IN VITRO FORMATION OF GAP JUNCTION VESICLES

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ABSTRACT

A method is described that uses trypsin digestion combined with collagenase-hyaluronidase which produces a population of gap junction vesicles. The hexagonal lattice of subunits ("connexons") comprising the gap junctions appears unaltered by various structural criteria and by buoyant density measurements. The gap junction vesicles are closed by either a single or a double profile of nonjunctional "membrane," which presents a smooth, particle-free fracture face. Horseradish peroxidase and cytochrome *c* studies have revealed that about 20% of the gap junction vesicles are impermeable to proteins 12,000 daltons or larger. The increased purity of the trypsinized junction preparation suggests that one of the disulfide reduction products of the gap-junction principal protein may be a nonjunctional contaminating peptide. The gap junction appears to be composed of a single 18,000-dalton protein, connexin, which may be reduced to a single 9,000-dalton peak. The number of peptides in this reduced peak are still unknown.

After the pioneering studies of Benedetti and Emmelot (1–3) a relatively pure preparation of hepatocyte gap junctions has been prepared from mouse liver (4–7, 10). The isolation protocol yields flat sheets of gap junctions, showing curving, stiff profiles in thin-sectioned material similar to the junctions' appearance *in situ*. Since these junctions no longer join adjacent cell compartments, it has not been possible to measure directly the permeability of the isolated gap junction membranes.

Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis of isolated junction proteins revealed one principal protein, connexin, with an apparent molecular weight of 18,000 daltons, flanked by peptides of higher and lower molecular weight (6). Reduction of disulfide bonds with dithiothreitol (DTT) in the presence of SDS resulted in the disappearance of most of the 18,000-dalton connexin band, and the appearance of two peptides at 11,000 and 9,000 daltons. The 18,000-dalton protein, connexin, was considered the structural unit in the gap junction. It was not known if the two low molecular weight polypeptides, called A and B, resulted from reduction of intermolecular disulfide bridges or from reduction of intramolecular disulfide bonds. If the principal protein were partially degraded by proteases during isolation, reduction of intramolecular disulfide bonds might have resulted in the observed low molecular weight polypeptides. There was, in addition, a considerable amount of material which failed to enter the polyacrylamide gels, and the effects of DTT on this material were unknown.

This communication reports a method using trypsin digestion to generate a mixed population of "open" and "closed" gap junction vesicles which may be useful for future permeability studies. These vesicles are characterized by electron microscope techniques, gel electrophoresis, and buoyant density measurements.

MATERIALS AND METHODS

Materials

Hyaluronidase (type I, Sigma Chemical Company, St. Louis Mo.); collagenase (Type CLS, Worthington Bio-

chemical Corp. Freehold, N. J.); trypsin (Sigma type III); horseradish peroxidase (Sigma, type II); cytochrome *c* (Sigma, type III); benzamidine hydrochloride, B grade (Calbiochem, San Diego, Calif.); phenylmethylsulfonyl fluoride (PMSF) B grade (Calbiochem); DFP-trypsin (Worthington Biochemicals) were used.

Methods

Gap junctions were isolated from mouse livers, as described previously (6), with the following modification. The crude plasma membrane pellet was digested with 0.1% hyaluronidase, 0.05% collagenase, and 0.02% trypsin overnight in the cold room and for 1 hr at 37°C. The remainder of the isolation protocol was identical to that published earlier (7). On some runs, trypsin activity was inhibited by substituting DFP-trypsin (0.02%) or by adding PMSF (0.1 mM) and benzamidine-HCl (0.1%) to the enzyme mixtures. On other runs, trypsin activity was terminated after digestion and before subsequent washes by adding PMSF and benzamidine-HCl.

Peroxidase Studies

The methods used were those of Graham and Karnovsky (11) and Karnovsky and Rice (12). Isolated gap junction vesicles were incubated with 0.1% horseradish peroxidase (HRP) or 0.1% cytochrome *c* for 1 h at 23°C. The junctions were pelleted from the solutions at 100,000 *g* for 1 h in a Beckman SW41 rotor at 4°C. The treated pellets were fixed for 30 min in 2% glutaraldehyde in 0.1 M phosphate, pH 6.5. The HRP-treated pellets were preincubated in 0.05% 3, 3'-diaminobenzidine (DAB) in 0.1 M phosphate buffer, pH 6.5, for 30 min; the cytochrome *c*-treated pellets were preincubated in 0.05% DAB in 50 mM citric acid-sodium citrate buffer, pH 3.9. The pellets were then incubated for 1 h at 23°C in the same media but with the addition of 0.01% H_2O_2 prepared freshly from a 30% H_2O_2 stock solution. Controls were treated identically, except that the HRP and cytochrome *c* were omitted.

After incubation, the pellets were washed twice with distilled water and fixed in 2% OsO_4 in water for 1 h at 0°C. Samples were poststained in 1% aqueous uranyl acetate for 1 h and dehydrated and embedded in epoxy plastics.

Negative Staining

Isolated junctions were negatively stained on carbon-coated Formvar grids with 1% aqueous uranyl acetate.

Freeze-Fracturing

Isolated junctions were equilibrated with 20% glycerol in water, concentrated by centrifugation, and frozen on paper disks in Freon 22. Freeze cleaving was carried out in a Balzer's BAF 301 (Balzer's High Vacuum Corp., Santa Ana, Calif.) according to standard techniques.

Polyacrylamide Gel Electrophoresis

Gel electrophoresis was done after solubilizing the junctions in 1% sodium dodecyl sulfate and 50 mM dithiothreitol as described previously (6).

RESULTS

Thin Sections of Trypsin-Treated Junctions

The trypsin digestion results in a mixed population of open and closed gap-junction vesicles 0.5–0.9 μm in diameter, illustrated in Figs. 1–7. Figs. 1 and 2 are micrographs of thin sections of the trypsin-treated pellets. Fig. 2 shows a low-magnification view; numerous circular profiles can be seen, intermixed with straight junction segments. Not all of these circular profiles are closed structures, however, as will be discussed with reference to ultrastructural tracers.

Fig. 1 shows a view of the vesicles at higher magnification. The characteristic parallel pair of junctional membranes, separated by a 2–3-nm "gap" (small apposed arrows), forms the wall of the junction vesicle. The vesicles are closed by either a single (large arrow, Fig. 1) or a double (inset arrows, Fig. 1) profile of nonjunctional "membrane." The chemical nature of this small segment of nonjunctional membrane is unknown.

