

JUNCTIONS BETWEEN INTIMATELY APPOSED CELL MEMBRANES IN THE VERTEBRATE BRAIN

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ABSTRACT

Certain junctions between ependymal cells, between astrocytes, and between some electrically coupled neurons have heretofore been regarded as tight, pentalaminar occlusions of the intercellular cleft. These junctions are now redefined in terms of their configuration after treatment of brain tissue in uranyl acetate before dehydration. Instead of a median dense lamina, they are bisected by a median gap 20–30 Å wide which is continuous with the rest of the interspace. The patency of these “gap junctions” is further demonstrated by the penetration of horseradish peroxidase or lanthanum into the median gap, the latter tracer delineating there a polygonal substructure. However, either tracer can circumvent gap junctions because they are plaque-shaped rather than complete, circumferential belts. Tight junctions, which retain a pentalaminar appearance after uranyl acetate block treatment, are restricted primarily to the endothelium of parenchymal capillaries and the epithelium of the choroid plexus. They form rows of extensive, overlapping occlusions of the interspace and are neither circumvented nor penetrated by peroxidase and lanthanum. These junctions are morphologically distinguishable from the “labile” pentalaminar appositions which appear or disappear according to the preparative method and which do not interfere with the intercellular movement of tracers. Therefore, the interspaces of the brain are generally patent, allowing intercellular movement of colloidal materials. Endothelial and epithelial tight junctions occlude the interspaces between blood and parenchyma or cerebral ventricles, thereby constituting a structural basis for the blood-brain and blood-cerebrospinal fluid barriers.

INTRODUCTION

Within the vertebrate central nervous system, examined by electron microscopy, the neuronal and glial cell membranes are generally separated by a distance of 100 Å or more. However, at certain places the outer leaflets of contiguous cell membranes come into close apposition (23) and these appositions have come to be known as tight junctions, external compound membranes, quintuple-layered units, or *zonulae occludentes* (5, 42, 45, 53). Such junctions are situated between the endothelial cells of capillaries (42, 48, 54), between

certain neuronal processes (55), between astrocytes (45), between the epithelial cells of the ependyma (5, 49) and choroid plexus (2, 8), and between adjacent myelin lamellae (53). Although these junctions were known to differ in that some are plaque-shaped and of limited extent while others are continuous belts between cells, they have been regarded as having a general structural plan consisting of five layers: two cytoplasmic dense leaflets and two lucent leaflets on either side of a single median dense leaflet representing

intimate apposition if not fusion between the outer leaflets of adjacent cell membranes (17, 32). This proposed unity in structure of tight junctions within the brain and elsewhere was in marked contrast to the multiplicity of functions ascribed to them. For example, they are purported to act as a barrier to the passage of material through the clefts between cells (17, 18), as a bond holding cells together (18, 58), and as the site at which substances move from one cell to another (33).

Another type of junction between adjacent cell membranes is a "close apposition" (12, 24, 50, 57, 63), which has a seven-layered rather than a five-layered appearance because it is bisected by a narrow lucent gap. With new electron microscopical techniques (51), certain tight junctions in organs other than the vertebrate brain have been demonstrated to be actually close appositions characterized by a lattice of polygonal subunits within a central lucent gap 20–30 Å wide. These close appositions have been classified as "gap junctions" (51, 52). The morphologist is now confronted with such problems as determining whether structural variation in junctions underlies functional differences. Also important, particularly within the nervous system, is whether a particular structural variation is representative of the living condition or of the vagaries in preparative techniques. For instance, intermembranous relationships in the brain may range from ubiquitous pentalamellar appositions (29) to an occasional interglial junction (45), depending on the choice of preparative methods (28). Under these circumstances, the reality of a junctional structure is at least probable if it persists unchanged in tissues fixed with different agents and subsequently prepared in different ways. Furthermore, the tightness of a junctional structure may be explored by exposing it before fixation to proteins which can subsequently be localized with the electron microscope. The following account uses this approach to reexamine, with a variety of electron microscopical techniques, a hopefully representative sample of the intercellular junctions in the vertebrate brain that have heretofore been regarded as tight.

The conclusion of this study is that some "tight" junctions in the vertebrate nervous system are actually seven-layered gap junctions. These configurations, not tight junctions, are found between astrocytes, ependymal cells, and neuronal processes at some electrical synapses. Evidence will be presented that the gap junction in brain serves primarily for adhesion between cells and possibly

for communication of ions from one cell to another. True tight junctions are found only between endothelial and certain epithelial cells, within myelin, and, in a few instances, between neuronal processes. Those found at epithelia and endothelia impede extracellular movement of proteins thereby exerting a profound influence on the passage of colloidal and perhaps smaller substances between blood and brain or cerebrospinal fluid.

The major findings presented here have been previously presented in preliminary form (8, 9). Fig. 41 is a schematic conception of junctions and spaces in the vertebrate brain based on these studies.

MATERIALS AND METHODS

Adult white mice, goldfish approximately 10 in length, and adult domestic chickens or chicks 7 (25) and 25 days after hatching were subjected to variations in fixation and tissue preparation for electron microscopy. In some specimens, horseradish peroxidase or lanthanum hydroxide was introduced into the systemic blood or cerebrospinal fluid. The full range of techniques was employed only in the mouse.

Most of the mice and all of the chickens and fish were fixed by perfusion through the heart. Mice were anesthetized by injecting intraperitoneally 1 g per kilogram body weight of tribromoethanol dissolved in tertiary amyl alcohol and diluted in water. For chickens, pentobarbital was given intravenously and for fish, tricaine methanesulfonate (40 mg %) was added to the ambient water. The aldehyde fixative was a mixture of formaldehyde and glutaraldehyde in cacodylate buffer used as previously described (48) except that the glutaraldehyde was further purified by fractional vacuum distillation. The fraction we selected had negligible absorbance at 235 m μ (16). A dilute initial fixative, which was perfused without any previous washing out of blood, contained glutaraldehyde 1.25%, formaldehyde 1.0% (made from paraformaldehyde, Matheson, Coleman, and Bell, Cincinnati, O.), sodium cacodylate 0.08 M, and calcium chloride 10⁻³ M. The initial fixation lasted approximately 5 min and was followed by perfusion, for approximately 2 min, of a concentrated fixative containing glutaraldehyde 5.0% and formaldehyde 4.0% (30). These fixatives were used at pH 7.1–7.2 and were found to work well, unaltered, for the chicken and the fish.

In some mice, 0.3–4.0% sodium chloride was added to the aldehyde fixatives, or phosphate-buffered formaldehyde was used alone in an attempt to manipulate the size of extracellular space in the brain. Some animals receiving intraventricular injections of peroxidase were fixed by perfusing the cerebral ventricles with 3% glutaraldehyde in sodium cacodylate buffer,

although the concentrated fixative gave better results. All aldehyde-fixed tissues were postfixed in cacodylate- or phosphate-buffered 2% osmium tetroxide. Fixation in mice by perfusion of sodium cacodylate- or phosphate-buffered osmium tetroxide was accomplished by procedures previously used in this laboratory (37). Fixation with potassium permanganate was by perfusion of the cerebral ventricles with 3% potassium permanganate in 0.15 M phosphate buffer.

To use lanthanum as an electron microscopic tracer (51), a 2% solution of lanthanum nitrate was adjusted to pH 7.7 and added to an equal volume of aldehyde fixative at pH 7.1 which was prepared so that the final concentrations of aldehydes and buffer were equal to those ordinarily used. The dilute and concentrated fixatives containing lanthanum were either perfused through the heart after washing out the circulating blood or perfused through the cerebral ventricles. The tissue was subsequently postfixed in 1.25% osmium tetroxide dissolved in sodium cacodylate buffer containing 1% lanthanum. For ventricular perfusion, an indwelling catheter, 2.5 mm long with an outside diameter of 0.3 mm, was placed through a burr hole situated 1.5 mm anterior to and 1.0-1.5 mm lateral to the bregma of mice weighing 20 g. The tip of the catheter thus entered a normally distended portion of the lateral ventricle. Fluid was pumped at 0.5 ml per hour through the ventricular system and, after entering the cisterna magna of the subarachnoid space, was permitted to escape through an incision in the atlantooccipital membrane. In other mice, the subarachnoid space was directly perfused in a similar manner by inserting a cannula over the frontal lobe and allowing fluid to escape through an incision in the atlantooccipital membrane.

For introducing peroxidase into the brain, 5-10 mg of type VI horseradish peroxidase (Sigma Chemical Co., St. Louis) was injected into the tail veins of unanesthetized mice up to 1/2 hr before fixation, or 0.5-3.0 mg of peroxidase dissolved in Elliot's solution (Baxter Laboratories, Morton Grove, Ill.) was perfused through the ventricles for 5-90 min before fixation. Fixed tissue was immersed in 15% sucrose, frozen, and sectioned at approximately 40 μ within a cryostat, or unfrozen, fixed tissue was sectioned with a tissue sectioner (60); incubation of the sections and subsequent tissue processing has been previously described (22, 48). When control blocks from brains not exposed to peroxidase were incubated in the appropriate substrate, or when areas of the brain to which peroxidase did not spread were examined, no extracellular peroxidase localization was evident.

