction with a spacing of 8 nm. These cells were also in block stained with 0.5% uranyl acetate. × 119,400.

FIGURE 2 Unstained section of gap junction infiltrated with lanthanum. Unstained intercellular bridges have a periodicity of approximately 8 nm (arrows). × 236,400.

FIGURE 3 Replica of the A face of freeze-fractured A/B_m-5 plasma membrane showing aggregation of particles characteristic of vertebrate gap junctions. The minimum center-to-center particle spacing is about 8 nm (arrows). × 98,900.

therefore, that the slowly curving, dense parallel membranes in unstained sections should be characterized in more specific ways. Alternate serial sections were stained with heavy metals, and several stained and unstained sections through the same presumed junction were compared. In such pairs, intercellular densities with a characteristic 8-nm periodicity were observed in the stained junctional profile, suggesting that these were gap junctions of the type described above (Fig. 8–11).

Calcium concentrations of 0.005, 0.05, 0.08, or 0.1 M all produced about the same size and frequency of deposits when fixation was carried out at room temperature. After cells were fixed at 0°C with calcium containing glutaraldehyde, however, a considerable reduction in size and number of deposits was observed. Only 18 of 100 junctional profiles possessed deposits after fixation on ice

with 0.05 M CaCl₂, compared with 67 of 100 junctional profiles from cells fixed at room temperature. The average number of deposits per junctional profile after fixation at 0°C was 0.54, compared with 5.12 after fixation at room temperature. Many junctions fixed with calcium at 0°C had only minute densities intimately associated with their membranes (Fig. 12). Deposits never appeared on cell membranes when calcium was omitted from the fixative and processing solutions.

Distribution of Deposits on Gap Junctions

In transverse sections of gap junctions, deposits were frequently paired precisely across the two cell membranes (Fig. 13). On several junctional profiles, deposits in one cell were paired with deposits of comparable size across the junctional mem-

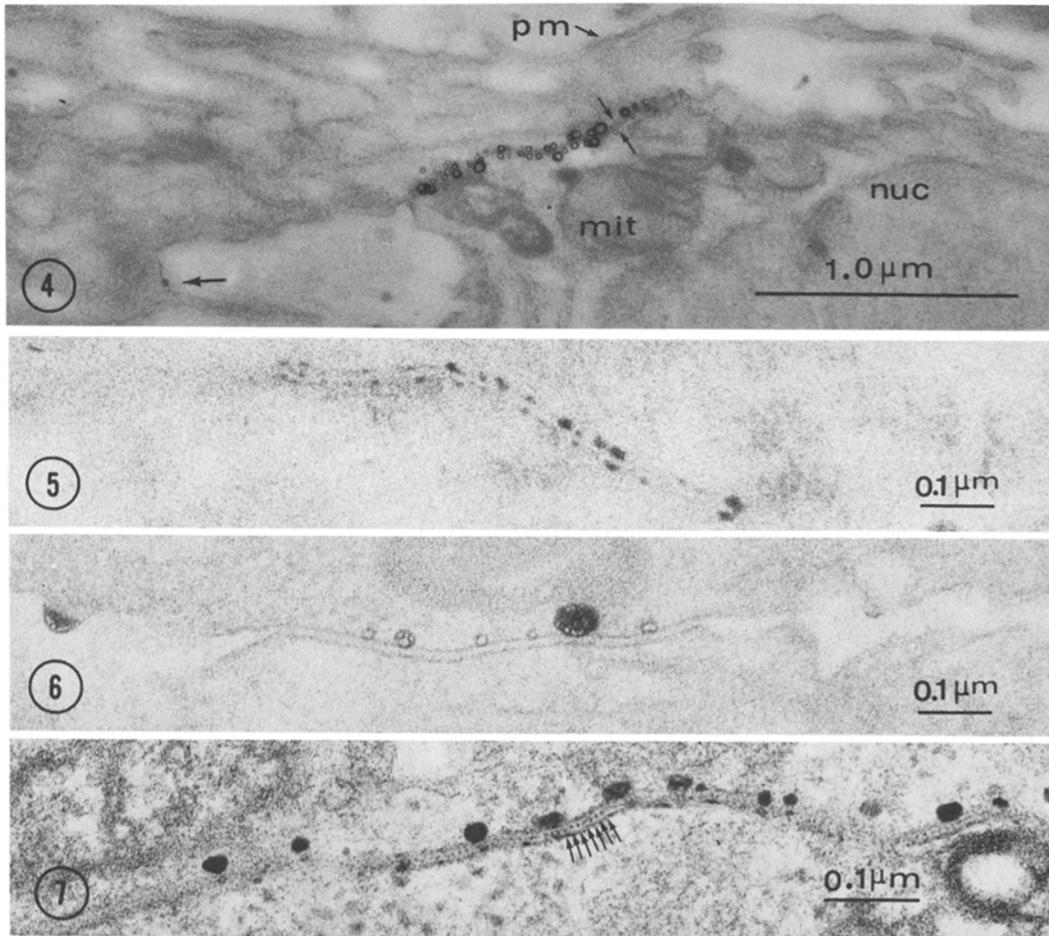


FIGURE 4 Osmicated, unstained section demonstrating large numbers of opaque deposits in an area of close cell contact characterized by dense parallel membranes (arrows). One nonjunctional deposit is indicated (large single arrow). *pm*, plasma membrane; *mit*, mitochondrion; *nuc*, nucleus. $\times 34,800$.

FIGURE 5 Unosmicated, unstained section demonstrating parallel rows of deposits. Directly apposed deposits are separated by a 10–12 nm space. $\times 89,700$.

FIGURE 6 Osmicated, unstained section demonstrating deposits on a cell junction characterized by dense, slowly curving parallel membranes. $\times 90,600$.

