Permeability and Structure of Junctional Membranes at an Electrotonic Synapse

Abstract. *The dye Procion Yellow M4RS crosses junctional membranes from cytoplasm to cytoplasm at electrotonic synapses between segments of the crayfish septate axon. The dye does not enter the cells from extracellular space. Thus permeability of junctional membranes is qualitatively different from that of nonjunctional membranes. Electron microscopy after fixation in the presence of lanthanum hydroxide indicates that these synapses are "gap junctions" and that there is a network of channels continuous with extracellular space between apposed junctional membranes. These channels must be interlaced with intercytoplasmic channels that are not open to extracellular space.*

Many cells are electrically coupled by way of junctions containing membranes of low electrical resistivity (*1, 2*). This low resistivity has raised the question whether there are channels between cells that can transmit large molecules. Junctional membranes can be highly permeable to K^+, a result predictable from the low resistivity as K^+ must in general be the major charge carrier in cytoplasm (*3, 4*). Other small ions and a few dye molecules have also been observed to pass between coupled cells (*2, 4, 5*). However, dyes such as fluoroscein can cross nonjunctional membranes and therefore could enter adjacent cells by way of extracellular space rather than by crossing intercellular junctions; also, appreciable electrical coupling could occur between cells if regions of low resistance membrane were opposed but separated by a small extracellular space (*6*).

Our experiments demonstrate that Procion Yellow M4RS (molecular weight, about 500) crosses an electrotonic junction from cytoplasm to cytoplasm but does not penetrate these cells when applied from the outside. These results indicate that the cells are coupled by junctions that are permeable to larger molecules than the nonjunctional membranes and that these junctions are not accessible to extracellular space.

Procion Yellow M4RS has been described as a useful dye for marking and determining the geometry of neurons (*7*), largely because of its retention within the cell and slow binding to cell proteins. Thus it can diffuse in axons and dendrites for considerable distances. The staining is preserved during conventional fixation and subsequent procedures for preparation of histological sections.

The experiments were performed on septate axons of the crayfish (*Procambarus*). These axons consist of a series of cells, one per segment, that are apposed along an oblique septum in each ganglion. Over most of the septum, the axons are separated by a compact fibrillar layer lined with thin layers of Schwann cell processes. There are small areas within the septum where the intervening material is absent and the two axons come into close apposition (*8, 9*). These regions have been thought to be electrotonic synapses, that is, sites of the electrotonic coupling that occurs between adjacent segments (*8–10*).

Nerve cords were prepared as described (*10*). Procion Yellow was injected into the lateral giant fibers of isolated nerve cords by iontophoresis. A microelectrode filled with dye (5 percent solution) was placed in the axon not more than 250 μ from the septum. Rectangular pulses of hyperpolarizing current, 50 or 100 msec in duration and 0.5 μa in amplitude, were applied at a frequency of about one per second. This electrode was also used for recording action potentials after external stimulation of the cord. A conventional microelectrode filled with KCl was inserted into the axon on the other side of the septum to monitor impulse transmission across the septum. The presence of dye fluorescence during injection was observed with a dissecting microscope with appropriate illumination and filters (*7*). After the dye was injected, tissue was fixed in 2.5 percent glutaraldehyde in 0.1*M* cacodylate buffer, *p*H 7.2, and embedded in Epon 812 or Maraglass 732; the tissue was then sectioned at 1