Negative Staining of Trypsin-Treated Junctions

Negatively stained images of the trypsin-treated junctions also reveal their vesicular shape. Figs. 3–5 show low and high magnification views of the flattened circular vesicles. The hexagonal array of junction subunits is not disrupted by the trypsin treatment (Fig. 4). The same "connexon" has been proposed for these hexagonally packed, 6-nm subunits (7). At the edges of the vesicles, negatively stained profiles of the gap junctions can be seen (arrows, Fig. 4) where the junction folds normal to the plane of the micrograph to join the upper and lower halves of the collapsed vesicles. At such folds the 2-nm gap is seen to be filled with negative stain (arrow *G*, Fig. 5) separating a pair of stain-excluding junctional membranes.

The stain within the 2–3-nm gap may be relatively homogeneous or periodic, depending on the tilt of the specimen. The periodically stained aspect (arrow G, Fig. 5 *inset*) is evident when the angle of view is parallel to the rows of junctional subunits (connexons).

Fig. 5 presents an interesting image of the

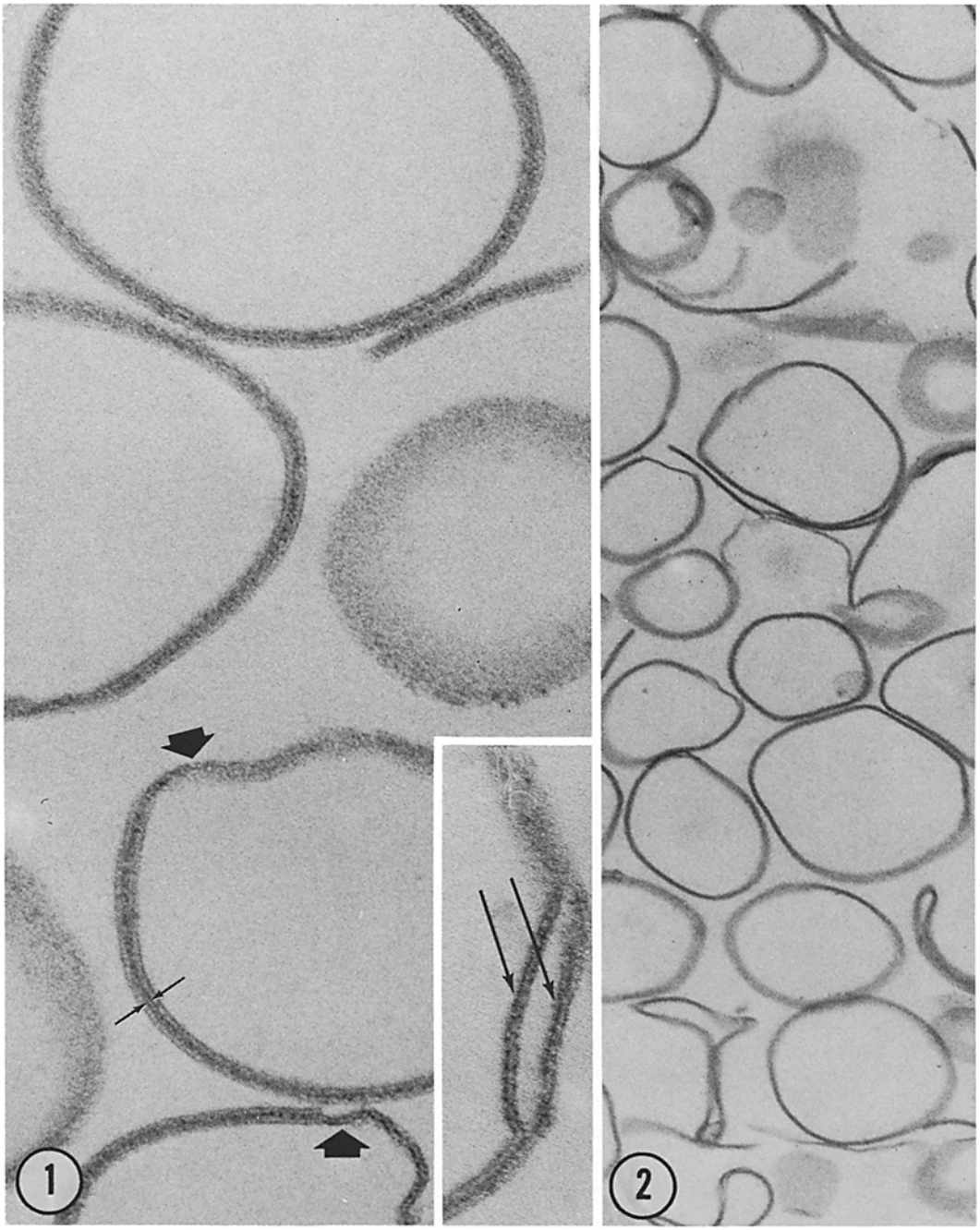


FIGURE 1 High magnification thin sections of pellets of gap junction vesicles reveal that the wall of each vesicle is composed of an intact gap junction, showing its characteristic 2–3-nm "gap" (small arrows). In favorable planes of section it is seen that the vesicles are closed by either a single trilaminar membrane profile (large arrows) or by a double profile (*inset*). × 200,000. *inset:* × 233,000.

FIGURE 2 Low magnification views of thin sections of gap junction vesicles allow a visualization of vesicle size and an estimation of the number of vesicles in relation to straight junction profiles. The very high purity of the preparation is also apparent. × 40,000.