FIGURE 7 Osmicated, stained section showing dense deposits on a gap junction. After staining, intercellular junctional densities with an 8 nm periodicity are clearly observed (arrows). $\times 122,500$.

branes (Fig. 14). In this micrograph, five such pairs are easily discernable. In stained and unstained sections normal to the junctional membranes, the maximum distance between the closest edges of apposed deposits is approximately 10–12 nm. Pairing of deposits, however, was usually not observed on short junctional profiles where rela-

tively large deposits (30–100 nm in diameter) were present only on one side of the junction (Fig. 15). Long junctional profiles, on the other hand, were associated with generally smaller deposits (4–45 nm in diameter) in both cells, and occasionally favorable sections revealed that many of these deposits were paired with deposits of equal size

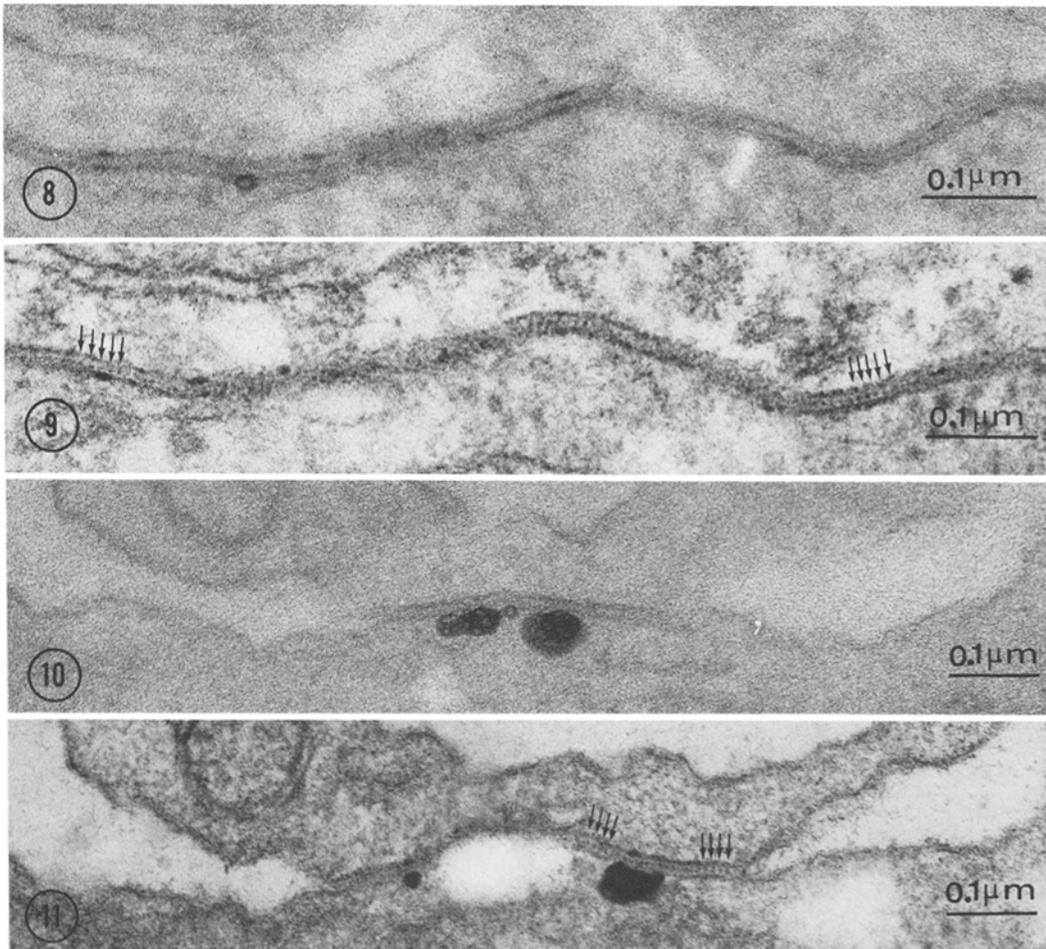


FIGURE 8 Osmicated, unstained section of dense, slowly curving parallel membranes in an area of close cell-to-cell apposition. $\times 142,500$.

FIGURE 9 Stained section through the same cell junction shown in Fig. 8 demonstrating intercellular densities (arrows) with a periodicity of 8 nm. $\times 142,500$.

FIGURE 10 Osmicated unstained section of dense parallel membranes associated with large opaque cytoplasmic deposits. $\times 114,600$.

FIGURE 11 Stained section through the same cell junction as shown in Fig. 10 demonstrating intercellular densities (arrows) with a periodicity of 8 nm. $\times 114,600$.

across the junction (Fig. 16). Observation of 44 junctional profiles measuring from $0.2 \mu\text{m}$ to $4.2 \mu\text{m}$ in length supported the idea that large asymmetrically distributed deposits are preferentially found on relatively short junctional profiles. 12 of 15 profiles measuring less than $0.7 \mu\text{m}$ possessed only large deposits on one side of the junction, while smaller deposits were distributed on both sides of all 29 junctional profiles $0.7 \mu\text{m}$ – $4.2 \mu\text{m}$

long. The lengths of those three junctional profiles shorter than $0.7 \mu\text{m}$ and possessing paired symmetrical deposits were $0.5 \mu\text{m}$ (Fig. 14), $0.65 \mu\text{m}$ (Fig. 5), and $0.65 \mu\text{m}$ (Fig. 17), respectively. Large asymmetrically distributed deposits were never found on junctional profiles longer than $0.7 \mu\text{m}$.

