THE PERMEABILITY OF ISOLATED AND *IN SITU* MOUSE HEPATIC GAP JUNCTIONS STUDIED WITH ENZYMATIC TRACERS

DANIEL A. GOODENOUGH and JEAN-PAUL REVEL

From the Department of Anatomy, Harvard Medical School, Boston, Massachusetts 02115. Dr. Goodenough's present address is The Cardiovascular Research Institute, University of California, San Francisco Medical Center, San Francisco, California 94122

ABSTRACT

We have studied the effects of phospholipase C from *Clostridium welchii* on gap junctions in the intact mouse liver and in a junction-rich fraction prepared from mouse liver. Treatment of the isolated junctions results in the disappearance of both the 20 A gap and of the polygonal lattice visible with lanthanum. The junctions are morphologically unaltered, however, when whole livers are perfused with phospholipase via the portal vein. These results suggest that extracellular phospholipase cannot diffuse into the junctional area, but that the enzyme may affect structures within the gap from its cytoplasmic surfaces which become exposed in the isolated preparations. Horseradish peroxidase, which has physical dimensions similar to those of *Clostridium* phospholipase is also denied access to the 20 A gap in whole liver, while peroxidase reaction product can be seen in the gap in isolated preparations. Beef liver catalase, however, a tracer molecule much larger than peroxidase, cannot penetrate even in isolated fractions. If the cytoplasmic approaches to the gap junction used by peroxidase and phospholipase are available in vivo, and have not been created during the process of mechanical isolation, they may play a role in cell-to-cell passage of molecules larger than ions.

INTRODUCTION

The gap junction in mouse liver, which appears in the electron microscope as an area of intimate cell-to-cell apposition, has been previously described in detail (8, 24). Use of colloidal lanthanum reveals a polygonal lattice of substructures in the 20 A gap which separates the outer leaflets of the apposed junctional membranes (24). The polygonal lattice may also be visualized after negative staining of isolated membrane preparations (1, 2), and by the freeze-cleave technique (11).

In an earlier paper (8), we provided evidence that the appearance of the 20 A gap and associated polygonal lattice could be altered by exposure to polar organic solvents, notably acetone. At a critical concentration of 60% aqueous acetone, both the 20 A gap and the polygonal lattice disappeared from thin-sectioned, freeze-cleaved, and negatively stained material. Thin-layer chromatography revealed that a complex group of phospholipids were solubilized by the acetone at the critical concentration.

To further investigate the role of phospholipids, we have studied the effects of phospholipase C from *Clostridium welchii* on the gap junction. The phospholipase, however, is a much larger molecule than the organic solvents which were used previously. As a control we have therefore made a parallel study with horseradish peroxidase, a

molecule of physical dimensions similar to *Clostridium* phospholipase C, and with catalase, a much larger molecule. The peroxidase serves as a tracer, outlining the routes available for the penetration of phospholipase into the gap junction.

MATERIALS AND METHODS

Mature mice obtained from the Charles River Breeding Labs., Inc., Wilmington, Mass., were used throughout.

Fixation and Embedding

All aldehyde fixation was carried out in 3% glutaraldehyde in 0.2 M s-collidine (3). Alcian blue 8GX (Allied Chemical Corp., New York.) was added to this fixative in 0.1% concentration when used (26). Postfixation was done in a 2:1 mixture of 2% aqueous OsO_4 and collidine.

Whole livers were fixed by perfusion as previously described (8). The livers were kept warm with an infrared lamp during the phospholipase perfusions. For the horseradish peroxidase experiments, livers were fixed by immersion at room temperature.

The livers were cut into 1 mm cubes and stained en bloc with 2% uranyl acetate for 1 hr (10) and then rapidly dehydrated in ethanol and embedded in Epon-Araldite (16). Silver sections were examined in a Siemens Elmiskop 1A.

Freeze-Cleave

Freeze-cleaved replicas of liver were obtained by using the method of Bullivant and Ames (5). These procedures were described previously (8).

Isolation of Junctions

Mouse livers were homogenized in 1 mM bicarbonate, and the membrane fractions were further purified by sucrose density centrifugation (8), using the method of Neville (18) as modified by Emmelot et al. (6). As has been illustrated previously, the isolated fragments are flat sheets bounded by a broken membrane that does not seem to reseal to form a closed sac (8).

Horseradish Peroxidase

To study the penetration of peroxidase into the gap junctions of intact livers, 3 mg of type II horseradish peroxidase (Sigma Chemical Company, St. Louis, Mo.) in 0.3 cc of isotonic saline were injected into the inferior vena cava of anesthetized mice. The animals were sacrificed within 5 min and the tissues were reacted for peroxidase according to the methods of Graham and Karnovsky (9).

In parallel experiments, isolated junctions in suspension were incubated for 1 hr in isotonic saline containing 1 mg/ml of horseradish peroxidase and 10 mg/ml of bovine serum albumin. The incubation medium was then centrifuged for 10 min at 10,000 rpm in a Sorvall SS-1 and fixed as a pellet with aldehyde. The peroxidase reaction was then carried out according to Graham and Karnovsky (9).