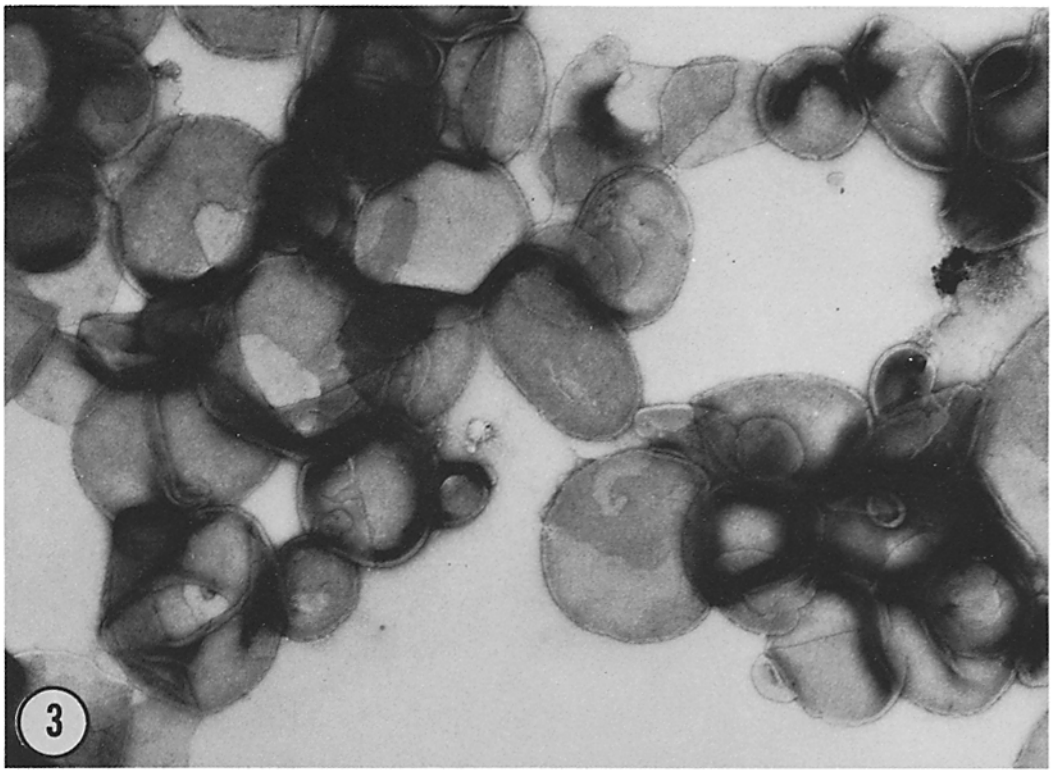


FIGURE 3 Low magnification views of negatively stained gap junction vesicles reveal that most of the spherical junctions have collapsed into circular profiles during the negative staining process. × 40,000.

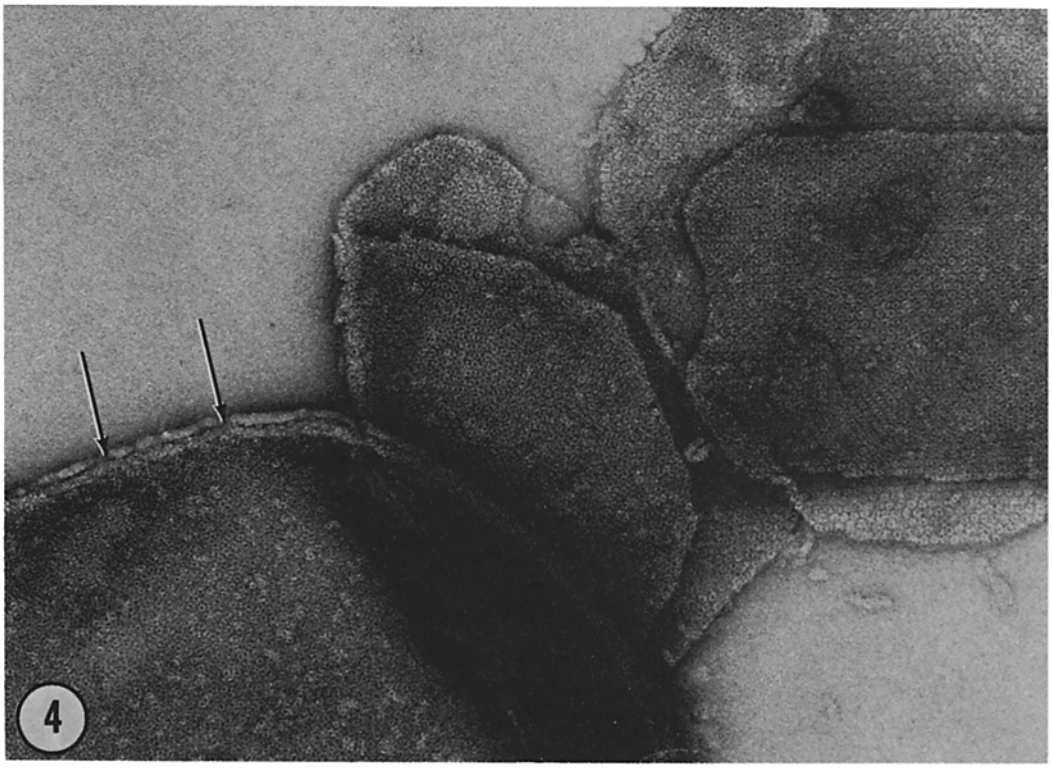
negatively stained junction profile. The dense, periodic stain in the 2-nm gap, corresponding to the spaces between the rows of connexons, alternates with stain-excluding regions, presumably the superimposed rows of connexons themselves. When the rows of connexons are precisely aligned parallel to the electron optical axis, a faint, 1-2-nm channel or pore may be discerned in the center of the stain-excluding connexon (pairs of arrows *P*, Fig. 5). This slender channel appears to extend across the full 15-nm profile, from one cytoplasmic face of the junction to the other. This channel is viewed end-on in the face view of the junction in Fig. 4, and corresponds to the 1-2-nm diameter dense "spot" in the center of each connexon. Similar micrographs of the slender transjunctional channels in negatively stained images of gap junctions have been presented by Gilula (5).

Freeze-Fracture of Trypsin-Treated Junctions

The vesicles may be seen in freeze-fracture replicas in Figs. 6 and 7. The particles and pits characteristic of the junction A and B fracture faces appear unaffected by the trypsin treatment. The large particles on the B fracture face in Fig. 6 represent an unusual contamination in this run which adhered only to gap junction B faces. Occasionally, the fracture plane passes through the area of nonjunctional membrane which closes the vesicles. These regions appear smooth (arrow, Fig. 7) and devoid of intramembrane particles. A fracture face of a small vesicle of nonjunctional membrane is evident in Fig. 7.