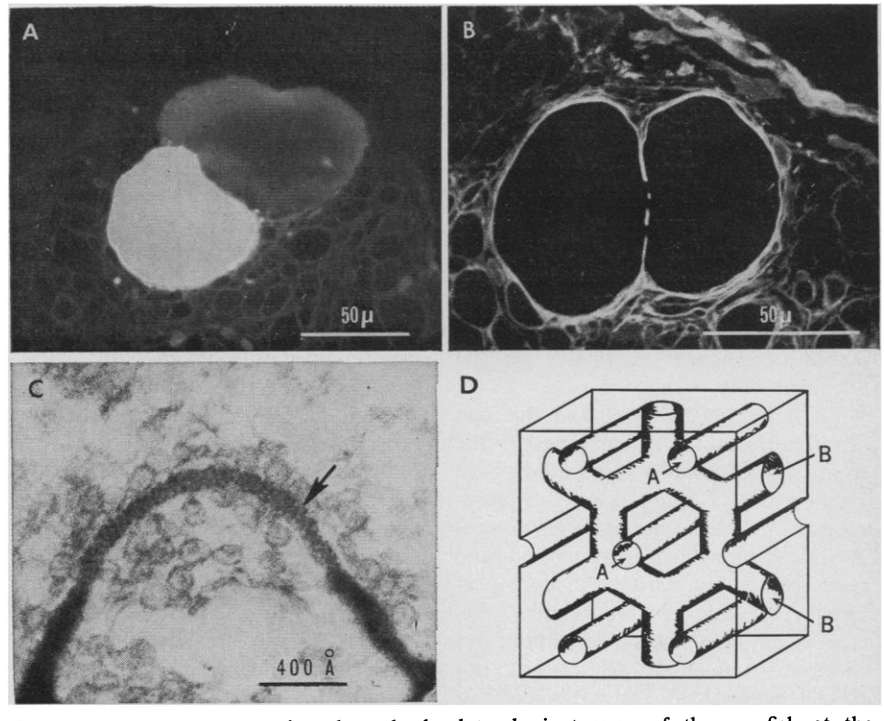

Fig. 1. (A) Transverse section through the lateral giant axon of the crayfish at the septum after injection of dye into one of the cells. Definite but less intense Procion fluorescence can be seen in the cell not injected. The fluorescence is more intense in the peripheral part of the injected cell. Neighboring axons not injected are visible because of their autofluorescence. (B) Staining of the lateral giant axon after soaking in a physiological solution containing 1 percent Procion Yellow. Schwann sheaths surrounding all the axons are heavily stained. The septum is also stained except for three small regions where septal connective tissue is presumably absent. A faint Procion fluorescence extended across these regions. (C) Electron micrograph of a tangential section through a "gap junction" after fixation in lanthanum and glutaraldehyde (*13*). Very dense lanthanum staining is seen in the extracellular space at the edge of the junction and a repeating pattern can be discerned within the junction (arrow). (D) Model illustrating possible distribution of intercellular channels (*A*) and extracellular channels (*B*) within the junctional membrane complex.

to 2 μ and observed by fluorescent microscopy (*7*).

Where conduction across the septum was maintained for an hour or more after iontophoretic injection was started, the pinkish-orange fluorescence of the dye could be detected on both sides of the septum under the dissecting microscope. The intensity of the fluorescence was much greater in the injected cells. Conduction across the septum and the excitability of both cells could often be maintained for 2 hours or more during iontophoresis of the dye. In some preparations septal conduction failed earlier, although 1 to 2 μ sections of such material (in three out of four experiments) still showed some evidence of spread of dye across the septum (Fig. 1A). In all sections the fluorescence of the dye was clearly distinguishable from the pale green autofluorescence of the tissue fixed in glutaraldehyde. No leakage of dye into adjacent tissues was observed. Functioning axons that showed cell to cell passage of the dye under the dissecting microscope were also fixed and sectioned, and the presence of dye in both cells was confirmed (*11*). Sections cut through the injected cells, which contained higher concentrations of the dye, showed a more intense fluorescence peripherally, an indication that the dye might be binding to the cell membranes (Fig. 1A). In some preparations, even though there was obvious Procion fluorescence in the cytoplasm of both cells, regions of the normal green autofluorescence were visible in the septum. This autofluorescence is ascribable to the Schwann cells.

To determine whether the dye can cross nonjunctional membranes of the axon, whole isolated nerve cords were immersed for 2 hours in crayfish physiological solution containing 1 percent Procion Yellow. This treatment blocked nerve conduction as determined by external recording and stimulation. Return to physiological saline without dye restored conduction. Sections of this rinsed material in the region of the nerve cord midway between the ganglia showed very little evidence of cytoplasmic staining within the axons. In contrast, the outlines of all the axons showed an intense Procion fluorescence. Sections through the ganglion did show cytoplasmic staining of some axons. It is possible that these axons ran in the segmental nerves and that dye diffused in from their cut ends. No evidence of cytoplasmic staining of the lateral giant fibers was seen, although there was marked staining of the Schwann cell sheath and the septum (Fig. 1B). Serial sections (2 μ thick) of the septal region revealed occasional areas where the septum was greatly reduced in thickness (Fig. 1B). Electron microscopic observations indicate that these are the regions where the septal connective tissue is absent and close apposition of axonal membranes occurs. In these regions there was a faint continuity of Procion fluorescence that was, however, difficult to photograph. This fluorescence suggests that the extracellular dye can penetrate between the axons at the junctions, although it does not enter the cells (*12*).