Catalase

Isolated junctions were incubated for 1 hr at 37°C with 1 mg/ml beef liver catalase (Sigma Chemical Co., St. Louis, Mo.) and 10 mg/ml bovine serum albumin. The crystalline protein was solubilized by sonication in isotonic saline with a Branson Sonifier Model LS-75 (Branson Instruments, Co., Stamford, Conn.). The sonication was done in short (5 sec) bursts at 20 KC, to avoid overheating, until the solution turned a clear deep green. The peroxidase reaction was carried out on the fixed pellets by using the BaO_2 dialysis technique of Venkatachalam and Fahimi (30).

Phospholipase C

Phospholipase C from *Clostridium welchii* (Sigma Chemical Co., St. Louis, Mo.) was used in concentrations of 1 mg/ml in isotonic saline pH 7.3 with 3 mg/ml $CaCl_2$. Digestions were done at 22°C and 27°C for intervals ranging from 5 min to 1 hr.

Pronase

Isolated junctions were digested for 15 min at 24°C or 37°C with 1 mg/ml pronase (B grade, Calbiochem, Los Angeles, Calif.) in isotonic saline pH 7.0.

RESULTS

Studies with Clostridium Welchii Phospholipase C

The isolated membrane preparation, rich in gap junctions, was digested for varying periods of time and at different temperatures with phospholipase C, resulting in the loss of the gap (Figs. 3 and 4). We found that in the isolated membrane preparation the reaction of phospholipase C takes place very rapidly. The 20 A gap typical of the intact junction disappeared in less than 5 min even when the temperature of the incubation medium was lowered to 20°C. When used after phospholipase C, the alcian blue-lanthanum method (26) failed to reveal the usual junctional specializations (Fig. 5). In no case could one find the normal junction's

20 A gap or the polygonal lattice of particles which should be evident in tangential sections.

Studies with Pronase

The commercially available preparations of *Clostridium* phospholipase C are not pure enzyme preparations (20) and may have proteolytic activity. In order to reveal the kinds of morphological alterations that could result from proteolysis, samples of the isolated membrane preparation were also digested with pronase, an enzyme with broad proteolytic activity.

The isolated junctions were digested for 15 min at 24°C and 37°C with pronase. Micrographs of pronase-digested gap junctions (Figs. 6 and 7) show that this treatment did not cause a disappearance of the 20 A gap. All of the membranes were adversely affected by proteolytic digestion, and only relatively few junctions could be found in the preparations. In every case, however, gaps were found in all the junctions that could be examined after such proteolytic digestion. It seems safe to assume that any proteolytic activity associated with the phospholipase preparations probably does not contribute to the loss of the 20 A gap. The ability of the phospholipase C preparation to effect a disappearance of the 20 A gap thus lends support to the notion that phospholipids play a role in the maintenance of gap junction structure.

Phospholipase Studies on Whole Mouse Liver

Phospholipase C at 37°C was perfused through the mouse liver *in situ* as described in the Materials and Methods section, the perfusion lasting for from 5 to 20 min. Fixatives were then perfused through the livers in the usual way. The results were strikingly different from those obtained on isolated junctions. The phospholipase had no effect

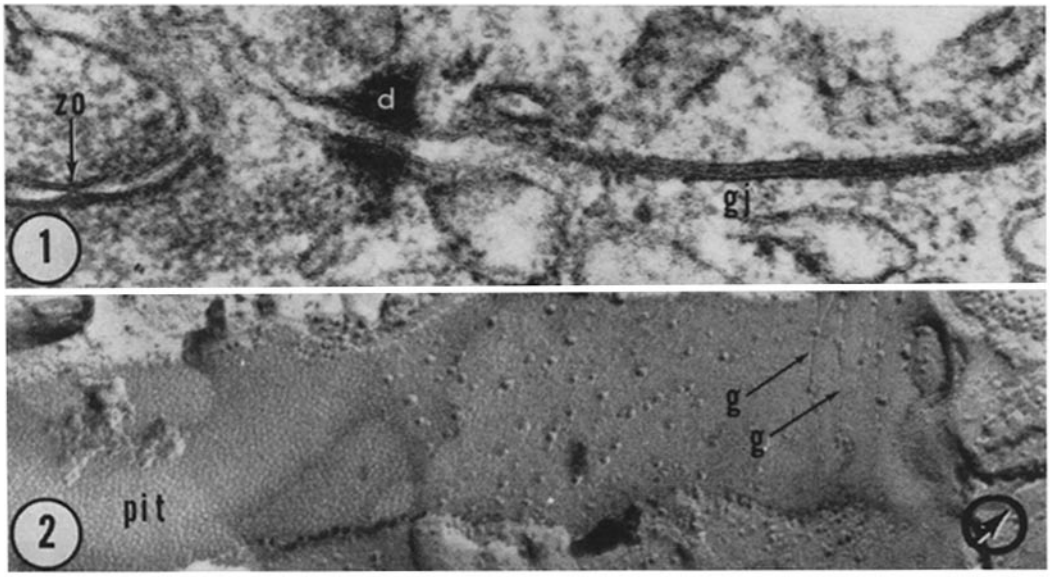


FIGURE 1 This electron micrograph shows a region of apposition between two hepatocytes in a mouse liver which has been perfused with phospholipase C at 37° for 20 min. A zonula occludens (*zo*), a desmosome (*d*), and a gap junction (*gj*) may be seen. None of the junctions appear disturbed by the enzyme. Note in particular that the 20 A gap of the gap junction is clearly visible. × 130,000.