Effects of Protease Inhibitors

In general, the addition of protease inhibitors to the enzyme reaction mixture suggests that proteolysis is a necessary condition for vesicle formation. The addition of PMSF and benzamidine-HCl to the enzyme incubation mixture, or the substitution of DFP-trypsin for trypsin results in typical junction sheets, with little or no tendency to form vesicles. The addition of PMSF and benzidine-HCl after trypsin digestion does not prevent vesicle formation.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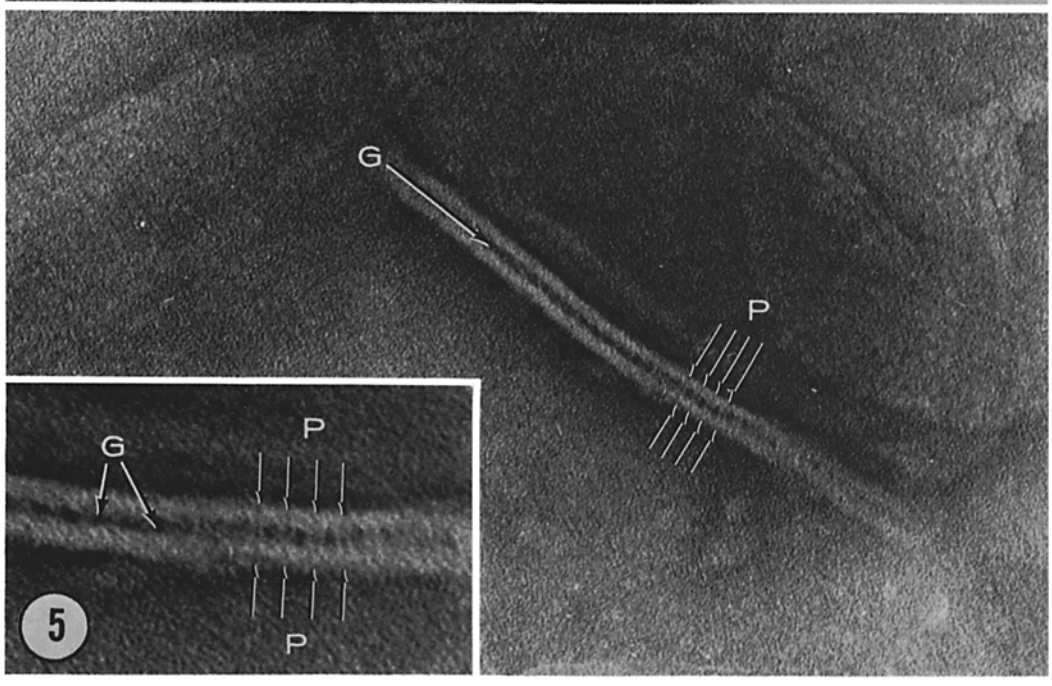
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Polyacrylamide Gels of Trypsin-Treated Junctions

Photographs of polyacrylamide gels of SDS- and DTT-solubilized gap junctions are shown in Fig. 8. Superimposed gel scans are included for direct comparison of peptide molecular weights. Gel A and the solid scan show the junction protein pattern after treatment, during the isolation protocol, with collagenase-hyaluronidase alone. The scan reveals a pair of low molecular weight peptides in position 2, and the 18,000-dalton "connexin" in position 1, similar to gel profiles described earlier (6). In addition, there is a significant amount of material which fails to enter the gel. Gel B and the broken scan in Fig. 8 show the protein profile after digestion with trypsin plus collagenase-hyaluronidase.

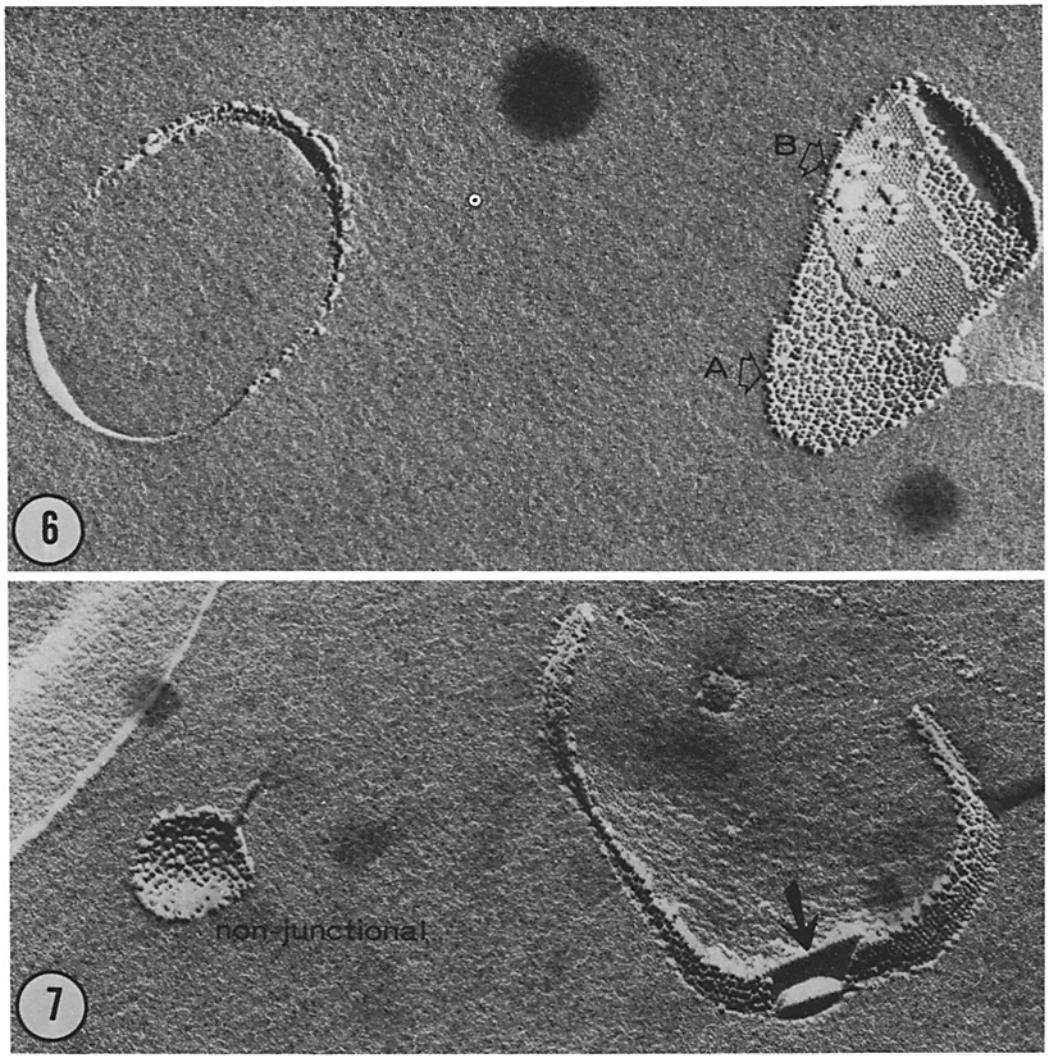
Four observations may be made in comparing these gels. First, after trypsin digestion the protein peak in position 1 appears shifted to a lower molecular weight. The second observation is that the heavier of the two peptides in position 2 (connexin A) is removed by the trypsin digestion, leaving a single peak. The mobility of the polypeptide remaining at position 2 does not appear to have changed relative to the lighter of the two 10,000-dalton peptides in the nontrypsin-treated control. The third observation is that almost all of the material at the top of the gel is removed by trypsin digestion. Finally, a high molecular weight peptide, about 110,000 daltons, may be seen about 2 cm from the top of each gel, in position 3.