With the exception of the experiments using lanthanum or potassium permanganate, the fixed blocks were usually treated with 0.5 or 2.0% solutions of uranyl acetate in sodium maleate at approximately 2°C (31), the pH being varied from 3.3 to 5.0. Some blocks were treated in permanganate after dehydra-

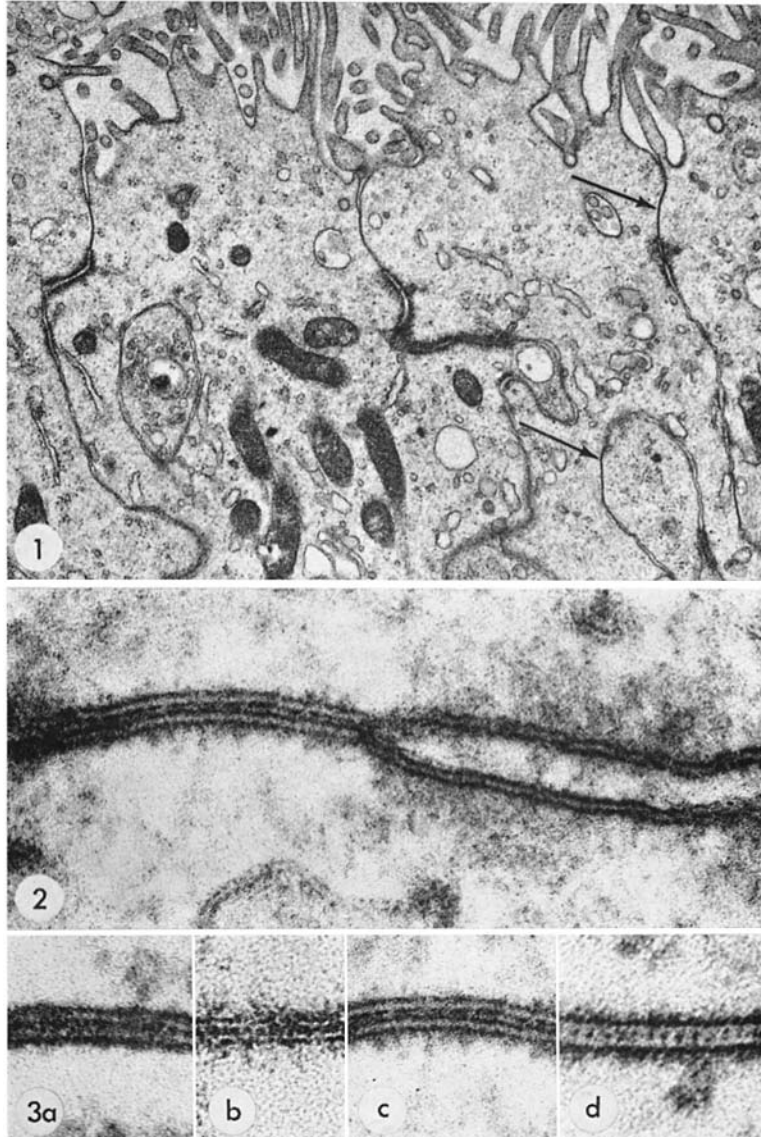
tion (44). Dehydration was in four 10-min steps in methanol or sometimes acetone, and embedding was in Araldite (Ciba Co., Oxford, England). Grids were stained with lead citrate or uranyl acetate and examined in an AEI-6B electron microscope.

OBSERVATIONS

We considered only those junctions in which adjacent cell membranes approached to within 30 A or less. Junctions of this type fell into three general classes: gap junctions, tight junctions, and labile appositions between cell membranes. In this respect, junctions within the central nervous system resemble junctions between cells lying outside of the nervous system (17, 41, 51). Because of certain structural differences interneuronal gap junctions are considered separately from interependymal and interglial gap junctions.

Interependymal and Interglial Gap Junctions

The gap junctions between ependymal cells and between astrocytes were structurally equivalent, and so they will be described together. When uranyl acetate treatment followed fixation either in osmium tetroxide or in aldehydes and osmium tetroxide, the gap junctions were immediately recognizable. They were found at the characteristic locations where tight junctions had been described previously (5, 45): at or near the apical ends and periodically along the lateral aspect of adjacent ependymal cells (Figs. 1-3, 10) and between astrocytes (Figs. 4-7, 11, 14), particularly those around blood vessels and at the surface of the brain. A general feature of the gap junctions in all of these locations was that they did not encircle cells. This point has already been recognized for ependymal (5, 6) and perivascular astrocytes (48) and, in this study, is illustrated for the marginal layer of astrocytic processes comprising the surface of the cerebellum and cerebral cortex (Figs. 12, 13). At gap junctions, the usual interspace of 100-200 A was abruptly narrowed to a constant width of 20-30 A (Figs. 2, 7). All seven component layers of the junctions were exactly coextensive and had a straight or cursive smoothly undulating contour. Like the rest of the plasmalemma, the cell membrane within gap junctions was approximately symmetrical although the outer leaflets within the gap junctions were perhaps narrower than the inner leaflets. These morphological features were identical in the gap junctions between astrocytes (Figs. 4 and 7) and ependymal cells of the goldfish, chicken, and mouse (Figs. 2 and 3 c).



Unless otherwise stated, all illustrations are of tissue treated with uranyl acetate before dehydration and stained on the grid with lead citrate.

FIGURE 1 The internal surface of the brain is formed by the folds of ependymal plasma-lemma projecting into the cerebral ventricle (top). The four contiguous ependymal cells are joined, at their apices and below, by junctions heretofore interpreted as "tight" (arrows). OsO_4 . Mouse. $\times 16,000$.

FIGURE 2 In tissue treated with uranyl acetate before dehydration such "tight" junctions appear as seven-layered gap junctions bisected by a median slit of constant width (left) that is continuous with the remaining interspace (right). OsO_4 . Mouse. $\times 310,000$.

FIGURE 3 A comparison in appearance of apical ependymal gap junctions after various preparative methods. **3 a**, Initial fixation in OsO_4 followed by immersion in potassium permanganate after dehydration. (No uranyl acetate treatment.) **3 b**, Potassium permanganate fixation alone. (No lead staining of sections.) **3 c**, Initial fixation in OsO_4 followed by immersion in uranyl acetate before dehydration. **3 d**, Initial fixation in OsO_4 . Lead and uranyl acetate staining of sections. (No uranyl acetate treatment.) Mouse. $\times 310,000$.

The over-all width of these junctions, that is, the distance between the cytoplasmic faces of adjacent membranes, averaged 135–150 Å.

When embedded sections rather than unembedded blocks were treated with uranyl acetate, the seven-layered gap junctions appeared five layered; instead of a median gap, a median lamina bisected the junction (Fig. 3 *d*). In some planes of section, this lamina appeared as a row of globules regularly spaced about 70 Å apart and about 40 Å in diameter (1). In these specimens, cell membranes adjacent to the junctions were asymmetric, the outer leaflets being thinner and less dense than the inner, and the cytoplasmic fuzz subjacent to the gap junctions was intensely stained. With either method of staining, the width of these junctions was 135–150 Å. The block treatment emphasized the outer leaflet of the cell membranes, while staining of sections rendered dense the gap between the cell membranes. The gap junctions, then, appear to consist of closely apposed cell membranes separated by a stainable material.

After fixation in potassium permanganate, which usually showed membranes rather poorly, the gap junctions again appeared five layered with a wide median lamina about the width of the gap in other specimens (Figs. 3 *b* and 6). However, the total width of the junction at many places was somewhat less than the total width of the gap junctions in brain fixed in aldehyde or osmium tetroxide, although the width of adjacent unit membranes was unchanged and the subjacent cytoplasmic fuzz or web was still apparent (Fig. 6).¹ A similar five-layered appearance without an over-all narrowing of the junction is obtained

¹The difference previously reported (10) was intended to be 30 Å rather than 50 Å.

after aldehyde fixation and block treatment in potassium permanganate, suggesting an affinity of the stainable material within the gap for permanganate (13) (Fig. 3 *a*).