FIGURE 2 Replicas of freeze-cleaved whole livers which have been perfused with phospholipase C reveal that the polygonal lattice of substructures associated with the gap junction are not affected by the enzyme. In this replica, which is from a preparation similar to that illustrated in Fig. 1, both the pitted lattice (*pit*) of the gap junction and the grooved aspect of the zonula occludens (*g*) show a normal appearance in spite of the experimental treatment. The circled arrow indicates the direction from which the specimen was shadowed. × 90,000.

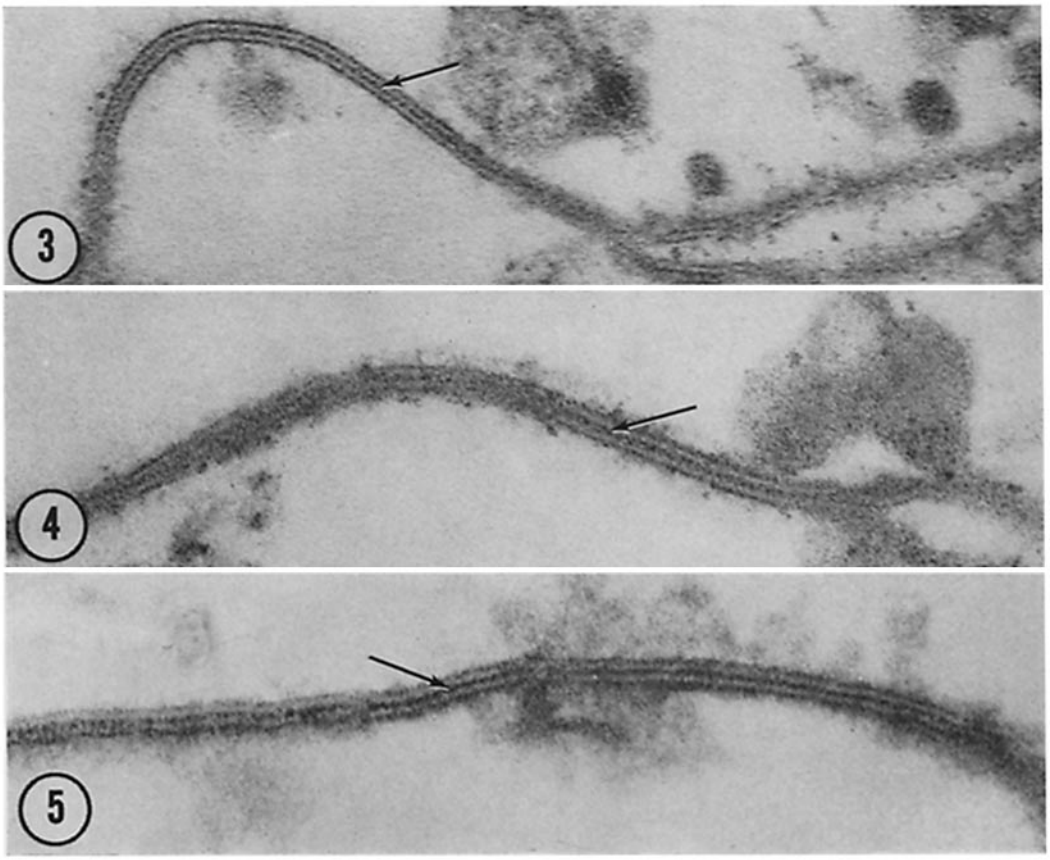
on the morphology of the gap junctions, failing both to collapse the 20 A gap and to disrupt the polygonal lattice of subunits (Figs. 1 and 2). At the longer time period, there was extensive evidence of damage to the cell membranes facing the intercellular space as well as those next to the space of the Disse. We believe therefore that the phospholipase C was active.

To make sure that molecules of the size of phospholipase could reach the gap by diffusion through the extracellular space, further studies were carried out with horseradish peroxidase (HRP), an enzyme which is similar in molecular weight to *Clostridium* phospholipase C, and which can be localized at the fine structural level.