Further evidence of the structure of the junctions was obtained by electron microscopy after fixation in the presence of lanthanum hydroxide (*13*). Lanthanum in this procedure has been used for determination of extracellular space at the ultrastructural level. In the septum, extracellular space was filled with an electron-dense deposit of lanthanum (Fig. 1C). No lanthanum penetrated the axons. Sections perpendicular to the junctional membranes revealed a thin layer of lanthanum, about 20 Å thick, extending between the membranes at their regions of closest apposition. Tangential sections showed that this thin layer was not a continuous sheet. It consisted of a network of electron-dense regions that, at some places, appeared to form a hexagonal array with a periodicity of about 100 Å (arrow, Fig. 1C). Similar results have been obtained at a number of sites of close membrane apposition and are characteristic of the "gap junction" (*13*).

The combination of intracellular and extracellular application of stain described here indicates that substances can pass from cell to cell without entering the extracellular space. The only sites at which such passage could occur are the close appositions between cells, the gap junctions. Small ions should also be able to pass between cells at these sites, and since the cells are separated by intervening material over most of the septum, it can be concluded that the gap junctions are also the sites of electrotonic coupling. The electron microscopic evidence indicates that there is a network of channels through the junctions that is continuous with extracellular space; this conclusion is supported by the faint Procion fluorescence in the junctional regions after extracellular application of the dye. The most likely site of passage of Procion Yellow between cell cytoplasms would then be in the center of the hexagonal regions outlined by the channels continuous with extracellular space. The low and fixed electrical resistance of the junctional membranes (*10*) suggests that there are patent channels between cell cytoplasms, a fact that is supported by the nonspecific permeability of the junctional membranes (*4*). From the evidence for two interlocking kinds of channels, it can be concluded that the most probable structure of the junctions is as diagramed in Fig. 1D. One complete hexagon of the array of extracellular channels is shown with connecting segments from the neighboring hexagons (*B*). Channels cross the membranes through the centers of the hexagons (*A*) and connect cytoplasms of the coupled cells. Presumably the channels interconnecting cytoplasm contain polar groups, and staining of a central region at the hexagons has been observed at a number of junctions (*13, 14*). This model of the junctional complex presents some difficulties in terms of membrane structure. The walls separating the two types of channels would have to be substantially less than the 75 Å of the "unit membrane" (*15*), and the organization of lipid and protein in these regions remains to be worked out.

Our results indicate that the gap junctions are relatively more permeable for intercytoplasmic passage of larger molecules than the nonjunctional membranes are permeable for exchange between cytoplasm and external medium. The data do not allow the assignment of relative permeabilities normalized to a specific ion such as K^+. But by comparing areas of junctional and nonjunctional membranes having equal resistance (*10*), it is clear that much more Procion Yellow crosses the junctions than penetrates the nonjunctional surface. In this respect we can conclude that the permeabilities of the two kinds of membrane are qualitatively different (*16*). We cannot give a figure for dye permeability of the walls of the proposed intercytoplasmic channels of the junctions, although it must be very low. For quantitative evaluation we would need to know the concentration of the dye at the edges of the junctions and the amount penetrating the axon, if any. The dye concentration in the septum was probably little below that of the external medium as indicated by the intense staining of the septal tissue. Moreover, we would not expect the dye concentration in the septum to be reduced, because the dye binds slowly and does not penetrate into the axoplasm.

The results presented here provide evidence that permeability of intercellular gap junctions is qualitatively different from that of nonjunctional membranes and that material can pass between cell cytoplasms by way of channels not connected to extracellular space. The data also support the hypothesis that gap junctions are a site of electrotonic coupling between cells.