In many cases, the pairing of deposits across the junction was not obvious. A stereo pair of a transected junction revealed that inherent pairing

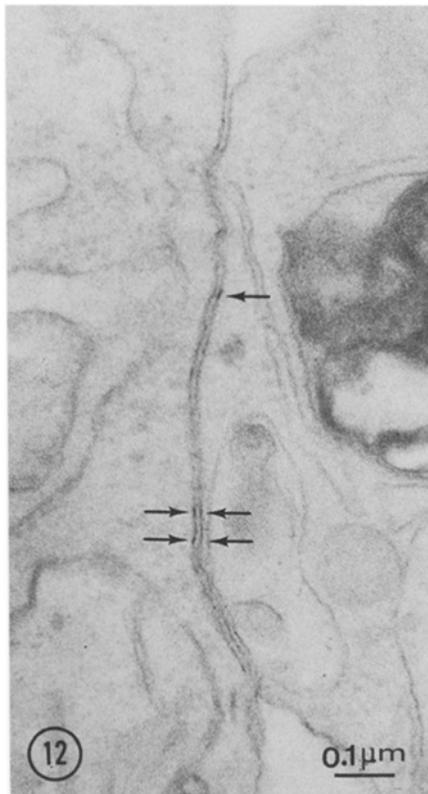


FIGURE 12 After fixation with calcium at 0°C most junctions possess only minute deposits intimately associated with the membrane (arrows). $\times 79,600$.

could be obscured because (a) deposits overlap at different levels of the section, (b) one member of a pair, tilted slightly at the surface of the section, may be excluded by sectioning, and (c) membrane definition may be lost in areas where the junction is sectioned more tangentially (Fig. 17).

Calcium Deposition on Nonjunctional Membrane

After fixing the A/B_m-5 cells with glutaraldehyde-calcium, opaque deposits were observed on nonjunctional membrane. These deposits were similar in appearance to those found on gap junctions but were usually widely separated (Fig. 18). In addition, they were always associated with a slight evagination of the membrane. Only infrequently were nonjunctional membranes observed with a comparatively high concentration of deposits (Fig. 19).

Staining of the Deposits with the von Kossa Technique

When cells fixed in calcium-containing glutaraldehyde were stained with silver by the von Kossa technique, light microscopy revealed scattered dense precipitates on nonjunctional membranes while aggregates of dense deposits were observed in regions where adjacent cells appeared to be in close apposition (Fig. 20). After processing for electron microscopy, unstained thin sections revealed extremely dense spherical deposits associated with nonjunctional membrane and areas of membrane apposition which appeared to be gap junctions (Fig. 21). When calcium was omitted from the fixative, however, no precipitates were observed after von Kossa staining (Fig. 22).

DISCUSSION

Composition of the Deposits

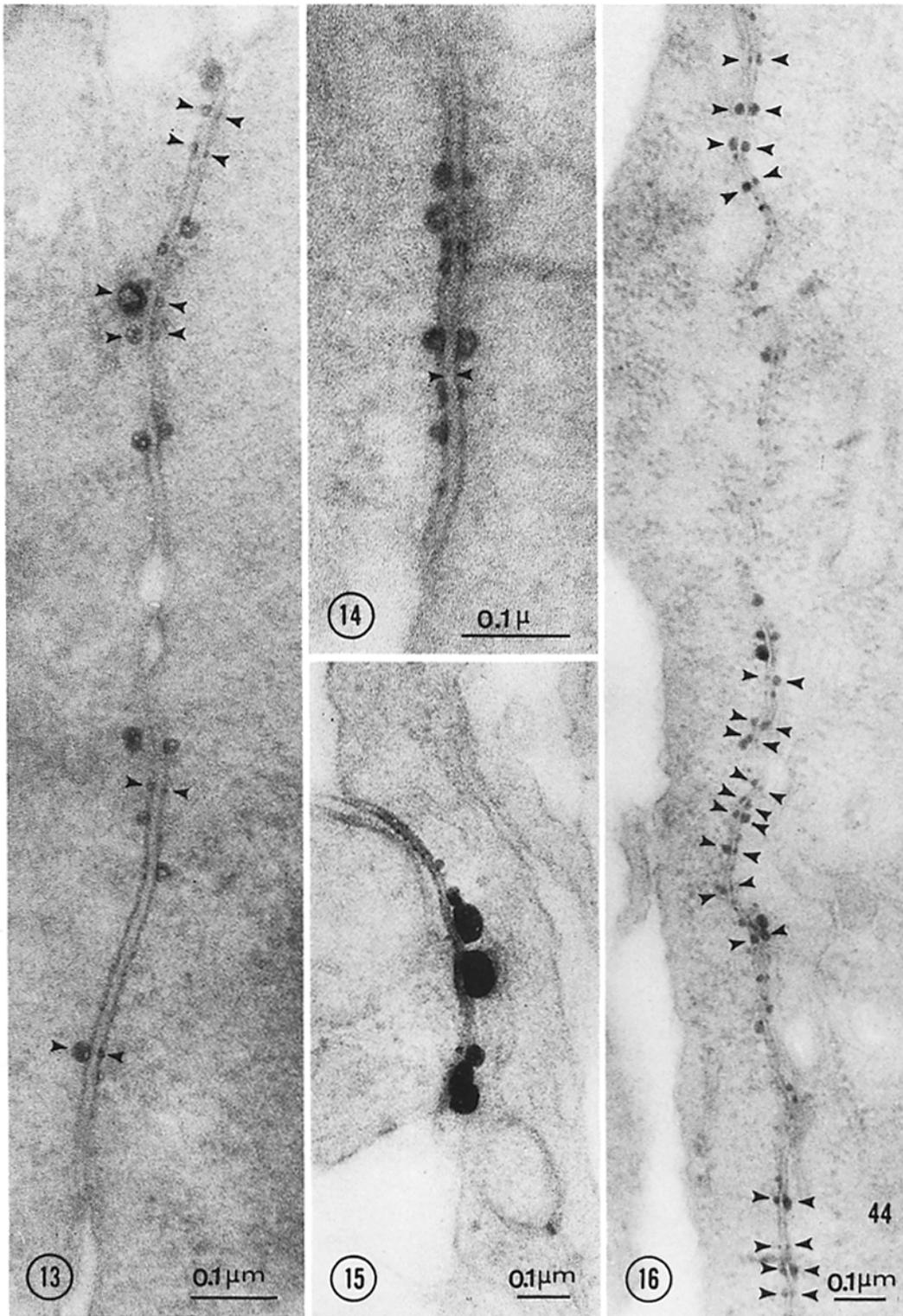
The composition of deposits formed on gap junctions by glutaraldehyde-calcium fixation is not known. Microprobe analysis, however, has shown that comparable deposits on membranes of squid

FIGURE 13 Unstained section of cell junction fixed in calcium-glutaraldehyde shows some pairing of deposits across the two membranes of the junction (arrow heads). $\times 132,000$.