Fig. 9 shows a combined buoyant density polyacrylamide gel experiment. Junctions were isolated with and without trypsin and then sedimented to equilibrium in a continuous gradient as previously described (10). The trypsin-treated and control junctions show the same buoyant density (approximately 1.17 g/cm^3) within the resolution of the gradients used. The trypsin-treated junctions show a broader density peak at equilibrium. This may be due to the fact that some of the vesicles trap water and detergent in their interiors and show a resulting lower buoyant density. It is also possible that some of the vesicles have lost protein due to the trypsin digestion and therefore show a lower density. The gels included in Fig. 9 are electropherograms in SDS and DTT of the two peaks after the centrifuge run. Gel A is trypsin treated, gel B the control. Note that there is little material at the tops of the gels, suggesting a higher degree of purity with continuous gradients than the two-step gradient previously described (6). Note also that with these highly purified specimens, the nontrypsin-treated control (gel B) shows only one band at 10,000 daltons.

Fig. 10 shows the results of various combinations of enzyme digestion of the gap junctions. All gels have been run with reduced specimens. Fig. 10 A is an electropherogram of the reduced and alkylated standard mixture. Fig. 10 B is the junction preparation digested with collagenase-hyaluronidase alone. The two low molecular weight peptides are not resolved from each other on this gel. Note the large amount of material that fails to enter at the top of the gel. Fig. 10 C is a preparation treated with trypsin after isolation with collagenase-hyaluronidase. Vesicles are not formed using this digestion sequence, but the gel top material is removed. Fig. 10 D is an electropherogram of the trypsin-collagenase-hyaluronidase-treated gap junction vesicles. Note the absence of material at the top of the gel. Fig. 10 E is a

FIGURE 4 Higher magnification negative-stain views of the collapsed gap junction vesicles, such as that indicated by the two arrows, show an apparent loss of the hexagonal lattice due to the moiré pattern caused by overlap of the front and back of the vesicle. The lattice is seen to good advantage, however, in the two gap junction vesicles in the upper right which have torn open and present nonoverlapped front and back halves. × 150,000.

FIGURE 5 At high magnification, the details of the negatively stained folded junctional profile can be seen. At a fold, the two stain-excluding junctional membranes are separated by the stain-penetrated gap (large arrow, *G*) which at favorable planes of tilt shows the expected periodicity resulting from superposition of the rows of junction subunits (connexons). Occasionally the connexons are exactly aligned, permitting visualization of the electron-dense core in the center of each connexon in profile view (arrows labeled *P*). In profile, the inset shows that these stain-penetrated cores appear as slender channels, approximately 2 nm in diameter, which appear to span the full 15-nm thickness of the junction profile. × 175,000. *inset*, × 300,000.



FIGURES 6 and 7 Freeze-fracture replicas of isolated, trypsinized gap junction vesicles reveal that the A- and B-face lattices of particles and pits appear unaltered by trypsin digestion. In Fig. 7 the area of closure of a vesicle has been fractured, showing a smooth fracture face similar to those faces displayed by pure lipid membranes. Occasional fracture faces of nonjunctional membrane contaminate the preparation. × 175,000.

preparation of junctions isolated without the use of any proteolytic enzymes. The resulting preparation is highly contaminated, but the low molecular weight components, characteristic of gap junctions, may still be seen (arrow).

Permeability Studies of Trypsin-Treated Junctions

The permeability of the junction vesicles to electron microscope tracers has been examined. Incubating the vesicles with horseradish peroxidase (HRP) or cytochrome *c* reveals two classes of gap junction vesicles (Figs. 11 and 12); those with reaction product on both sides of the junction profile (vesicles labeled, "open"), and those with the reaction product on the outside only (vesicles labeled "closed"). Those vesicles stained both inside and out are interpreted to be vesicles open to the surrounding medium due to incomplete closure, and those stained on the outside only to be vesicles closed by one or two layers of nonjunc-

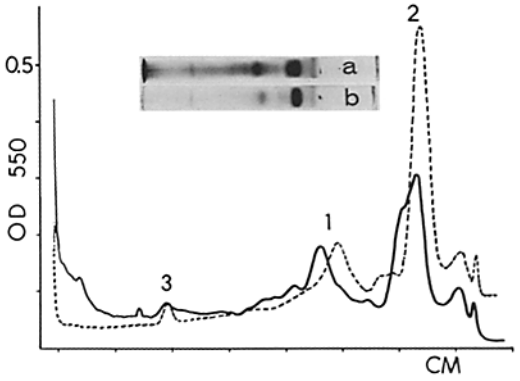


FIGURE 8 A comparison of gap junction protein profiles on SDS-polyacrylamide gels with and without trypsin digestion. Gel A and the solid scan show the protein profile of the control collagenase-treated junctions; gel B and the broken scan show a similar specimen which was treated with trypsin during the isolation protocol. The trypsin digestion removes one of the two low molecular weight peptides (position 2); it changes the molecular weight of the nonreducible 18,000-dalton component (position 1); and it removes the contaminating material which fails to enter the gel. The component at position 3 does not appear affected by the trypsin.