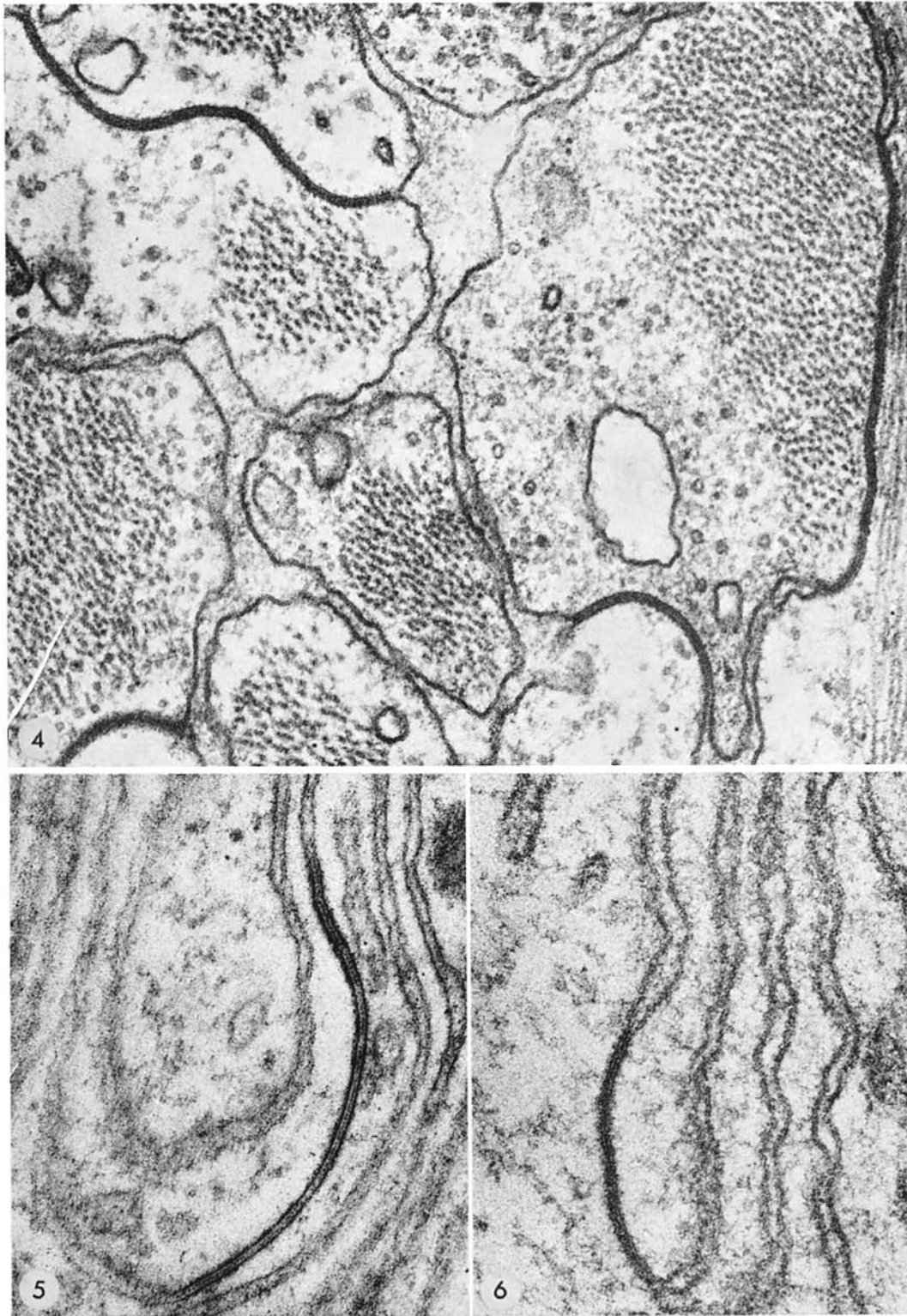
The use of lanthanum provided a means of outlining the substructure of the stainable material within the gap. After aldehyde fixative containing lanthanum had been introduced into the cerebral ventricles, an extremely dense mass occupied the clefts between adjacent ependymal cells and, in certain regions, the intercellular spaces of the subependymal neuropil. Within gap junctions sectioned normally, the median gap, owing to its lanthanum content, appeared as a black line about 40 Å wide (Fig. 8). This increased width of the median gap may be due to staining or infiltration of the outer leaflets (51). The lanthanum within clefts between cells appeared in sharp visual contrast to the cytoplasm of the cells. When viewed *en face*, the gap appeared to be occupied by an array of polygonal subunits, each outlined faintly by a thin rim of lanthanum approximately 20 Å wide (Figs. 8, 9). The period from center to center of each subunit was variable, averaging about 100 Å. Unlike the subunits of gap junctions in other organs or in gap junctions between neuronal processes, no perceptible density marked the center of the units, their size and shape were not as regular, and it could not be determined whether they were hexagonal.

The distribution of peroxidase provided a demonstration that the gap was continuous with the rest of the interstitial space prior to fixation. Following perfusion of peroxidase through the cerebral ventricles, the interspaces between ependymal, glial, and neuronal cells throughout the adjacent neuropil contained peroxidase reaction

FIGURE 4 Astroglial processes are characteristically joined by gap junctions, the median slits of which communicate with surrounding interspaces. This field is near the synaptic bed of a Mauthner cell where the interspaces contain a moderately dense "filler." Aldehydes. Goldfish. $\times 90,000$.

FIGURE 5 Adjacent astrocytic processes connected by a gap junction. Preparative techniques make this gap junction appear pentalaminar, resembling the ependymal junction in Fig. 3 *d*. The width, regular contour, and density of the component leaflets distinguish these junctions from the adjacent areas of parallel membrane apposition. OsO_4 . Mouse. $\times 110,000$.

FIGURE 6 Appearance of a similar field of astrocytic processes after fixation in potassium permanganate and no staining on the grid. This gap junction again appears pentalaminar resembling the ependymal junction in Fig. 3 *b*. Mouse. $\times 115,000$.



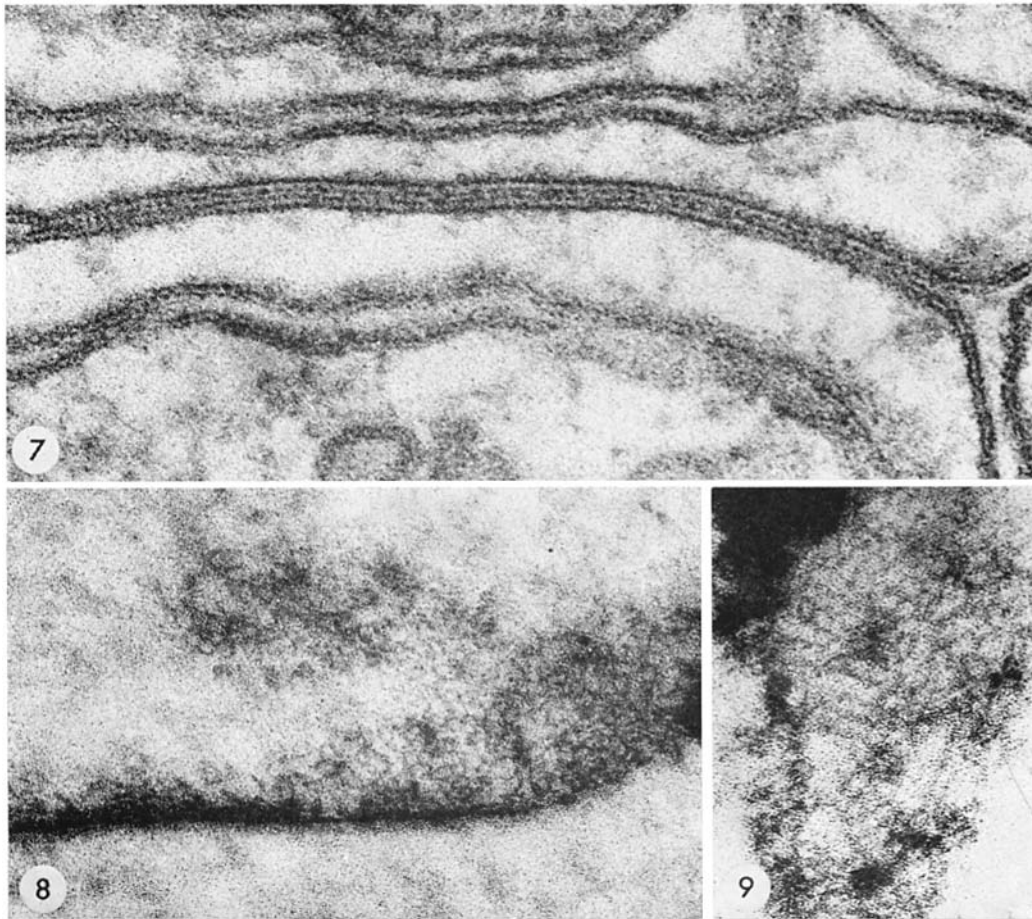


FIGURE 7 Adjacent astrocytic processes connected by a gap junction. The striations at the left end of the junction are probably due to structures crossing the gap which are seen only in particular angles of section. OsO₄. Mouse. $\times 315,000$.

FIGURE 8 Gap junctions between ependymal cells after intraventricular perfusion of lanthanum hydroxide showing penetration of lanthanum into the gap. From left to right, the junction is rotating into a tangential plane revealing, within the gap, a grid of irregular polygonal subunits. Aldehydes. Mouse. $\times 215,000$.