FIGURE 14 Unstained section; most of the deposits on this junctional profile are paired cell-to-cell, and members of each pair are nearly equal in diameter. The distance between arrow head points is approximately 10 nm. $\times 166,400$.

FIGURE 15 This unstained short junctional profile is typical of most short transactions since the relatively large deposits are confined to one side of the junction (see also Figs. 10 and 11). $\times 85,000$.

FIGURE 16 Very long unstained junctional profile after fixation with glutaraldehyde-calcium. Most of the deposits appear to be paired in areas of the junction where the membranes are perfectly transected (arrow heads). $\times 72,600$.



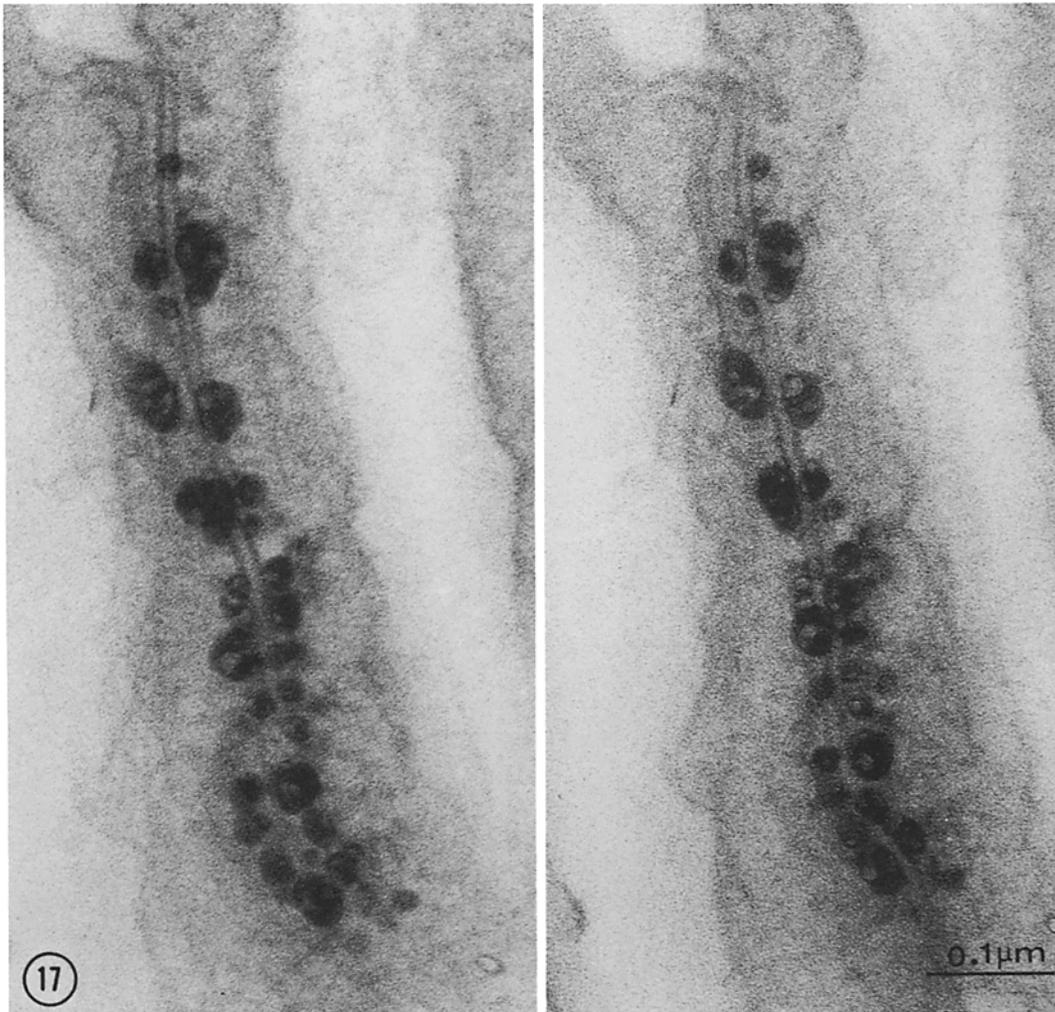


FIGURE 17 Stereomicrograph of unstained junction with a large number of opaque deposits. Several single deposits near the section surface can be seen, and the lower end of the junction is sectioned tangentially. Three areas of deposit overlapping are more clearly visualized when the two micrographs are merged. These two micrographs were taken at angles 12° apart. $\times 165,200$.

axon contain calcium and phosphorus (21, 33). Calcium is also a likely constituent of the deposits on gap junctions, since (a) it is necessary to add calcium to the fixative to produce the deposits, and they are present even when osmium, lead, and uranium are not used to stain the specimen, (b) the deposits are morphologically similar to those obtained when phosphate or oxalate are used to precipitate calcium transported into sarcoplasmic reticulum (10, 15), and (c) the positive von Kossa reaction suggests the presence of an insoluble calcium salt (23).