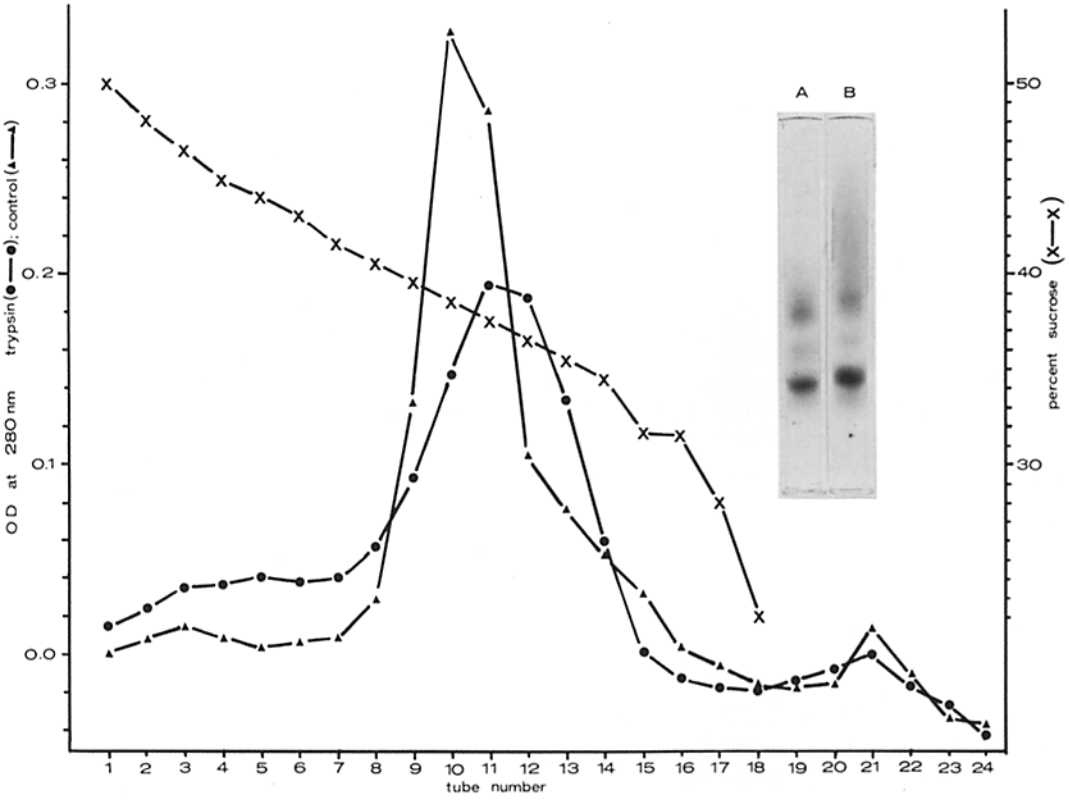


FIGURE 9 The buoyant densities of collagenase-hyaluronidase (triangles) and trypsin-collagenase-hyaluronidase (closed circles) are compared in this figure. The purified junctions were centrifuged at 280,000 *g* for 40 h in a continuous gradient. A profile of the sucrose gradient (×—×) was determined by refractometry of a gradient loaded with distilled water which was centrifuged and collected in aliquots identical to those of the junction-containing gradients. The material in each peak was then lyophilized, dissolved in SDS-DTT, and electrophoresed. Gel A contains the trypsin-treated junctions, gel B the control. The trypsin-treated junctions show a broader equilibrium position on the gradients, perhaps due to low sucrose permeability of some of the vesicles. The two preparations appear very similar.

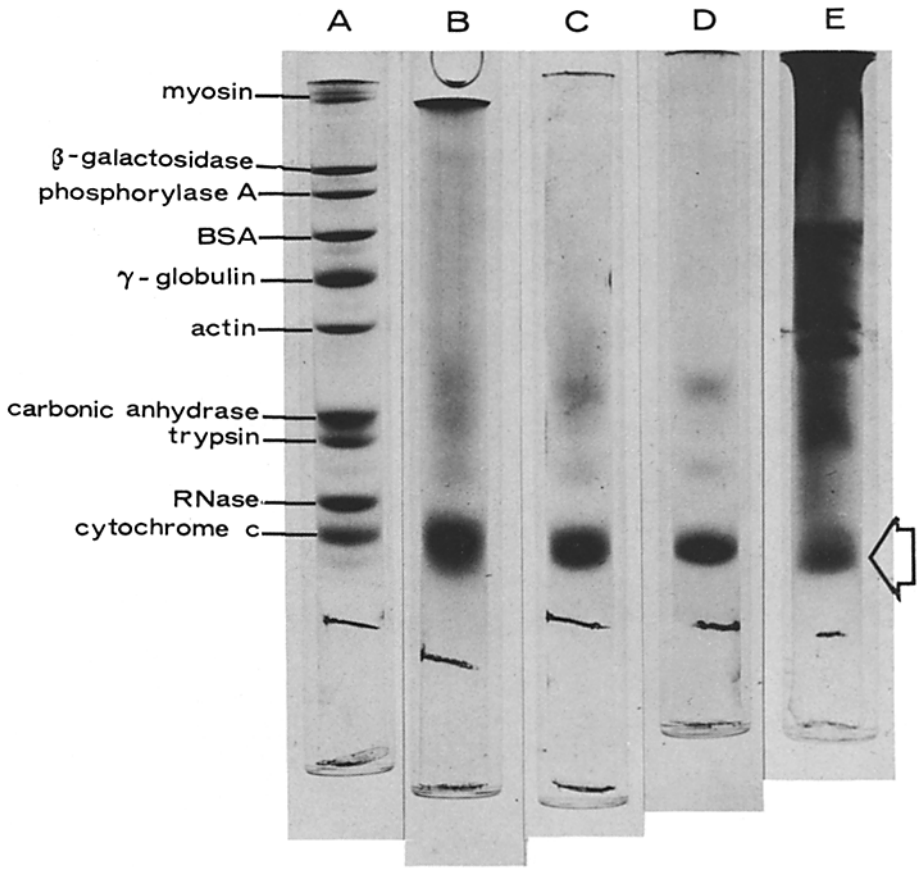


FIGURE 10 Electropherograms of gap junctions after various enzyme digestion sequences. All specimens have been treated with SDS and DTT. A, Reduced and alkylated standards; B, digestion with collagenase-hyaluronidase, followed by remainder of isolation protocol; C, identical to B, except digested with trypsin at the end of the isolation protocol; D, digestion with trypsin-collagenase-hyaluronidase, followed by remainder of isolation protocol; E, no enzyme digestion during isolation protocol. Arrow indicates 10,000-dalton position.

tional membrane. Closed vesicles represent about 20% of the population.