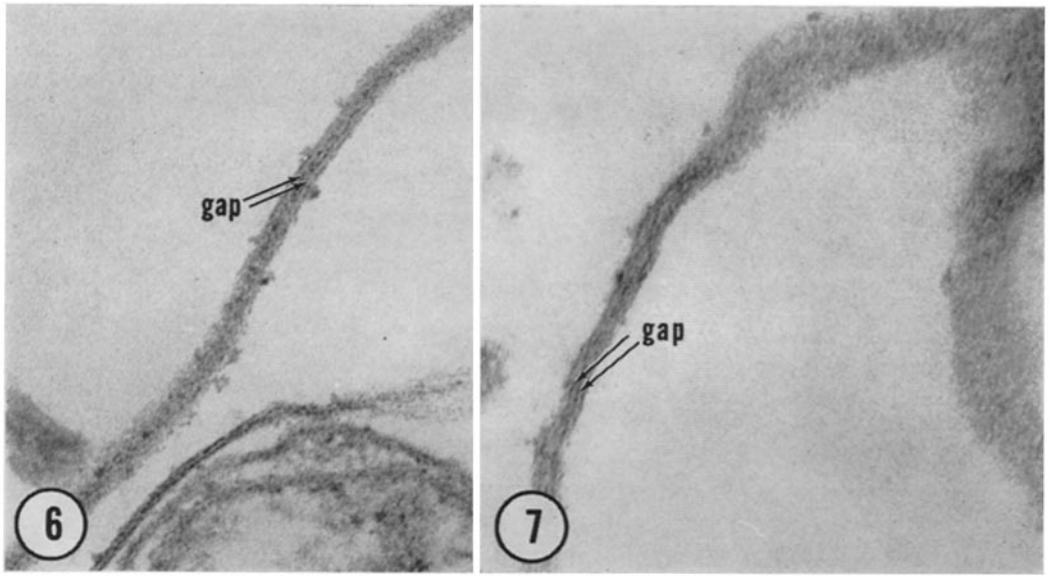
Peroxidase Studies of Whole Liver

Exogenous HRP was injected intravenously into anesthetized mice as outlined in the Materials and Methods section. Micrographs from such an experiment are shown in Figs. 8–12. Gap junctions may be seen even at low magnification, but it is not possible to tell if the peroxidase has penetrated the 20 A gap (Fig. 8). At higher magnification (Figs. 9–12), however, HRP is seen in the intercellular spaces but is stopped at the edge of the gap junctions (arrows) and fails to penetrate into the 20 A gap.

Due to its macular shape, the gap junction offers no barrier to the extracellular passage of peroxidase, and the tracer is seen on both sides of the junction (Figs. 9 and 12).



FIGURES 3–5 These micrographs show three examples of isolated gap junctions which have been digested with phospholipase C. Unlike the gap junctions in whole tissue, the isolated junctions have lost their 20 A gap following phospholipase treatment (arrows). The junction in Fig. 5 has been stained with the alcian blue-lanthanum method as outlined in the text. Tangential views of junctions stained in this manner do not reveal the polygonal lattice of subunits evident in control preparations. × 200,000.



FIGURES 6–7 The isolated gap junctions illustrated in these figures were digested for 15 min with pronase at 24°C and 37°C, respectively. The membrane preparations appear adversely affected by proteolytic digestion, but it does not result in the loss of the 20 A gap (compare with Figs. 3–5). Pronase digestion at 37°C (Fig. 7) results in a disruption of the usual smooth contours of the gap junction, but here again the 20 A gap can still be resolved. × 200,000.