B. W. PAYTON*
M. V. L. BENNETT, G. D. PAPPAS
Department of Anatomy,
Albert Einstein College of Medicine,
Bronx, New York

References and Notes

1. W. R. Loewenstein, S. J. Socolar, S. Higashino, Y. Kanno, N. Davidson, *Science* **149**, 295 (1965); M. V. L. Bennett, *N.Y. Acad. Sci.* **137**, 509 (1966); ———, G. D. Pappas, M. Giménez, Y. Nakajima, *J. Neurophysiol.* **30**, 236 (1967).
2. E. J. Furshpan and D. D. Potter, *Curr. Top. Develop. Biol.* **3**, 95 (1968).
3. S. Weidmann, *J. Physiol. (London)* **187**, 323 (1966).
4. M. V. L. Bennett, P. B. Dunham, G. D. Pappas, *J. Gen. Physiol.* **50**, 1094 (1967).
5. Y. Kanno and W. R. Loewenstein, *Nature* **212**, 629 (1966); G. D. Pappas and M. V. L. Bennett, *N.Y. Acad. Sci.* **137**, 495 (1966).
6. M. V. L. Bennett and A. A. Auerbach, *Anat. Rec.* **163**, 152 (1969).
7. E. A. Kravitz, A. O. W. Stretton, J. Alvarez, E. J. Furshpan, *Fed. Proc.* **27**, 749 (1968); A. O. W. Stretton and E. A. Kravitz, *Science* **162**, 132 (196→ M. Remler, A. Selverston, D. Kennedy, *ibid.*, p. 281.
8. K. Hama, *Anat. Rec.* **141**, 275 (1961).
9. G. D. Pappas, Y. Asada, M. V. L. Bennett, *ibid.* **157**, 297 (1967); Y. Asada, G. D. Pappas, M. V. L. Bennett, *Fed. Proc.* **26**, 330 (1967); ———, in preparation.
10. A. Watanabe and H. Grundfest, *J. Gen. Physiol.* **45**, 267 (1961).
11. M. Remler, A. Selverston, and D. Kennedy (*7*) failed to observe passage of Procion Yellow across the septum of the crayfish septate axon except in a few instances attributable to rupture of the septum. Since they did not monitor impulse conduction and used a fairly large electrode for injection by means of pressure, we anticipate that they had injured their axons, which is known to cause separation of the septal junctions (*9*).
12. In contrast to the crayfish axon, Procion Yellow does enter some other cells. It entered the intrafusal muscle fibers of frog in the region of the sensory endings, although it did not enter undamaged extrafusal muscle fibers. In desheathed frog sciatic nerve, myelin and Schwann cell cytoplasm were stained although no axoplasmic staining was observed. In both tissues, results consistent with a reversible local anesthetic action of the dye were found. Some of the staining results observed in these and other tissues also suggest that Procion Yellow binds to cell membranes.
→ J. P. Revel and M. J. Karnovsky, *J. Cell Biol.* **33**, C7 (19(→ M. W. Brightman and T. S. Reese, *ibid.* **40**, 648 (1969).
→ J. D. Robertson, *ibid.* **19**, 201 (1963).
15. ———, in *Nerve as a Tissue*, K. Rodahl, Ed. (Harper & Row, New York, 1966).
16. It has now been found that the septum is permeable for intercytoplasmic passage of sucrose. The surface membrane is, as in most cells, relatively impermeable to sucrose (M. V. L. Bennett and P. B. Dunham, unpublished results).
17. We thank S. B. Waxman for assistance with some of these experiments and Dr. P. G. Model for helpful discussion. Supported in part by PHS grant 5 P01 NB 07512 and NSF grant GB-6880.
* Present address: Faculty of Medicine, Memorial University, St. John's, Newfoundland, Canada.

23 July 1969; revised 27 October 1969

Hemoglobin as a Tracer in Hemodynamic Pulmonary Edema

Abstract. *Stroma-free hemoglobin is an electron-opaque molecule useful as a tracer for the ultrastructural study of pulmonary capillary permeability. After this tracer was infused into the isolated pulmonary lobe of the dog, the endothelial junctions of the capillaries, as revealed by electron microscopy, act like distensible pores, thus allowing the tracer to escape when the pulmonary artery pressure was raised above 50 millimeters of mercury.*

Shortly after it is injected intravenously into the mouse, horseradish peroxidase (molecular weight, 40,000) passes through the clefts between endothelial cells of lung capillaries, traverses potential sites of obstructions within these clefts (cell junctions) (*1*), and enters the pericapillary interstitial space (*2*). It has been proposed that the junctions within the clefts between the capillary endothelial cells correspond to the "small pore system" and the plasmalemmal vesicles to the "large pore system" referred to by physiologists (*2, 3*).