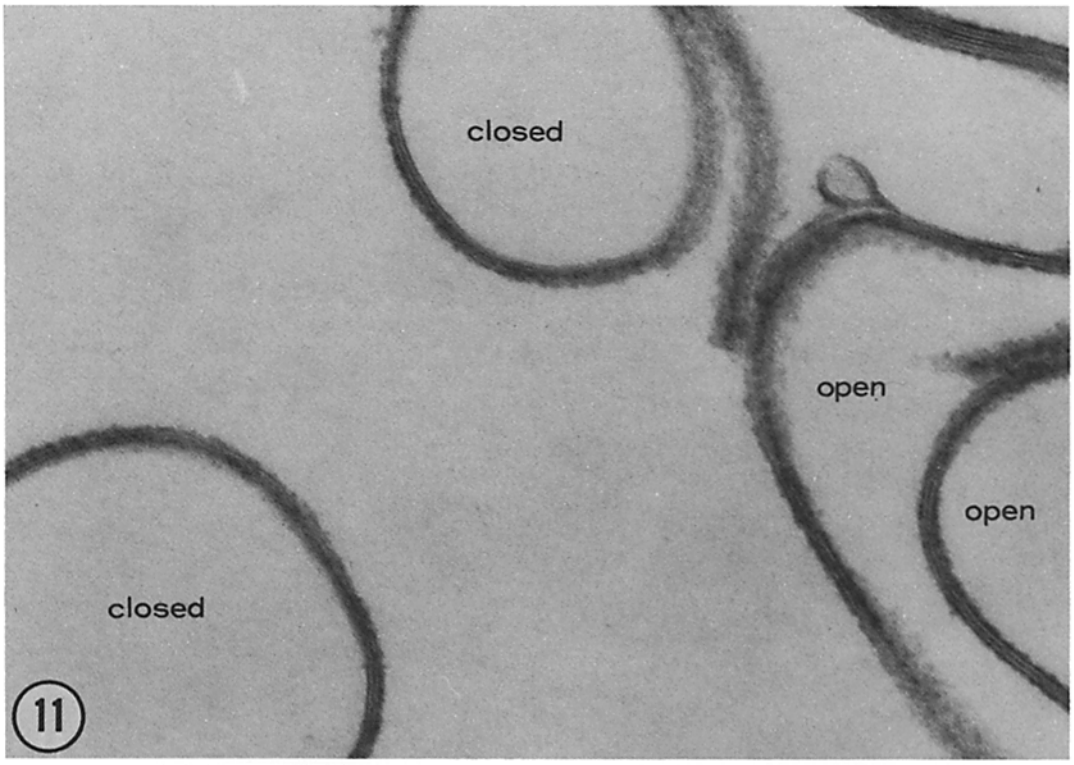
Both open and closed vesicles may show electron density in the 2–3-nm gap of the junction after HRP incubation. This observation provides evidence that the presence of density in the 2–3-nm gap is not related to junctional permeability, suggested earlier by Goodenough and Revel (9).

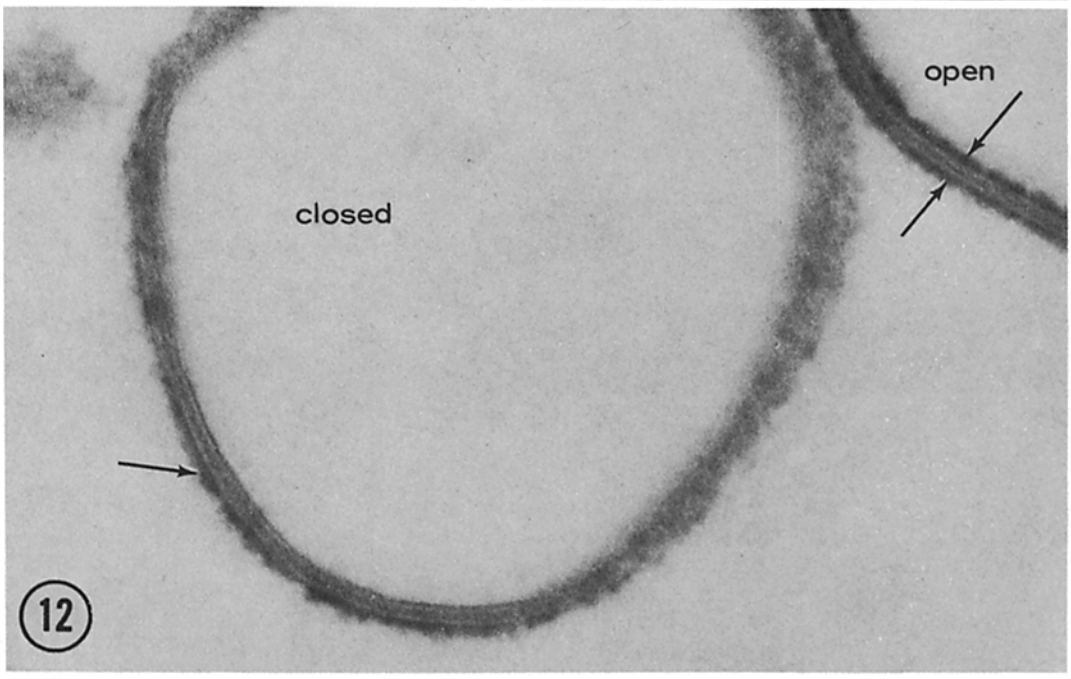
Thus the closed gap junctions appear impermeable to proteins of 12,000-dalton molecular weight or larger. Studies are in progress to determine whether the closed vesicles are permeable to smaller molecules.

DISCUSSION

Digestion of crude liver membranes with trypsin during the isolation of mouse hepatocyte gap junctions results in the production of gap junction vesicles 0.5–0.9 μm in diameter. Studies with ultrastructural tracers reveal that these vesicles consist of two populations: one which is permeable to HRP and cytochrome *c*, and another which is impermeable. The open vesicles are probably permeable to the ultrastructural tracers through large holes in the junctional vesicles where there was incomplete sealing.

Except for vesicle formation, the ultrastructure of the junction does not appear detectably altered by the trypsin digestion compared to controls. The lattice of connexons appears normal by negative stain and freeze-fracture criteria. In addition, X-ray diffraction analysis of junction vesicles shows no change in structure of the connexons due to the trypsin digestion, although the hexagonal





FIGURES 11 and 12 Studies with cytochrome *c* (Fig. 11) or horseradish peroxidase (Fig. 12) reveal that the trypsin-generated gap junction vesicles are composed of two populations, one which is permeable to the tracers (open) and one which is impermeable (closed). The open vesicles are characterized by reaction product on both sides of the junction profile (apposed arrows, Fig. 12), and the closed vesicles by reaction product confined to the outside surface of the vesicle (single arrow, Fig. 12). The ratio of closed to open vesicles is about 1:5. Fig. 11, × 105,000; Fig. 12, × 175,000.

lattice increases in crystallinity and may become more tightly packed (D. L. D. Caspar, L. Makowski, and D. A. Goodenough, manuscript in preparation).

Benedetti and Emmelot (3) reported tryptic digestion of isolated gap junctions. They reported that trypsin reduced the diameter of the connexon about 30% to 55 Å, and they reported a decrease in the lattice constant to 55 Å. These extreme lattice contractions due to trypsin digestion have not been observed in this study.