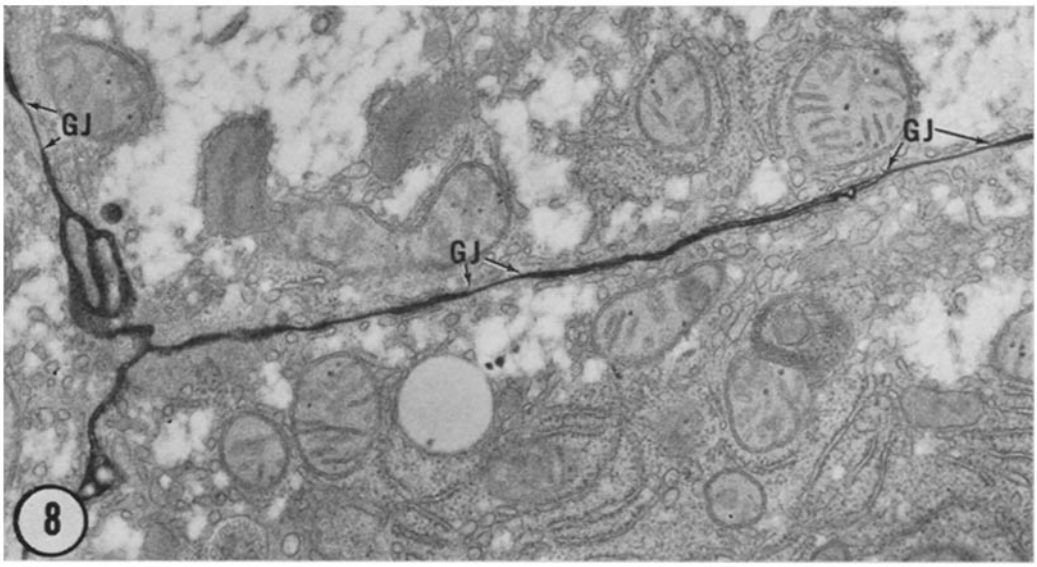
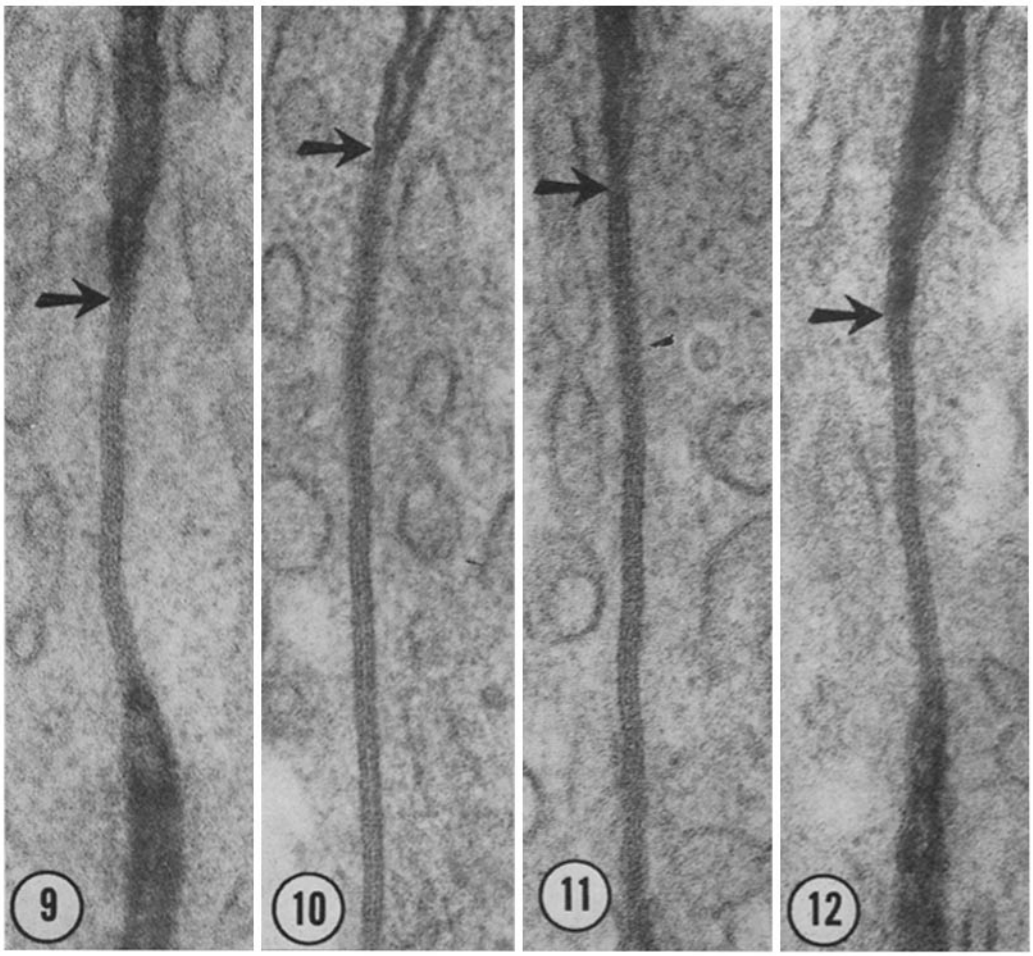


FIGURE 8 The intracellular spaces separating the three hepatocytes illustrated in this micrograph have been impregnated with horseradish peroxidase and are made electron opaque by the accumulation of reaction product. Three gap junctions may be seen between the liver cells (*GJ*) as areas of close cell-to-cell apposition. It is not possible at this low magnification to determine whether or not the peroxidase has penetrated the 20 A gap of the junctions. Due to their macular shapes, the gap junctions offer no barrier to the intercellular diffusion of the horseradish peroxidase, and thus the tracer may be seen on both sides of each junction. × 25,000.