However, there is no consensus concerning the proposed role for the junctions. Indeed, it has been claimed that most of the junctions are sealed by fusion of their walls and that only few form open channels, approximately 50 Å in diameter (*4*). Accordingly, plasmalemmal vesicles have been proposed (*4*) as the anatomic counterpart of both the small (*5, 6*) and large (*7, 8*) pore systems of the physiologists.

Since it is known that, in pulmonary edema, proteins of greater molecular weight than that of horseradish peroxidase do pass from the vascular lumen to the interstitial space (*9*), we undertook to examine the ultrasonic basis for this increased permeability of the alveolo-capillary membrane in experimental pulmonary edema. Using stroma-free hemoglobin (molecular weight, 64,500) as a tracer molecule, we found that high luminal pressures seem to widen the calibers of the interendothelial junctions in pulmonary capillary walls and allow the passage of hemoglobin from the capillary lumen into the interstitial tissue.

Ten mongrel dogs, weighing 12 to 15 kg, were anesthetized with pentobarbital

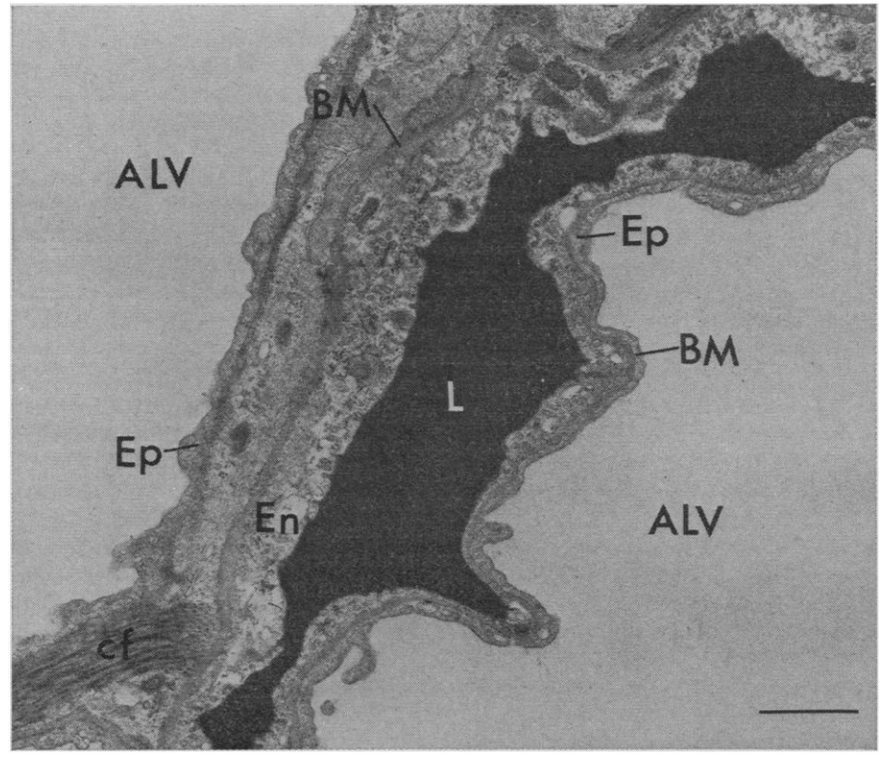

Fig. 1. Dog lung perfused with hemoglobin at normal pulmonary arterial pressure for 20 minutes. In the peroxidase-reacted sections, hemoglobin appears as an electron-opaque reaction product which uniformly fills the vascular lumen (*L*); *En*, endothelium; *Ep*, epithelium; *BM*, basement membrane; *ALV*, alveolus; *cf*, collagen fibers. Section reacted with peroxidase, stained with uranyl acetate and lead citrate. The bar at the lower right corner indicates 1 μm.