Significance of the Deposits

The role of cell-to-cell contact in the control of cell growth and differentiation constitutes one of the central problems in developmental biology. Recent evidence has correlated deficiencies in the ability to form gap junctions with a loss of growth control in several tissue culture lines (3, 18) and in an intact tissue (29). Other studies have implicated calcium in the control of the movement of ions from cell to cell (27, 32, 44). The presence at gap junctions of opaque deposits that are probably composed in part of calcium may provide a clue

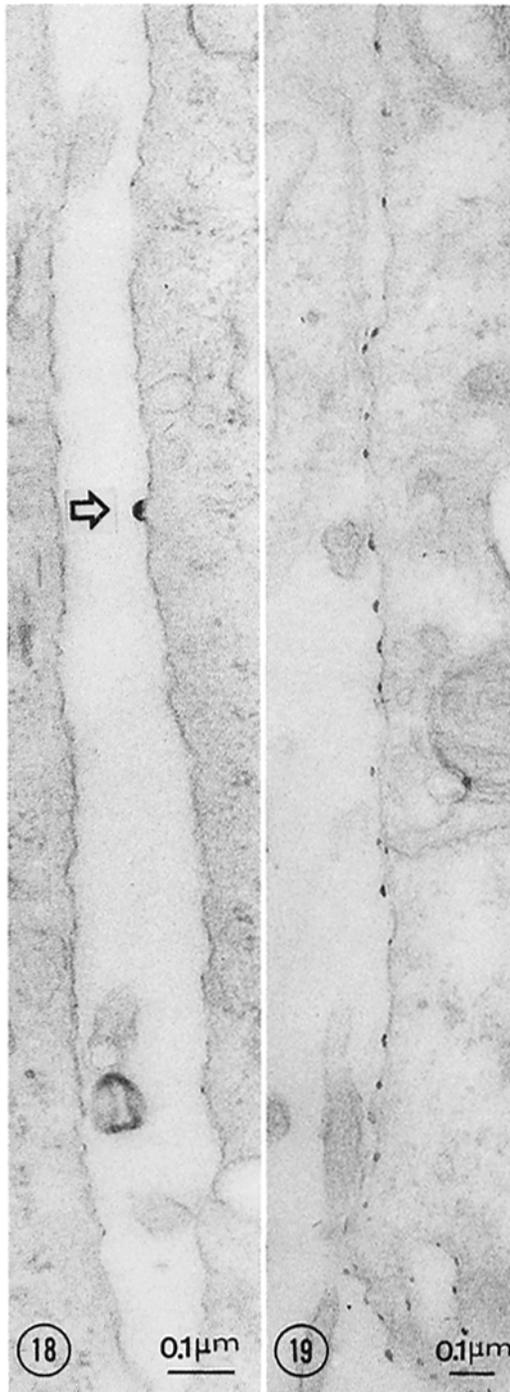


FIGURE 18 Unstained section demonstrating a nonjunctional deposit (arrow). These deposits are usually widely separated. $\times 66,600$.

FIGURE 19 Unstained section demonstrating nonjunctional membrane with an unusually high density of deposits. $\times 55,000$.

about the processes occurring at this region of cell contact. Although we do not know how the deposits form at gap junctions, we feel it is worthwhile to discuss briefly some possible alternative explanations which have arisen during this and previous studies.

Many studies have demonstrated that ions and small molecules can move from cell to cell across the junctional membranes (6, 27, 45) and that high intracellular concentrations of calcium inhibit this process (32). Recent experiments of Rose and Loewenstein (44) have utilized aequorin, a molecule that fluoresces in the presence of calcium, to probe interactions of calcium with the junctional membranes. They found that calcium injected into a chironomid salivary gland cell prevented the movement of small ions between cell interiors only when the injected calcium diffused to the junctional membrane. Thus it is conceivable that deposits on gap junctions of A/B_m-5 cells may localize calcium binding sites that regulate the movement of small molecules from one cell to another. Binding of calcium to fixed sites has been postulated to explain the formation of deposits on several other membranes (21, 34, 35, 42).

An alternate explanation for deposit formation is that calcium entering the cell acts as a trapping agent, precipitating anions produced by the hydrolysis of endogenous substrates such as ATP. Calcium (19), strontium (16, 17), and lead (47) have been used to localize alkaline phosphatase and ATPase. Such a mechanism has been proposed to explain deposit formation on the neurilemma of squid axon (33). The finding that gap junction deposits are smaller and less frequent when the cells are fixed at 0°C suggests that the deposits may be formed enzymatically. Binding of calcium to fixed membrane sites would not be expected to be so temperature sensitive.

It is also conceivable that calcium may precipitate with pyrophosphate produced from endogenous ATP by adenylate cyclase. Recent studies by Cutler and co-workers (see footnote one) have indicated that deposits produced on the cytoplasmic face of plasma membrane of a slime mold by glutaraldehyde-calcium fixation are similar in morphology and distribution to the reaction product obtained when the technique of Howell and Whitfield (22) was used to localize adenylate cyclase activity (12).

In addition, a previous study has suggested that adenylate cyclase may be activated at the sites of cell-to-cell contact through interactions of contact-sensing molecules on the plasma membrane (28).