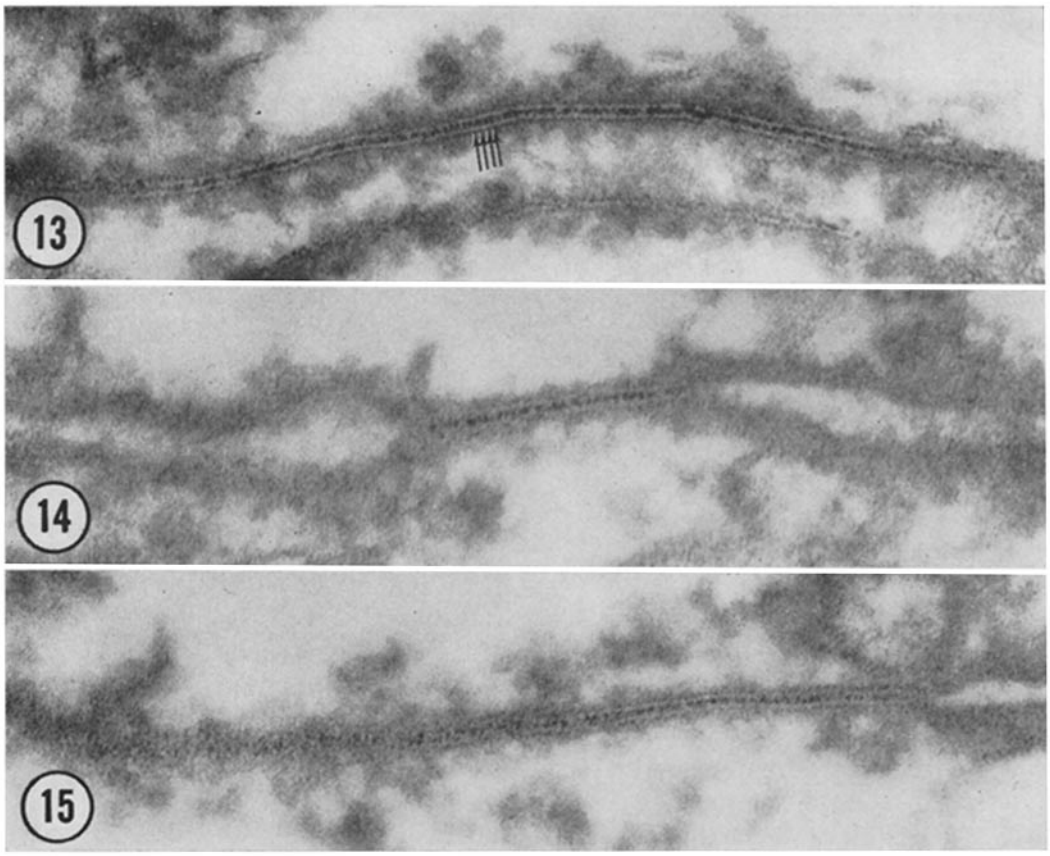
Peroxidase Studies of Isolated Junctions

After incubation with peroxidase the isolated gap junctions present a dramatically different picture. These results are shown in Figs. 13–15. Here, in each case, the 20 A gap is filled with the electron-opaque reaction product. In certain areas (arrows), this reaction product shows a periodic or striated appearance, suggesting that some of the periodic structures typical of the gap had been stained. It has not been possible to obtain satisfactory images of sections that are cut frankly *en face*. This is due, in part, to the relatively low contrast obtainable with horseradish peroxidase, and to the fact that even thin sections (50 nm) of junctions include much adherent amorphous material. This electron-opaque coating is present only after incubation with horseradish peroxidase or catalase. It is absent from untreated isolated junctions (8) or from fractions treated with diaminobenzidine and hydrogen peroxide in the absence of tracer enzyme. We presume that this amorphous material may represent enzyme protein bound by glutaraldehyde to the internal (cytoplasmic) face of the junctions.

The results obtained with peroxidase, in addition to indicating possible access routes available to phospholipase C, also imply that the gap junction is permeable to a 44,000 molecular weight



FIGURES 9–12 The four examples of gap junctions in whole tissue illustrated here reveal that the peroxidase is denied access to the 20 A gap. In each case, the peroxidase may be seen to stop abruptly at the periphery of the gap junction (arrows), leaving the 20 A gap free of tracer. × 160,000.