Efforts aimed at understanding the effects of trypsin digestion on the gap junction protein chemistry are as yet far from conclusive. Polyacrylamide gel electrophoresis in SDS reveals that the trypsin digestion usually results in the removal of the 11,000-dalton peptide (connexin A) and the material which fails to enter the gel. Polyacrylamide gels of highly purified junctions support the conclusion that connexin A is not a junction peptide but may be released from the nonjunctional contaminant at the top of the gels by reduction of disulfide bonds. The remaining 9,000-dalton band, previously called connexin B, appears to be a true junction peptide(s). This band does not appear to be grossly affected by the trypsin digestion within the resolution of the technique, a conclusion supported by the buoyant density measurements, X-ray diffraction, and electron microscope data.

The prior assertion (6) that there are two low molecular weight junction polypeptides, connexin A and B, must be revised in the light of these new data. It would appear, then, that reduction of disulfide bonds in the presence of SDS results in the disappearance of most of the 18,000-dalton protein, connexin, and the appearance of a low molecular weight peptide at 9,000 daltons. Not all of the 18,000-dalton band may be reduced, however, suggesting the coelectrophoresis of similar but nonidentical proteins.

It must be emphasized that the presence of one band on SDS electropherograms does not demonstrate only one polypeptide. It is also possible that the heavier of the two low molecular weight peptides has been digested to coelectrophorese with the lighter peptide in the 9,000-dalton position; end-group analysis may shed light on this question. It is also possible that enzymatic digestion may nick the junction polypeptide during the isolation protocol. Due to intramolecular disulfide bonds, the enzymatically "nicked" connexin molecule might hold together in SDS until reduced.

The production of gap junction vesicles has revealed many profile views in negatively stained preparations, since the vesicles collapse on the support film during drying, producing numerous folds. In these preparations, it has been possible to photograph slender channels, 15 nm long by 1–2 nm in diameter, possibly spanning the full profile thickness of the gap junction. These channels correspond to the electron-dense spot in the center of the connexon seen in negatively stained face views, and may be a direct visualization of the cell-to-cell hydrophilic channel postulated by Payton et al. (15), McNutt and Weinstein (13), and Pappas et al. (14). Zampighi and Robertson (16) sought images of the hydrophilic channels in fragments of synaptic disks viewed edge-on, but they were not found. The absence of the channels may have been due to the treatment with EDTA and deoxycholate that is necessary for the disk fragmentation.

There are as yet no firm data available which would provide an understanding of the mechanism by which the trypsin digestion results in gap junction vesicle formation. It is possible that the digestion exposes hydrophobic groups at the edge of the junction to the aqueous medium, but this may only be postulated. It is unclear why small segments of nonjunctional membrane which close the vesicles are retained while in routine gap junction sheets the naked ends of the junction are content to face the aqueous environment. It is not possible to form the gap junction vesicles by digesting routinely isolated junctions with trypsin. The combination of trypsin digestion with sarcosyl treatment seems to be necessary.

It is hoped that the closed vesicle population will provide a system to study directly the permeability of gap junction membranes to ions and small molecules. While the gap junction channel may be "uncoupled" due to cell death, manipulation of the ionic environment of the isolated junction vesicles may permit in vitro ion flux studies.

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REFERENCES

1. BENEDETTI, E. L., and P. EMMELOT. 1965. Electron microscope observations on negatively stained plasma membranes isolated from rat liver, *J. Cell Biol.* **26**:299–305.
2. BENEDETTI, E. L., and P. EMMELOT. 1968. Hexagonal array of subunits in tight junctions separated from isolated rat liver plasma membranes. *J. Cell Biol.* **38**:15–24.
3. BENEDETTI, E. L., and P. EMMELOT. 1968. Structure and function of plasma membranes isolated from liver. *In* The Membranes. A. J. Dalton and F. Haguenau, editors. Academic Press, Inc., New York. 33–120.
4. EVANS, W. M., and J. W. Gurd. 1972. Preparation and properties of nexus and lipid enriched vesicles from mouse liver plasma membranes. *Biochem. J.* **128**:691–700.
5. GILULA, N. B. 1974. Isolation of rat liver gap junctions and characterization of the polypeptides. *J. Cell. Biol.* **63**(2, Pt. 2):111a. (Abstr.).
6. GOODENOUGH, D. A. 1974. Bulk isolation of mouse hepatocyte gap junctions. Characterization of the principal protein, connexin. *J. Cell Biol.* **61**:557–563.
7. GOODENOUGH, D. A. 1975. Methods for the isolation and structural characterization of hepatocyte gap junctions. *In* Methods in Membrane Biology, Vol. III. E. D. Korn, editor. Plenum Press, New York.
8. GOODENOUGH, D. A., and N. B. GILULA. 1974. The splitting of hepatocyte gap junctions and zonulae occludentes with hypertonic disaccharides. *J. Cell Biol.* **61**:575–590.
9. GOODENOUGH, D. A., and J. P. REVEL. 1971. The permeability of isolated and *in situ* mouse hepatic gap junctions studied with enzymatic tracers. *J. Cell Biol.* **50**:81–91.
10. GOODENOUGH, D. A., and W. STOECKENIUS. 1972. The isolation of mouse hepatocyte gap junctions. Preliminary chemical characterization and x-ray diffraction. *J. Cell Biol.* **54**:646–656.
11. GRAHAM, R. C., and M. J. KARNOVSKY, 1966. The early stages of injected horseradish peroxidase in the proximal tubules of the mouse kidney: ultrastructural cytochemistry by a new technique. *J. Histochem. Cytochem.* **14**:291–302.
12. KARNOVSKY, M. J., and D. F. RICE. 1969. Exogenous cytochrome *c* as an ultrastructural tracer. *J. Histochem. Cytochem.* **17**:751–753.
13. MCNUTT, N. S., and R. S. WEINSTEIN. 1970. The ultrastructure of the nexus. A correlated thin-section and freeze-cleave study. *J. Cell Biol.* **47**:666–688.
14. PAPPAS, G. D., Y. ASADA, and M. V. L. BENNETT. 1971. Morphological correlates of increased coupling resistance of an electrotonic synapse. *J. Cell Biol.* **49**:173–188.
15. PAYTON, B. W., M. V. L. BENNETT, and G. D. PAPPAS. 1969. Permeability and structure of junctional membranes at an electrotonic synapse. *Science (Wash. D. C.).* **166**:1641–1643.
16. ZAMPIGHI, G., and J. D. ROBERTSON. 1973. Fine structure of the synaptic discs separated from the goldfish medulla oblongata. *J. Cell Biol.* **56**:92–105.