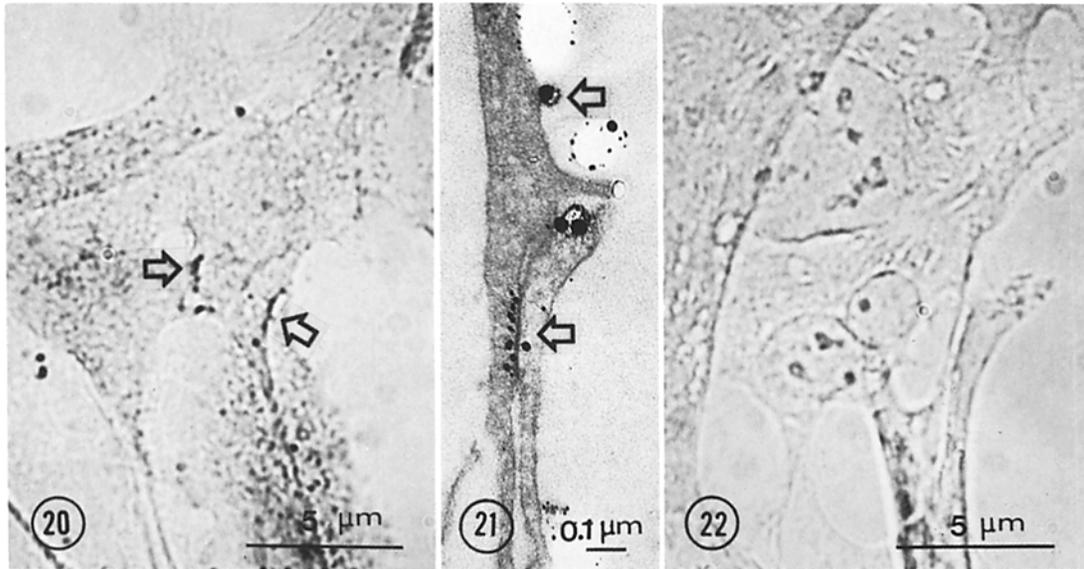


FIGURE 20 Light micrograph of A/B_m-5 cells after fixation with calcium-containing fixative and staining with the von Kossa technique. Dense deposits appear on cell surfaces and in areas of cell-to-cell apposition (arrows). $\times 4,400$.

FIGURE 21 Electron micrograph of cells fixed in the presence of calcium and subsequently stained with the von Kossa technique. Dense spherical deposits appear on nonjunctional membrane and are also clustered on an apparent gap junction (arrows). $\times 12,500$.

FIGURE 22 Light micrograph of A/B_m-5 cells fixed with glutaraldehyde without calcium and subsequently stained with the von Kossa technique. No obvious deposits are observed. $\times 4,400$.

Several recent studies have provided support for this hypothesis and suggest the possibility that gap junctions could be involved in this process when normal cells in tissue culture make contact with one another.

Although several tumor cells have been shown to possess gap junctions (24, 29, 41), in one quantitative study cells in an intact tumor were shown to possess significantly fewer gap junctions than those cells in corresponding normal tissue (29). Cells derived from certain tumors are apparently unable to make these structures (3, 18). Other studies have shown that many tumor cell lines in culture have low intracellular levels of c-AMP (20, 37, 46) or low adenylate cyclase activities (1, 2, 9, 26, 31). These cells reach relatively high densities in tissue culture. Cultured normal cells, on the other hand, form gap junctions and exhibit "contact inhibition" of cell growth. In addition, there is now evidence that confluence of normal cells in culture is accompanied by an increase in intracellular c-AMP levels (2, 5, 13, 20, 37). In 3T3 cells (5), intracellular c-AMP levels begin to rise when

contact is first observed and then plateau shortly after the cells become confluent.

Distribution of Deposits

Freeze-fracture studies (25, 24, 48) have shown that gap junctions begin to form as small aggregations of intramembranous particles. These aggregates probably enlarge by the addition of single particles or groups of particles as the cells remain in contact. We have observed larger, asymmetrically distributed deposits on shorter junctional profiles. Some of these shorter profiles may arise from sections cut near the periphery of large junctions (the last part of the junction to be formed) while others may arise from sections through short "younger" junctions. Deposits seem to be more symmetrically distributed on larger, more mature junctions. This symmetry could develop over time through an interaction between gap junctional elements in the membranes of adjacent cells.

The finding that paired deposits are separated

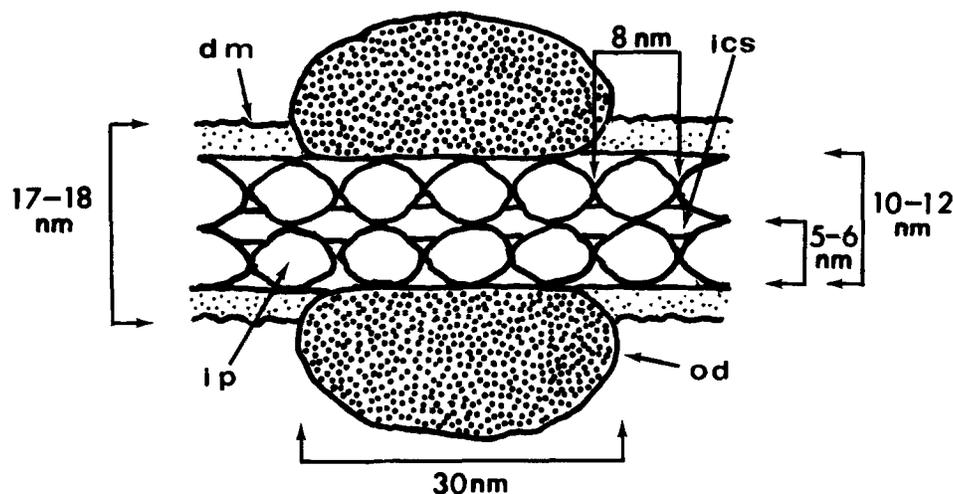


FIGURE 23 Diagram depicting diameters and relationships of intramembranous particles and opaque gap junctional deposits suggested by measurements of distance between paired deposits, total junctional thickness in stained thin sections, and gap junctional particles in freeze-fracture replicas. For illustrative purposes, large diameter-paired deposits are represented. It is not known whether deposit centers are strictly aligned with the central axis of one intramembranous particle pair. Note that the opaque deposits are embedded in the dense material associated with the cytoplasmic leaflets of the junctional membranes. *ip*, intramembranous particle; *od*, opaque deposit; *ics*, intercellular space; *dm*, dense material.

by a distance of only 10–12 nm is somewhat surprising in view of the usually reported measurement for the total thickness of the junction (17–18 nm) determined from stained thin sections (30). Since several electron microscope studies have shown that intramembranous particles are paired precisely cell-to-cell within the gap junction (11, 29, 38, 39), the distance between deposits must represent the maximum thickness of two particle diameters in the axis normal to the junction. Assuming that membrane thickness is not altered by the fixation procedure, and that intramembranous particles do not protrude into the cytoplasmic deposits, the maximum diameter of a single particle along this axis would be only 5–6 nm (Fig. 23).