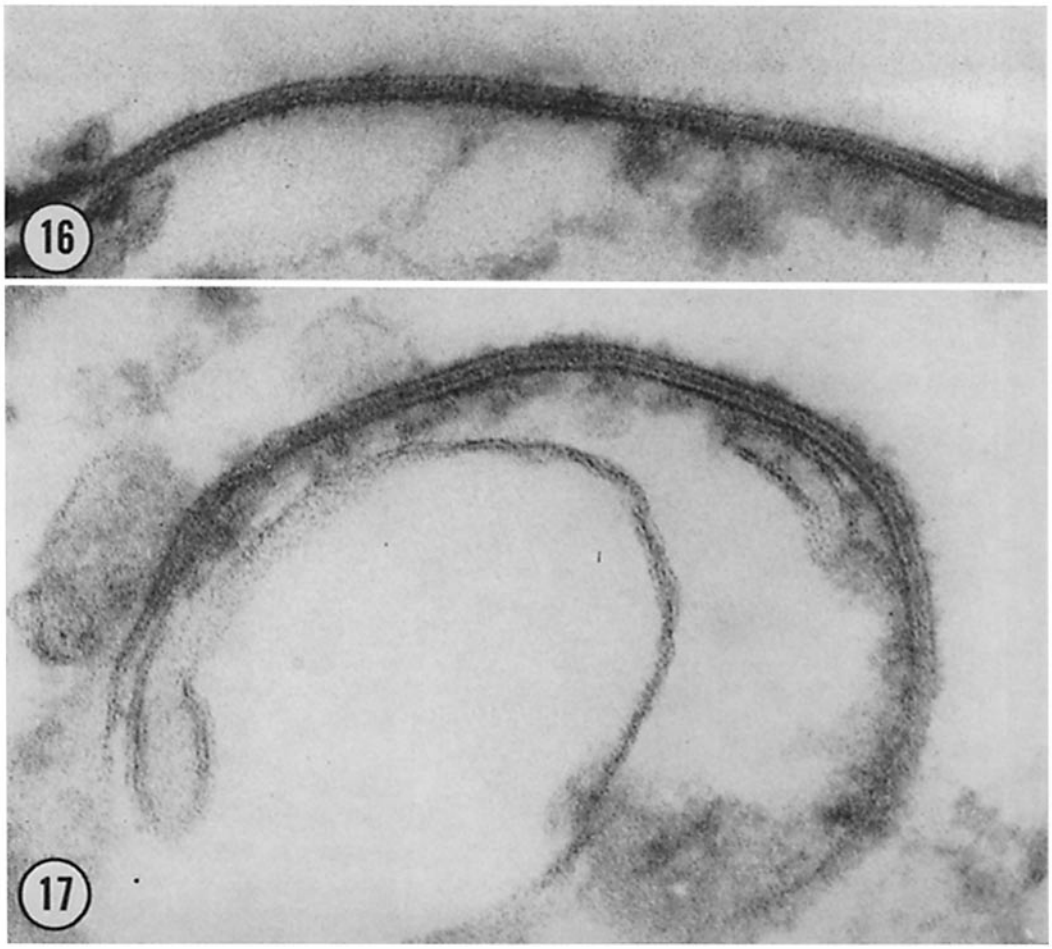
FIGURES 13–15 In the isolated preparation, in contrast to the results in whole tissue, the peroxidase is free to penetrate into the 20 A gap of the gap junction. In certain areas, the reaction product in the gap has a striated appearance (arrows). Due to the large amounts of peroxidase protein adhering to the inner leaflets of the junctional membranes, it has not yet been possible to determine where the peroxidase is situated in the 20 A gap with respect to the lattice of particles visible with lanthanum or negative stain. If the peroxidase has penetrated into the 20 A gap via pathways which exist in the in vivo junctions, then these pathways may represent intercellular channels for passage of molecules much larger than ions. × 200,000.

protein *across* its junctional membranes. We cannot tell, however, if this permeability of the isolated junctions reflects an in vivo permeability or damage to the gap junction membranes. Such damage could consist of opening or enlarging access pathways on the cytoplasmic faces of the junctions, or it may affect the passage of material through the intercellular space. It must be emphasized, however, that no evidence for morphological alterations of the isolated junctions can be obtained by any of the several methods used.

In order to determine an upper limit for the molecular weight of a protein which can penetrate into the 20 A gap, isolated gap junctions were incubated with beef liver catalase according to the method of Venkatachalam and Fahimi (30). This molecule is a heme protein with a molecular weight of 240,000 (25). The results are shown in Figs. 16 and 17, and it appears that the catalase does not penetrate the 20 A gap in a manner similar to the HRP.

DISCUSSION

In order to test further the previously proposed hypothesis that phospholipids are involved in the structure of the gap junction, membrane pellets were digested with phospholipase C from *Clostridium welchii* (8). The 20 A gap, as well as the polygonal lattice, disappeared following incubation



FIGURES 16–17 These two micrographs illustrate gap junctions in the isolated preparation which have been incubated with beef liver catalase. As can be seen here, the catalase does not penetrate the 20 A gap of the gap junctions in the same manner as the horseradish peroxidase. These data thus set an upper limit of 240,000 mol wt for the size of the molecule which can penetrate into the 20 A gap of the gap junction. The upper limit is likely to be found to be much lower than this when tracers of intermediate molecular weight become available. × 200,000.

with this enzyme. These data parallel those reported previously for acetone and other polar organic solvents (8).

Since the phospholipase C used was a commercially available crude enzyme extract, the criticism could be made that the changes in junctional morphology were due to contaminating proteolytic enzymes in the preparation. For this reason, isolated junctions were digested with pronase, a proteolytic enzyme of broad specificity. After such treatment it was difficult to obtain well preserved specimens, as most cell membranes were adversely affected. In the remaining gap junctions, however, one could still always observe the 20 A gap and its associated polygonal lattice. Phospholipase C was also perfused through the intact, unfixed liver via the portal vein and, surprisingly, under these conditions the gap junctions remained intact.

An hypothesis which it is tempting to use in explaining these results is that the phospholipase is too large to diffuse into the 20 A gap. The observation that phospholipase can attack the gap junction in the isolated preparation suggests, however, that access to the gap is actually possible from the cytoplasmic surfaces of the junctional membranes.

These cytoplasmic surfaces would of course be available to the enzyme in the isolated preparation but not in the intact liver.