FIGURE 9 Similar grid of polygonal subunits from a gap junction between ependymal cells, cut completely *en face*. The average center to center period of these units is 90–100 Å. Aldehydes. Mouse. $\times 315,000$.

product (Fig. 39) as did both ends or the entire length of the median slit in gap junctions (Fig. 10–12, 14). This apparent entry of peroxidase into the median slit did not alter the total width of the gap junction. The absence of any extracellular reaction product, when injection of peroxidase was omitted, suggests that the deposition of reaction product is specifically due to the presence of exogenous peroxidase although the possibility that

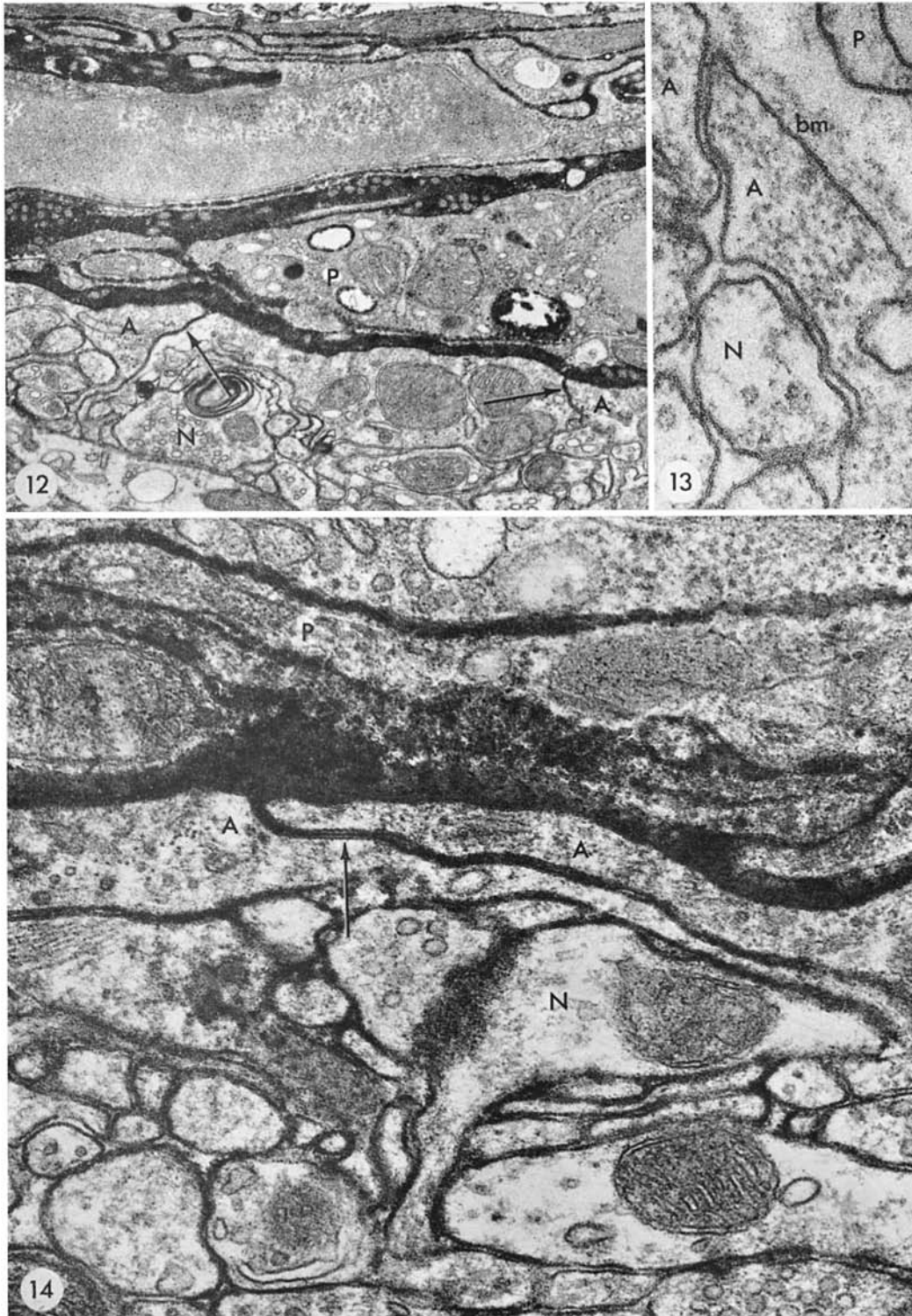
enzyme or reaction product diffused into the gaps during or after fixation has not been completely excluded.

The most superficial layers of the brain consisted of a network of interdigitating sheetlike astrocytic processes limited by a basement membrane. Lying next to this basement membrane were the flat mesothelial cells of the pia (Fig. 13). Adjacent mesothelial cells were connected by gap junctions



FIGURE 10 The patency of the median slit bisecting ependymal gap junctions is demonstrated by the localization within these slits (arrows) of peroxidase that had been perfused through the cerebral ventricles (*V*). Mouse. $\times 80,000$.

FIGURE 11 Peroxidase localized within interglial gap junctions after intraventricular perfusion. Reaction product surrounds most neuronal processes (top), fills the median slit of a gap junction between perivascular astrocytic processes (*A*), and permeates the basement membrane around a capillary whose lumen appears at bottom. Mouse. $\times 95,000$.



FIGURES 12-14 External surface of the brain after subarachnoid perfusion of peroxidase. The external surface of the brain is delimited by marginal astrocytic processes (*A*) which are either separated by open slits (Fig. 12, arrows; Fig. 13, without peroxidase) or joined by gap junctions (Fig. 14, arrow) and capped by a basement membrane (*bm*, Fig. 13). Peroxidase passes from the subarachnoid spaces to the parenchyma of the brain by going through open spaces between leptomeningeal cells (*P*), crossing the astrocytic basement membrane, and going through the open slits (Fig. 12, arrows) or gap junctions (Fig. 14, arrow) between astrocytes (*A*) to penetrate the interspaces of the neuropil (*N*). Mouse. Fig. 12, $\times 24,000$; Fig. 13, OsO_4 , $\times 120,000$; Fig. 14, $\times 70,000$.

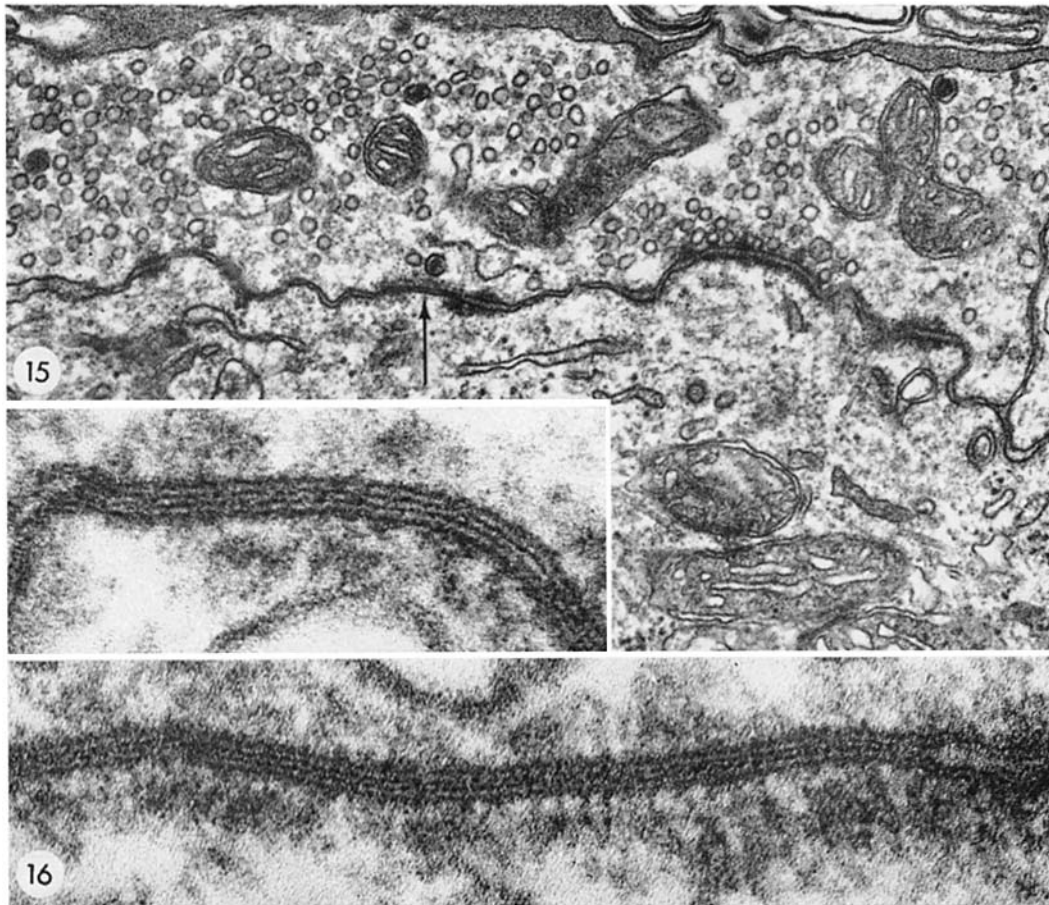
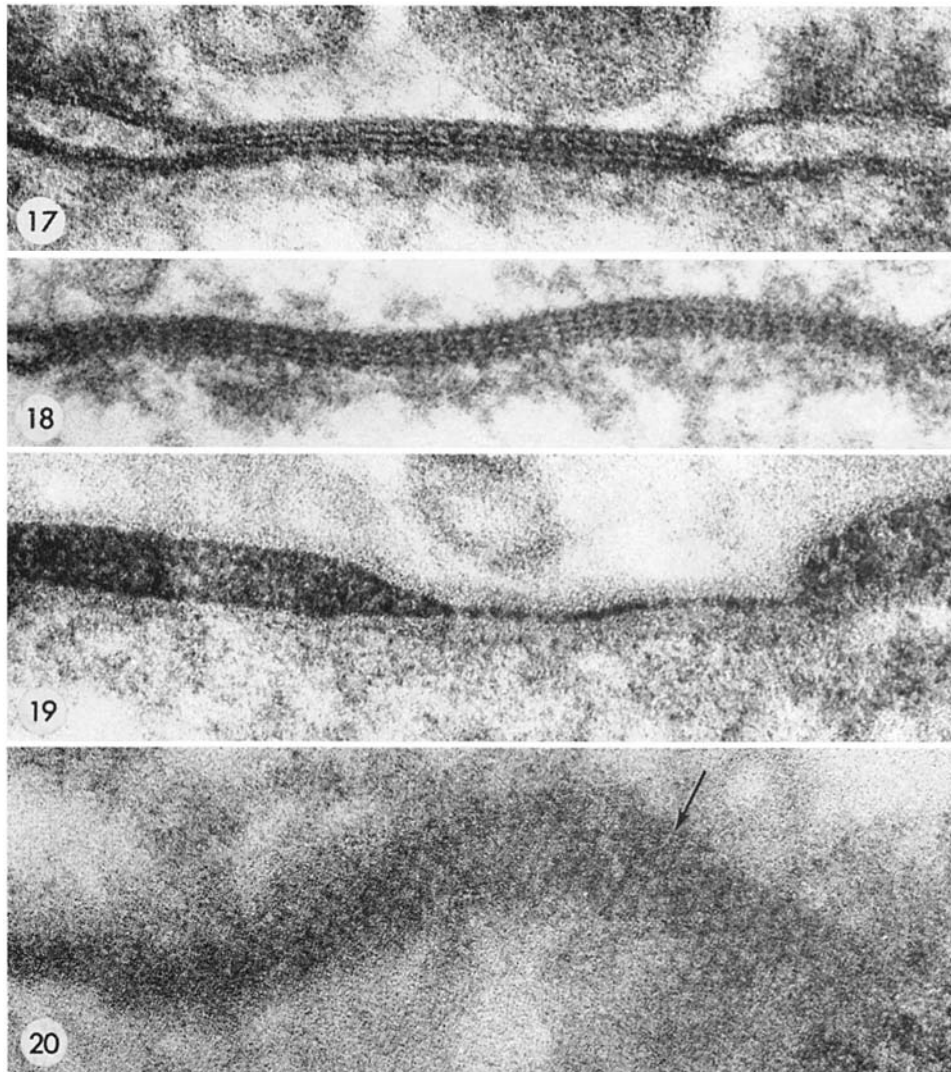