CONCLUSIONS

Deposits have been observed on the cytoplasmic faces of gap junction and nonjunctional membrane in A/B_m-5 cells fixed with glutaraldehyde-calcium shortly after attaining confluence. The evidence for the presence of calcium in the deposits and several alternative mechanisms of deposit formation have been discussed. It was suggested that the pairing of deposits of equivalent size across the membranes of the gap junction implies an interaction between junctional elements of adjacent cells and that this

interaction develops during a period of junctional growth.

We are indebted to Doctors R. Azarnia, D. L. Garrison, and R. Ho for valuable discussions. We also thank Dr. J. L. Oschman, Dr. B. J. Wall, and Dr. T. Shipley for critical reading of the manuscript. We are especially grateful to Dr. Oschman and Dr. Wall for their interest and criticism. We are also indebted to Dr. R. Azarnia and Mrs. Fermina Varacalli for the culturing of the A/B_m-5 cells, to Mrs. Mary Losa and Mrs. Connie Johnson for technical assistance, and to Dr. D. S. Smith for use of the Balzer's apparatus.

This work was supported in part by the United States Public Health Service research grant, CA 14464, and the National Science Foundation grant, GB 36763 X 1 awarded to W. R. Loewenstein, and the National Institutes of Health postdoctoral fellowship 1 F02 GM51417-01.

Received for publication 3 February, and in revised form 21 August 1975.

BIBLIOGRAPHY

- ANDERSON, W. B., G. S. JOHNSON, and I. PASTAN. 1973. Transformation of chick-embryo fibroblast by wild type and temperature-sensitive Rous sarcoma virus alters adenylate cyclase activity. *Proc. Natl. Acad. Sci. U. S. A.* **70**:1055.

2. ANDERSON, W. B., T. R. RUSSELL, R. A. CARCHANAN, and I. PASTAN. 1973. Interrelationship between adenylate cyclase activity, adenosine 3':5' cyclic monophosphate phosphodiesterase activity, adenosine 3':5' cyclic monophosphate levels, and growth of cells in culture. *Proc. Natl. Acad. Sci. U. S. A.* **70**:3802.
3. AZARNIA, R., W. J. LARSEN, and W. R. LOEWENSTEIN. 1974. The membrane junctions in communicating and non-communicating cells, their hybrids, and segregants. *Proc. Natl. Acad. Sci. U. S. A.* **71**:880.
4. AZARNIA, R., and W. R. LOEWENSTEIN. 1973. Parallel correction of cancerous growth and of a genetic defect of cell-cell communication. *Nature (Lond.)*. **241**:455.
5. BANNAI, S., and J. R. SHEPARD. 1974. Cyclic AMP, ATP, and cell contact. *Nature (Lond.)*. **250**:62.
6. BENNETT, M. V. L. 1973. Function of electrotonic junctions in embryonic and adult tissues. *Fed. Proc.* **32**:65.
7. BJERSING, L., and S. CAJANDER. 1974. Ovulation and the mechanism of follicle rupture. LV. Ultrastructure of membrana granulosa of rabbit Graafian follicles prior to induced ovulation. *Cell Tiss. Res.* **153**:1.
8. BOHAN, T. P., A. F. BOYNE, and P. S. GUTH. 1973. Electron-dense particle in cholinergic synaptic vesicles. *Nature (Lond.)*. **244**:32.
9. BÜRK, R. R. 1968. Reduced adenyl cyclase activity in a polyoma virus transformed cell line. *Nature (Lond.)*. **219**:1272.
10. CARSTEN, M. E., and M. K. REEDY. 1971. Cardiac sarcoplasmic reticulum: chemical and electron microscopy studies of calcium accumulation. *J. Ultrastruct. Res.* **35**:554.
11. CHALCROFT, J. P., and S. BULLIVANT. 1970. An interpretation of liver cell membrane and junction structure based on observation of freeze fracture replicas of both sides of the fracture. *J. Cell Biol.* **47**:49.
12. CUTLER, L. S., and E. F. ROSSOMANDO. 1975. Localization of adenylate cyclase in *Dictyostelium discoideum* II. Cytochemical studies on whole cells and isolated plasma membrane vesicles. *Exp. Cell Res.* In press.
13. D'ARMIENTO, M., G. S. JOHNSON, and I. PASTAN. 1973. Cyclic AMP and growth of fibroblasts: effect of environmental pH. *Nat. New Biol.* **242**:78.
14. DECKER, R. S., and D. FRIEND. 1974. Assembly of gap junctions during amphibian neurulation. *J. Cell Biol.* **62**:32.
15. DE MEIS, L., W. HASSELBACH, and R. D. MACHADO. 1974. Characterization of calcium oxalate and calcium phosphate deposits in sarcoplasmic reticulum vesicles. *J. Cell Biol.* **62**:505.
16. ERNST, S. A. 1972. Transport adenosine triphosphatase cytochemistry. I. Biochemical characterization of a cytochemical medium for the ultrastructural localization of ouabain-sensitive, potassium-dependent phosphatase activity in the avian salt gland. *J. Histochem. Cytochem.* **20**:13.
17. ERNST, S. A. 1972. Transport adenosine triphosphatase cytochemistry. II. Cytochemical localization of ouabain-sensitive, potassium-dependent phosphatase activity in the secretory epithelium of the avian salt gland. *J. Histochem. Cytochem.* **20**:23.
18. GILULA, N. B., O. R. REEVES, and A. STEINBACH. 1972. Metabolic coupling, ionic coupling, and cell contacts. *Nature (Lond.)*. **235**:262.
19. GOMORI, G. 1941. The distribution of phosphatase in normal organs and tissues. *J. Cell Comp. Physiol.* **17**:71.
20. HEIDRICK, M. L., and W. L. RYAN. 1971. Adenosine 3',5'-cyclic monophosphate and contact inhibition. *Cancer Res.* **31**:1313.
21. HILLMAN, D. E., and R. LLINAS. 1974. Calcium-containing electron-dense structures in the axons of the squid giant synapse. *J. Cell Biol.* **61**:146.
22. HOWELL, S. L., and M. WHITFIELD. 1972. Cytochemical localization of adenyl cyclase activity in rat islets of Langerhans. *J. Histochem. Cytochem.* **20**:873.
23. HUMASON, G. L. 1962. Animal Tissue Techniques. W. H. Freeman and Co., San Francisco, Calif.
24. JOHNSON, R., M. HAMMER, J. SHERIDAN, and J. P. REVEL. 1974. Gap junction formation between reaggregated Novikoff hepatoma cells. *Proc. Natl. Acad. Sci. U. S. A.* **71**:4536.
25. JOHNSON, R., and D. PREUSS. 1973. Gap junction formation in a reaggregating system: an ultrastructural study. *J. Cell Biol.* **59**:158 a. (Abstr.).
26. LINCOLN, T. M., and G. L. VAUGHAN. 1974. A lesion in membrane adenylate cyclase affecting transformation in mouse melanoma cells. *J. Cell Biol.* **63**:194 a. (Abstr.).
27. LOEWENSTEIN, W. R. 1966. Permeability of membrane junctions. *Ann. N. Y. Acad. Sci.* **137**:441.
28. MCMAHON, D. 1973. A cell contact model for cellular position determination in development. *Proc. Natl. Acad. Sci. U. S. A.* **70**:2396.
29. MCNUTT, N. S., R. A. HERSHBERG, and R. S. WEINSTEIN. 1971. Further observations on the occurrence of nexuses in benign and malignant human cervical epithelium. *J. Cell Biol.* **51**:805.
30. MCNUTT, N. S., and R. WEINSTEIN. 1973. Membrane ultrastructure at mammalian intercellular junctions. *Prog. Biophys. Mol. Biol.* **26**:45.
31. MUKERJEE, H., and A. GOLDFEDER. 1974. Adenylate cyclase activity in mouse tumors of diverse growth rates. *J. Cell Biol.* **63**:235 a. (Abstr.).
32. OLIVEIRA-CASTRO, G. M., and W. R. LOEWENSTEIN. 1971. Junctional membrane permeability: Effects of divalent cations. *J. Membrane Biol.* **5**:51.