The molecular weight of phospholipase C from *Clostridium welchii* is not known, although Shemanova et al. (27) reported a sedimentation constant of 3.8 S for phospholipase C from *Clostridium perfringens*. This corresponds to a molecular weight of approximately 42,000. Horseradish peroxidase also has a sedimentation constant of 3.8 S and is known to have a molecular weight of 44,000 (19). Due to the similarity in S value, which reflects both the molecular weight and the shape of the proteins, horseradish peroxidase would seem to be a suitable tracer with which to examine the hypothesis that phospholipase C cannot approach its substrate in whole tissue due to its size. Accordingly, HRP was either injected into whole mice or incubated with isolated junctions to see if there was a difference in the extent to which it could penetrate into the jap junction. As reported in the Results section, the peroxidase cannot be found in the gap junctions in the whole tissue, but the enzyme is seen on the cytoplasmic face and in the 20 A gap of incubated isolated junctions. These results lend support tc the theory that HRP, as well as *Clostridium* phospholipase C, is too large to penetrate into the 20 A cleft from the extracellular spaces, but that access routes into the gap might be available on the cytoplasmic surfaces on the junction. As we have mentioned already in the Results section, it is not possible at this point to determine if these access routes are created during isolation, or if they exist also in vivo.

The failure of peroxidase, a protein of 40,000 mol wt, to penetrate a 20 A space is not surprising. Egg albumin and β-lactoglobulin have molecular weights of 42,000 and 40,000, respectively. The short axes of these ellipsodial proteins are greater than 30 A (17). With these dimensions as guides, it is difficult to imagine that HRP (mol wt 44,000) could diffuse freely through a 20 A wide space.

Reports in the literature indicate that peroxidase may be able to penetrate into some gap junctions (4, 15). Matter et al. (15) claim that they have found reaction product in the hepatic gap junctions, but they do not illustrate their findings. The observation of Brightman and Reese (4) on the gap junctions of the ependyma may indicate differences in gap junctions, depending on anatomical location. It is, however, difficult to judge the extent of peroxidase pentration in this case because the published illustrations are of relatively low magnification.

Due to the binding of HRP by glutaraldehyde at the cytoplasmic surfaces of the junctions, as well as in the 20 A gap, it has not yet been possible to determine the appearance of *en face* views of peroxidase-filled gap junctions. For this reason, the location of the peroxidase in the 20 A gap with respect to the polygonal lattice is not yet known.

The suggestion that can be made from the phospholipase C and HRP studies is that the two enzymes have used an access route which is available in vivo. If this suggestion is correct, then these routes may form intercellular channels which could allow molecules of up to 44,000 mol wt to penetrate into the junctions, and perhaps even to pass from cell to cell. These routes do not permit passage of beef liver catalase, hence a molecular weight of 240,000 represents an upper limit for the size of the molecule which can penetrate into the gap junction. It is unlikely that staining of the gap is due to a migration of the reaction product rather than enzymes, into the junction, since the gap was not stained in experiments involving catalase, in which essentially the same reaction product is believed to form.

There is evidence that certain vital dyes of up to 1000 mol wt can pass through certain electrical junctions (7, 14, 21, 23). Recently Payton et al. (22) reported the passage of Procion Yellow (molecular weight, 500) between segments of *Procambarus* septate axons. These investigators also demonstrated a polygonal lattice of subunits in these synapses with lanthanum.

It should be emphasized that low electrical resistance does not necessarily imply intercellular permeability of larger molecules. Slack and Palmer (28) have demonstrated that fluorescein does not pass between electrically coupled cells of State 7 *Triturus* embryos.

In addition to these studies, Subak-Sharpe et al. (29) have reported metabolic cooperation between a variant line of PyY cells and the parent line. This cooperation may involve the intercellular passage of substances larger than ions.

Loewenstein (12) suggested that molecules as large as serum albumin (69,000) can pass between insect salivary epithelium cells, although this figure has recently been reduced to 10,000 (13). Whether or not the peroxidase studies reported here bear directly on the problem of cell-to-cell communication must await further study.

In conclusion, the phospholipase studies have complemented the acetone extraction experiments reported earlier (8) by demonstrating that the 20 A gap and associated polygonal lattice are phospholipase sensitive. The observation that *in situ* gap junctions are immune to phospholipase attack suggests the presence of access reoutes which are available in isolated junctions on the inner cytoplasmic surfaces. These observations are supported by the distribution of horseradish peroxidase in intact liver and the isolated junctions. The HRP can gain access to the 20 A gap in isolated but not in *in situ* junctions.

Although there exists a complex polygonal lattice of substructures associated with the gap junction, it has not yet been possible to give a clear morphological demonstration of the presence of a "channel" or "pore" through the junctional membranes. The presence of a 15 A electron-opaque region ("dots") after lanthanum staining (24) and the existence of "pits" on the freeze-cleaved junctional surfaces (11) have inspired the notion that a pore through the junction can be recognized morphologically (22). Although this is a tempting hypothesis, there is as yet no experimental evidence for the true appearance of these channels in terms of their distribution within the lattice or their dimensions.

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