FIGURE 15 Between the vesicle-laden, presynaptic calyx (above) and a neuronal cell body (below), the typical neurohumoral type of synaptic contacts alternate with desmosome-like junctions and gap junctions (arrow). The interneuronal gap junctions (inset) are bisected by a median slit with a constant width of about 20 Å. Ciliary ganglion of a chick. Aldehydes. $\times 45,000$. Inset, $\times 340,000$.

FIGURE 16 The presynaptic (above) and postsynaptic (below) membrane of certain synapses on cell bodies in the tangential nucleus are joined by gap junctions, the median slit of which communicates with the rest of the interspace, as on the right. Note the periodicity in the cytoplasmic fuzz. Chicken. Aldehydes. $\times 280,000$.

as were underlying astrocytic processes. The intercellular relationships of the limiting glial sheath of the brain were thus the same as those of the astrocytic processes constituting the perivascular sheaths. In both instances, the underlying extracellular clefts of the parenchyma were in communication with the overlying basement membrane, either directly through open, relatively wide clefts or by way of the narrow slits in gap junctions (Figs. 12–14). Peroxidase, perfused through the subarachnoid space, was able to reach

the interstitial spaces of the brain parenchyma by passing through the clefts and through or around the gap junctions between the superficial glial processes (Figs. 12, 14). Usually the penetration of peroxidase was not so deep as after ventricular perfusion. A possible interpretation of this finding is that channels crossing the surface of the brain are less frequent than those crossing the ependyma, although other possibilities, such as a difference between the pressures used for ventricular and subarachnoid perfusions, have not been ruled out.



FIGURES 17-20 Gap junctions between club endings and the lateral dendrite of Mauthner's cell in the goldfish. Aldehydes.

FIGURE 17 In this plane of section, the median slit, continuous with the rest of the interspace (at either end), is mured by outer leaflets scalloped by periodic, shallow indentations. $\times 340,000$.

FIGURE 18 In another plane of section, regularly spaced patches of increased density in one outer leaflet are in register with those in the opposite outer leaflet. $\times 285,000$.

FIGURE 19 Lanthanum hydroxide, injected intraventricularly, enters the intercellular spaces at either end of a gap junction and appears, in transverse section, to be periodically interrupted within the median gap. $\times 340,000$.

FIGURE 20 Cut *en face*, the lanthanum deposit within the gap appears as an ordered lattice outlining closely packed, polygonal subunits which are more ordered than those in ependymal and glial gap junctions. (Compare with Fig. 9.) The center to center period is about 90 A. Arrow indicates a dense core within lucent subunit. $\times 340,000$.

Appreciable amounts of peroxidase were incorporated into cytoplasmic vacuoles of some pial cells (Fig. 12).

Interneuronal Gap Junctions

The three interneuronal gap junctions considered in this study had previously been considered to be tight junctions (15, 26, 56) or close approximations (63), but are shown here to be typical gap junctions. These interneuronal gap junctions are found at the club endings on the lateral dendrite of the Mauthner cell in the goldfish (Figs. 17–20), at the afferent endings in the chicken tangential nucleus (Fig. 16), and at the calyceal endings on cell bodies of the ciliary ganglion in the chicken (Fig. 15). After fixation with aldehydes and osmium tetroxide followed by uranyl acetate block treatment, gap junctions but no tight junctions were found between the neuronal processes at these locations. These gap junctions were characterized by structural features common to nonneuronal gap junctions, but the over-all width of the neuronal junctions was somewhat narrower, averaging 130–140 Å.

Gap junctions between axonal endings and the lateral dendrite of the Mauthner cell corresponded in extent and location to the tight junctions or “synaptic disks” which have been thought to mediate electrical transmission at these endings (55, 56). However, as in the ependyma, their reported over-all width after primary osmium tetroxide or formalin fixation is frequently greater than the over-all width after permanganate fixation (56). Our measurements of this junction fixed with aldehydes agreed with the figure previously published for primary formalin fixation (56). When peroxidase was perfused through the cerebral ventricles of the goldfish in order to demonstrate entry into the neuronal gaps, a dense substance was consistently present within the gap. However, the gap material was also somewhat darkened by diaminobenzidine in the absence of peroxidase so that differentiation from gaps filled with reaction product was difficult.

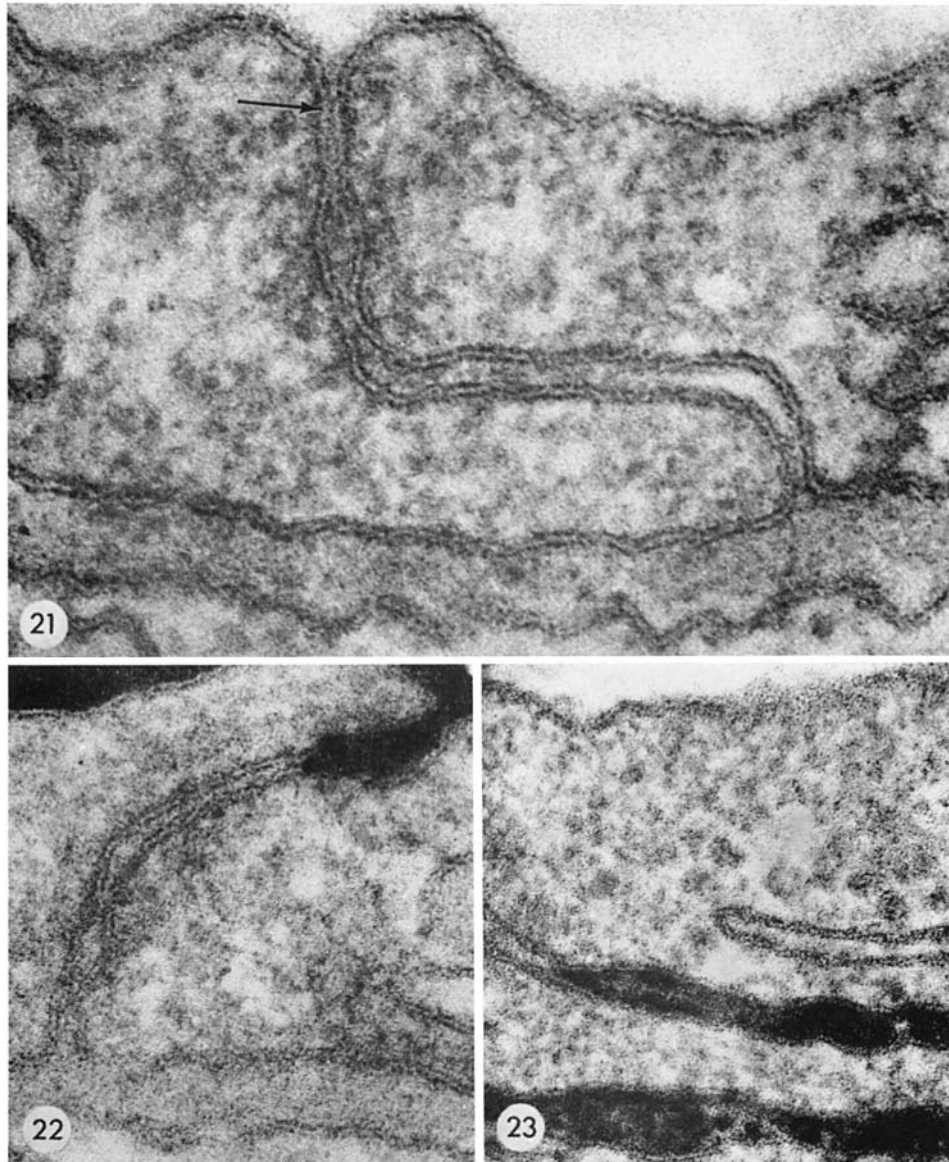
A prominent structural feature of the synaptic disk in some planes of section was a series of parallel dense lines about 100 Å apart and running perpendicular to the long axis of the gap junction across both of its component cell membranes and the median slit (Fig. 18). Lanthanum, introduced by immersing fixed slices in a mixture of strong aldehyde fixative and lanthanum hydroxide solu-