33. OSCHMAN, J. L., T. A. HALL, P. D. PETERS, and B. J. WALL. 1974. Association of calcium with membranes of squid giant axon. *J. Cell Biol.* **61**:156.
34. OSCHMAN, J. L., and B. J. WALL. 1972. Calcium binding to intestinal membranes. *J. Cell Biol.* **55**:58.
35. OSCHMAN, J. L., and B. J. WALL. 1973. Binding of calcium to cell membranes. In *Transport Mechanisms in Epithelia*, Alfred Benzon Symposium V. Munksgaard, Copenhagen, and Academic Press, Inc., New York. 392.
36. OSCHMAN, J. L., and B. J. WALL. 1974. Association of divalent cations with membranes of squid giant axon. *Biol. Bull. (Woods Hole)*. **147**:493.
37. OTTEN, J., G. S. JOHNSON, and I. PASTAN. 1971. Cyclic AMP levels in fibroblasts: Relationship to growth rate and contact inhibition of cell growth. *Biochem. Biophys. Res. Commun.* **44**:1192.
38. PERACCHIA, C. 1973. Low resistance junctions in crayfish. I. Two arrays of globules in junctional membranes. *J. Cell Biol.* **57**:54.
39. PERACCHIA, C. 1973. Low resistance junctions in crayfish. II. Structural details and further evidence for intercellular channels by freeze fracture and negative staining. *J. Cell Biol.* **57**:66.
40. PINTO DA SILVA, P., and N. B. GILULA. 1972. Gap junctions in normal and transformed fibroblasts in culture. *Exp. Cell Res.* **71**:393.
41. PINTO DA SILVA, P., and A. MARTINEZ-PALOMO. 1975. Distribution of membrane particles and gap junctions in normal and transformed 3T3 cells studied in situ, in suspension and treated with concanavalin A. *Proc. Natl. Acad. Sci. U. S. A.* **72**:572.
42. POLITOFF, A. L., S. ROSE, and G. D. PAPPAS. 1974. The calcium binding sites of synaptic vesicles of the frog sartorius neuromuscular junction. *J. Cell Biol.* **61**:818.
43. REVEL, J. P., and M. J. KARNOVSKY. 1967. Hexagonal array of subunits in intercellular junctions of the mouse heart and liver. *J. Cell Biol.* **33**:C7.
44. ROSE, B., and W. R. LOEWENSTEIN. 1975. Permeability of cell junction depends on local cytoplasmic calcium activity. *Nature (Lond.)*. **254**:250.
45. SATIR, P., and N. B. GILULA. 1973. Fine structure of membranes and intercellular communication in insects. *Annu. Rev. Entomol.* **18**:143.
46. SHEPARD, J. R. 1972. Difference in the cyclic adenosine 3',5'-monophosphate levels in normal and transformed cells. *Nat. New Biol.* **236**:14.
47. WACHSTEIN, M., and E. MEISEL. 1957. Histochemistry of hepatic phosphatases at a physiologic pH. *Am. J. Clin. Pathol.* **27**:13.
48. YEE, A. G. 1972. Gap junctions between hepatocytes in regenerating rat liver. *J. Cell Biol.* **55**:294 a. (Abstr.).