tion, filled the intercellular spaces including the narrow gap of the synaptic disk (Figs. 19, 20). In most planes of section, this dense bar of lanthanum was crossed by lighter lines, presumably corresponding to the dense parallel lines positively stained in other preparations (Fig. 19). These light bands were also spaced at approximately 100-Å intervals (cf. Fig. 3 *d*) and, when the lanthanum bar was cut *en face*, the lanthanum defined a regular, honeycomb-shaped structure (Fig. 20) identical to that previously described after permanganate fixation of this junction (56) and after lanthanum infiltration of the gap junctions between heart muscle cells (51).

Tight Junctions

Within the mouse brain, treated before dehydration with uranyl acetate, tight pentalaminar junctions were observed consistently in only a few locations: between the endothelial cells of parenchymal blood vessels (Figs. 21–23), between epithelial cells of the choroid plexus (Figs. 24–27), and between ependymal cells overlying the median eminence and area postrema (49). The only site where tight junctions connected neuronal cells with nonneuronal cells was outside the central nervous system, in the olfactory epithelium of the frog (Fig. 31). Junctions included in this group, because they consist of closely approximated cell membranes, were those between neuronal cell bodies of the mesencephalic nucleus of the trigeminal nerve in mice (Fig. 28) and between adjacent lamellae of myelin in mice and fish (Figs. 29, 30). Except for the last two examples, which are discussed separately, the structure of tight junctions in different locations was remarkably uniform.

In locations such as those near luminal surfaces, the outer leaflets of contiguous cell membranes came together at several discrete points to form a median lamina no denser or wider than a single outer leaflet of a single cell membrane (Figs. 21, 24). The over-all width of these junctions was, therefore, less than would be expected at a simple apposition of two cell membranes (48). In cross-section, they appeared as a series of punctate membrane fusions which periodically obliterated the interspace along the apical-to-basal axis of the intercellular apposition (Figs. 21, 22, 24). One or more such punctate fusions could be found at every level of cross-section of appositions between endothelial cells, suggesting that the fusions are of

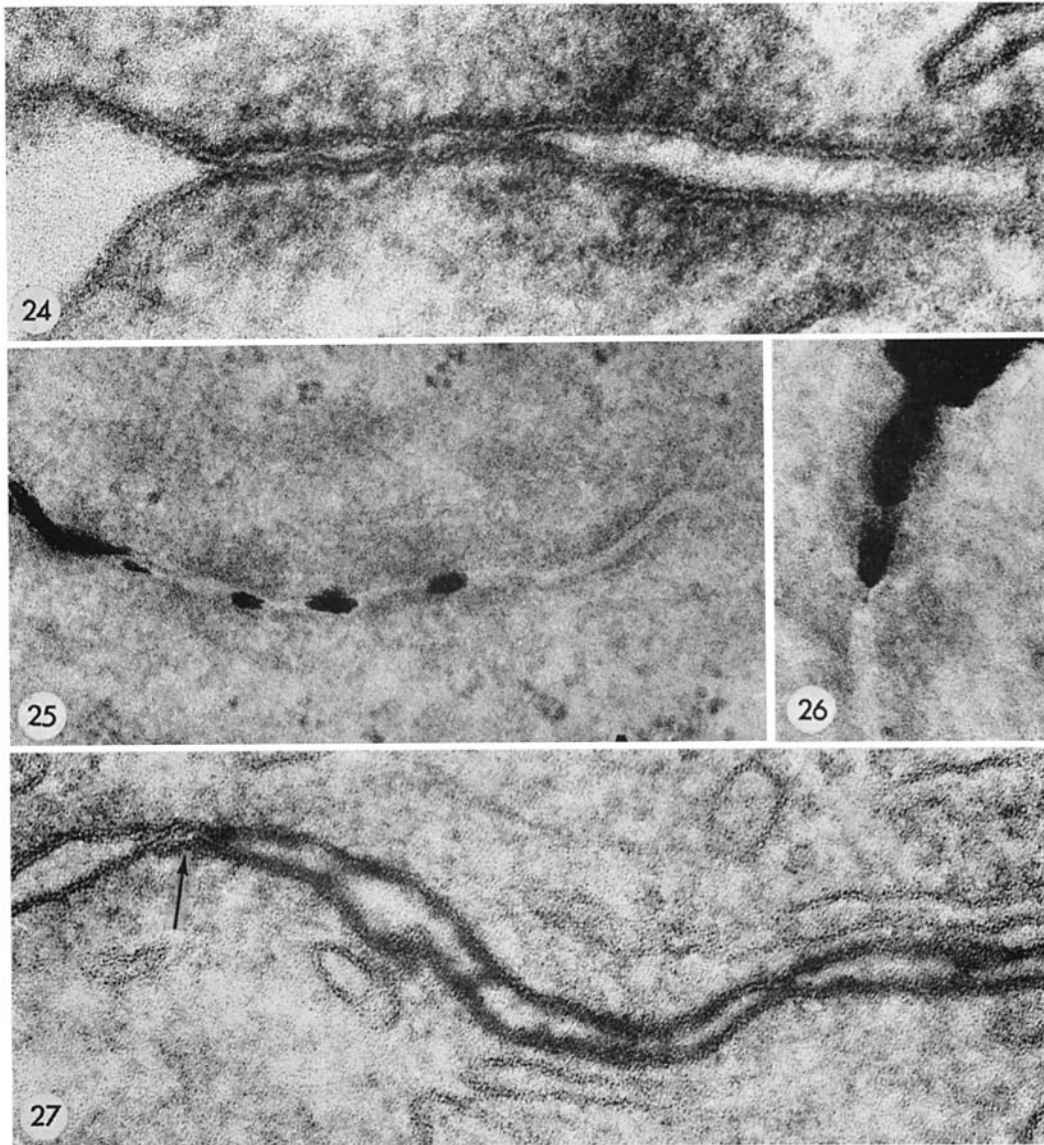


FIGURES 21-23 Tight junctions between endothelial cells in the cerebral capillaries of the mouse. The vessel lumen is at the top in each figure. Aldehydes.

FIGURE 21 A series of three punctate, pentalaminar junctions delimit two compartments within the inter-endothelial cleft. The median lamina (second dense lamina to right of arrow) is narrower than would be expected if the two outer leaflets were merely apposed. The narrowness of this lamina suggests that the membranes are fused at these points. $\times 340,000$.

FIGURE 22 The passage of intravascularly injected lanthanum between endothelial cells is blocked by a tight junction so that the remainder of the cleft and basement membrane (bottom) remain free of deposit. $\times 250,000$.

FIGURE 23 Peroxidase, injected intraventricularly, has passed from the basement membrane (bottom) of a capillary into the cleft between the endothelial cells until stopped by a tight junction. This distribution is, therefore, the mirror image of that in Fig. 22. $\times 190,000$.



FIGURES 24-27 Tight junctions between choroid plexus epithelial cells in the mouse. Aldehydes.

FIGURE 24 A row of four punctate, pentalaminar junctions cut transversely separate the cerebral ventricle (left) from the epithelial interspace (right). Compare with Fig. 21. $\times 315,000$.

FIGURE 25 Lanthanum, injected intraventricularly, circumvents three tight junctions but is eventually stopped by a fourth. $\times 95,000$.

FIGURE 26 Intraventricularly injected lanthanum stops at the tight junction between epithelial cells. No lanthanum has penetrated deeper into the epithelial interspace at this point, suggesting that this junction extends for a long distance around these cells. $\times 190,000$.

FIGURE 27 Intravascular peroxidase has escaped from the choroidal vessels and moved toward the ventricle (left) by passing between epithelial cells until stopped by a tight junction (arrow). The reaction product, in this instance, lines the cleft rather than filling it (cf. Fig. 23). $\times 210,000$.

greater extent in their longitudinal dimension. In this respect, they resemble the *zonulae occludentes* which have been found in a variety of epithelia outside of the nervous system (17).

The use of peroxidase provided supportive evidence that the tight junctions extended around the circumference of the cells, sealing off the ventricular and vascular lumina from the underlying interspaces (Figs. 23, 27). For instance, intravenously administered peroxidase does not penetrate the tight junctions between endothelial cells of leptomeningeal or parenchymal blood vessels (48). In choroid plexus, where the protein could readily cross the fenestrated vascular endothelium, it did not pass any of the tight junctions between the overlying epithelial cells (Fig. 27). Conversely, when peroxidase was perfused through the cerebral ventricles, the same interendothelial and interepithelial tight junctions blocked its movement from cerebral ventricles toward parenchymal (Fig. 23) or choroidal blood.

The pericellular arrangement of the tight junctions was further elucidated by the use of lanthanum hydroxide which presumably also behaved as a tracer (Figs. 22, 25, 26) (51). In contrast to the gap junctions, the amorphous lanthanum deposit at tight junctions did not outline any pattern of subunits. After intravascular injection along with aldehyde fixative, lanthanum characteristically stopped at the apical or first tight junction between vascular endothelial cells (Fig. 22) but occasionally was found on both sides of the first or a subsequent tight junction, perhaps because the colloidal lanthanum is smaller than peroxidase. However, one of the junctions ulti-

mately prevented lanthanum from reaching the basement membrane. Conversely, when administered intraventricularly, lanthanum sometimes passed one or two tight junctions at the apical surfaces of the epithelial cells of the choroid plexus (Figs. 25, 26). In some instances, a narrow column of lanthanum corresponded in position to a tight junction (Fig. 26). This "trickle" of lanthanum suggested that some tight junctions were not fused but, instead, perforated by a narrow pore. However, it is emphasized that even where lanthanum had moved beyond two or more tight junctions, it never crossed the whole series of junctions. As with peroxidase, after either route of administration of lanthanum, tight junctions accounted for the known impermeability of an endothelium or epithelium.

Five-layered junctions were also found between cells of the mesencephalic nucleus of the trigeminal nerve (27) where contiguous cell bodies pressed close together at flat sides of mutual contact. Along these flat sides were desmosome-like specializations and a few five-layered junctions about 110 Å wide (Fig. 28). A cytoplasmic fuzz symmetrically coextensive with these neuronal-neuronal junctions set them apart and, since they occurred after primary osmium tetroxide fixation, there is reason to believe that they are distinct from the labile intermembranous appositions which are discussed below. The function of these interneuronal junctions is unknown.

Within the myelin sheath, the intraperiod lamella is formed by the union of the outer leaflets of contiguous processes belonging to oligodendrocytes (Figs. 29, 30). The resulting lamella was not

FIGURE 28 A pentalaminar tight junction connects the cell bodies of two neurons in the mesencephalic nucleus of the trigeminal nerve. A fuzz (*f*) is associated with the cytoplasmic aspects of these junctions. Mouse. OsO_4 . $\times 255,000$.

FIGURES 29 and 30 Longitudinal sections near the nodes in central myelin sheaths. The intraperiod line (short arrow) of myelin (Fig. 29, bottom left; Fig. 30, top arrow) appears to be "tight" in contrast to the usual junction between oligodendroglia processes outside of myelin (Fig. 29, long arrow; Fig. 30, bottom arrow) which appear to be a variety of gap junction. In Fig. 29, astrocytic processes (*A*) come close to but do not form a specialized contact with an axon (right) at a central node. Thus, a gap junction between two astrocytic processes, the more open type of junction between oligodendroglial processes (arrow), and the intraperiod line of myelin occur in the same field. Goldfish. Fig. 29, $\times 220,000$; Fig. 30, $\times 205,000$. Aldehydes.

FIGURE 31 A row of discrete pentalaminar tight junctions connecting an olfactory bipolar neuron with a supporting cell (nonneuronal). Part of a ciliary basal body at left is in a bipolar neuron of the olfactory epithelium. Frog. Aldehyde. $\times 255,000$.



split to form a gap. However, at the node, outside of the myelin sheath, adjacent outer leaflets of oligodendroglial processes were not united to form a single lamella but were instead separated by a narrow gap of 10–20 Å. Thus, two types of junctions are possible between oligodendroglial processes in the fish: one approximately 120 Å wide with an apparent gap (Fig. 30), and another approximately 90 Å wide which appears to repre-

sent actual fusion between the outer leaflets of apposed glial processes (Fig. 29). Both of these junctions lack the cytoplasmic fuzz typically associated with other tight and gap junctions. Neither lanthanum nor peroxidase in the mouse and fish penetrated the myelin sheath, although, in a few instances, lanthanum did penetrate the space between the myelin sheath and the axon. The failure of these tracers to penetrate myelin suggests a

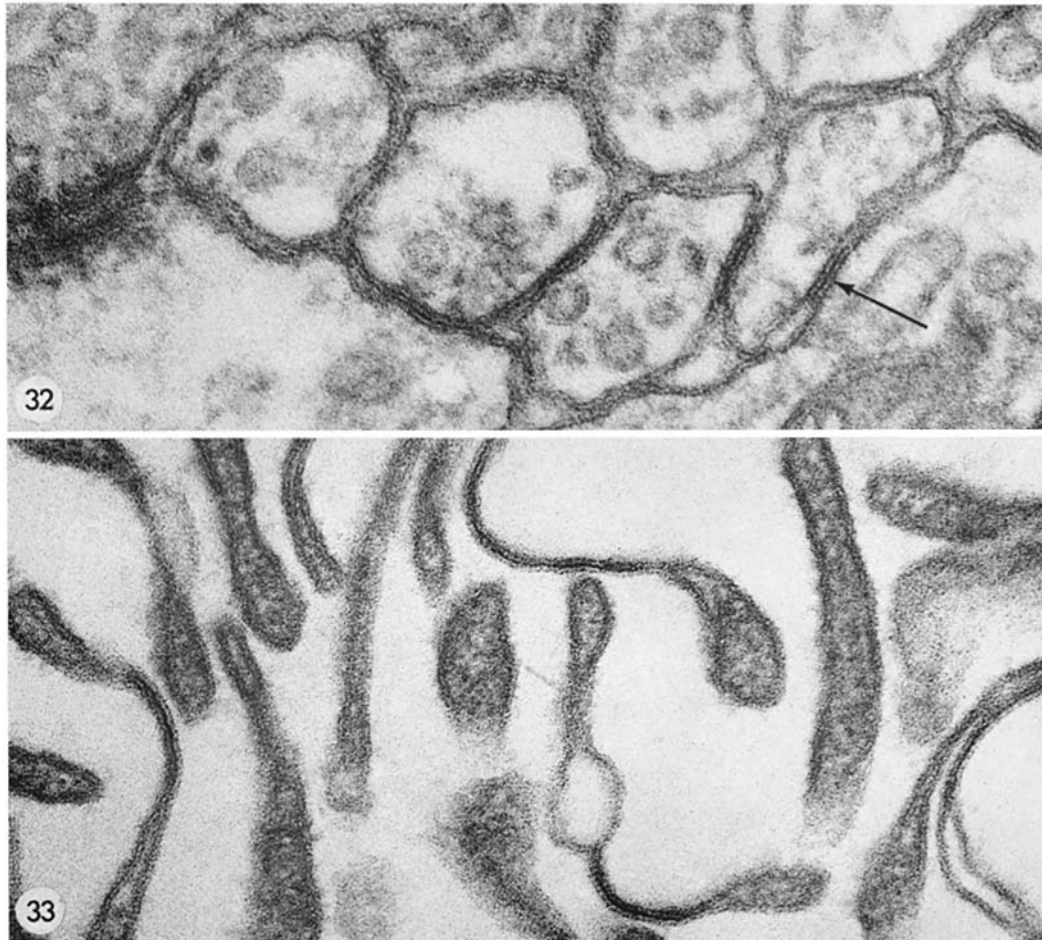


FIGURE 32 With certain preparative methods, pentalaminar configurations appear between outer leaflets of neuronal processes (arrow) and between glial processes. These “labile appositions” are approximately 120 Å wide, are bisected by a dense and relatively thick median lamina, and lack any associated cytoplasmic fuzz (cf. Fig. 28). Formalin-perfused cerebral cortex from mouse. No uranyl block treatment. $\times 170,000$.

FIGURE 33 Pentalaminary appositions between the cytoplasmic leaflets of the shrunken polypoid evaginations of the choroid plexus epithelium after fixation in aldehydes with 4% sodium chloride. The over-all width of the apposition is about 110 Å. Mouse. Aldehyde. $\times 170,000$.

resemblance with respect to "tightness" as well as to size between the external compound membrane and the tight junctions at endothelium and epithelium in the brain.

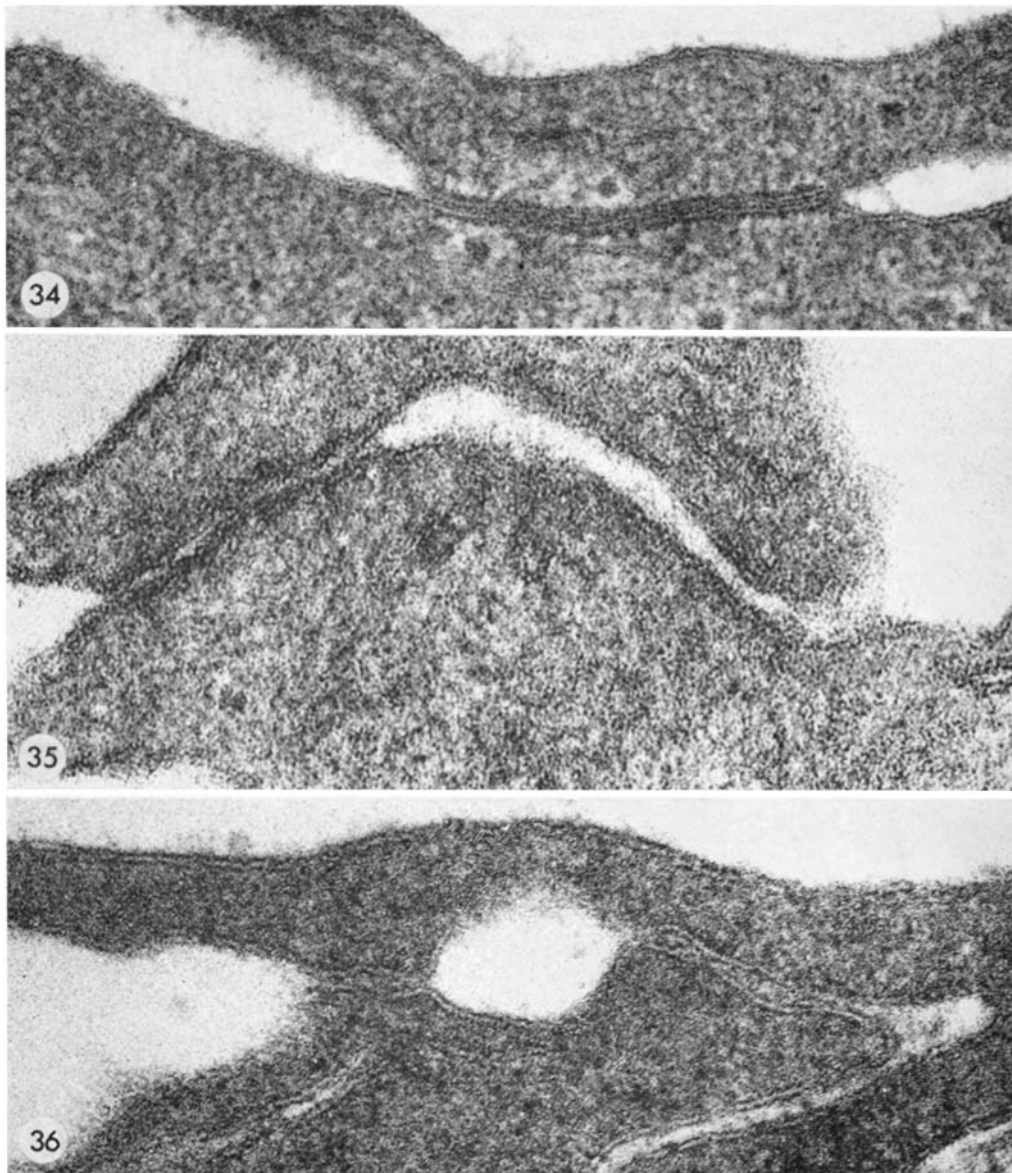
Labile Apposition of Cell Membranes

In the cerebral parenchyma of appropriately prepared mouse brains, extensive five-layered structures resembling tight junctions occurred at glial-glial, glial-neuronal, and neuron-neuronal appositions (Fig. 32). Whether or not the tissue was treated with uranyl acetate, the structure as well as the distribution of these five-layered appositions differentiated them from tight junctions. They lacked any associated cytoplasmic fuzz, their median laminae were as dense as but wider than an individual outer leaflet of adjacent cell membranes, and their over-all width was approximately twice that of an individual cell membrane (Fig. 32). Thus their structure was consistent with their being mere appositions rather than fusions of cell membranes. The extent of these appositions varied from brain to brain and between different areas in the same brain. They were less frequent in cerebellar and cerebral cortex and most frequent in the lateral geniculate nucleus (28, 29). Both the type of fixative and the quality of the fixation appeared to be significant; appositions were most frequent after aldehyde fixation in which there was a high incidence of swollen processes, that is, processes having no discernible cytoplasmic matrix and containing dilated cisternae of endoplasmic reticulum. This state of preservation was coincidental with a high frequency of appositions in brains fixed in formalin and dehydrated in acetone, procedures exactly following a previously published protocol (29). An increase in frequency of labile appositions has been reported to depend on using acetone alone for dehydration (28). However, if aldehyde-fixed tissue was treated with aqueous uranyl acetate before acetone dehydration, there were only a few appositions regardless of the quality of fixation. Labile appositions were very rare in brains fixed initially in osmium tetroxide and subjected to uranyl acetate treatment.

None of the variety of methods influencing the frequency of these appositions affected the frequency or location of gap and tight junctions. The variable incidence of labile appositions was systematically compared to the constant frequency of gap and tight junctions by including various

concentrations of sodium chloride (up to 4%) with the initial aldehyde fixative. In all experiments, gap junctions were found with their expected frequency at their usual locations near the apical ends of ependymal cells although the *fasciae adherentes* were separated or broken (Fig. 34). The width of the gap was unchanged although the rest of the interspace between ependymal cells was widely distended. Tight junctions between endothelial cells (Fig. 36) and between choroid epithelial cells (Fig. 35) also persisted, although the pockets of interspace between individual junctions were now widely dilated. However, no labile intercellular appositions were found anywhere in the parenchyma of the brain under hypertonic conditions, although the intercellular space was only slightly enlarged and retained its customary size only at the various recognized types of interglial junctions and at the neurohumoral type of synaptic junctions. The experiments using hypertonic fixation revealed an interesting alternative to the intercellular labile apposition. Five-layered appositions between cytoplasmic leaflets of the cell membrane were frequent in the villiform processes of the choroid epithelium (Fig. 33) and were present also within some glial processes. These labile intracellular appositions appear to be due to shrinkage of a process to the point of excluding its cytoplasmic content, resulting in apposition of two areas of the same membrane. This configuration is analogous to the major period of myelin (53).

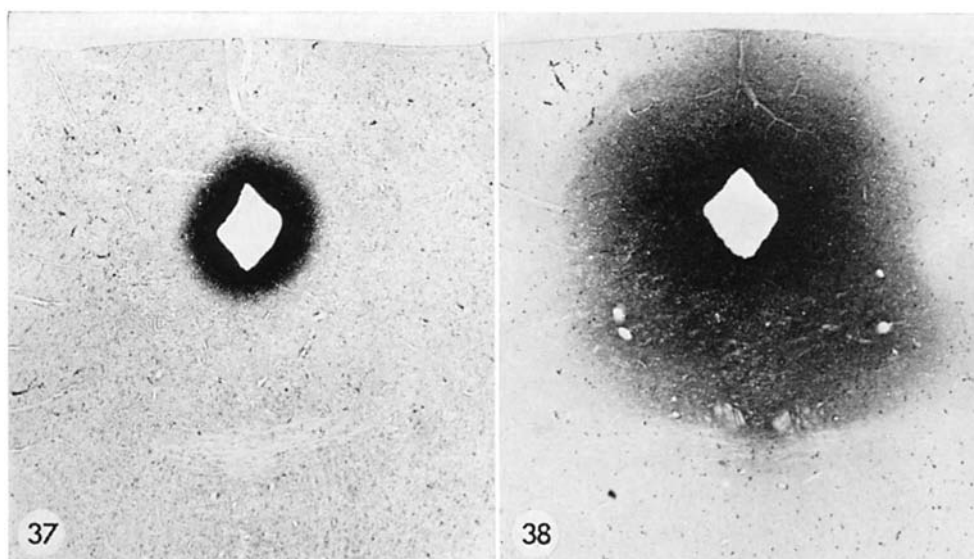
Regardless of their lability, the intercellular labile appositions might represent a structure of some functional significance albeit not that of intercellular adhesion. It would be arbitrary to dismiss them as artifactitious because of their dependence on certain methods of fixation, even though these methods produce what is generally accepted as poor fixation. If labile appositions are present in life they would be expected to obliterate interstitial space, making it unavailable to large molecules. This supposition was studied by intraventricular perfusion of peroxidase. The first approach was to determine whether any parenchymal extracellular space was open to large molecules and whether peroxidase spread through this space before or during fixation. That the over-all localization of peroxidase is due to movement of this protein before fixation was shown by the fact that the distance that the protein moved from the ventricle depended on the



FIGURES 34-36 Effect of fixation in aldehydes containing 4% sodium chloride on the structure of tight gap junctions. The gap junction between the apical processes of two shrunken ependymal cells (Fig. 34) retains its normal configuration and dimensions in spite of marked widening of adjacent intercellular spaces. The series of tight junctions between the apical processes of two shrunken choroid epithelial cells (Fig. 35, ventricle at left) and between two vascular endothelial cells (Fig. 36, lumen at top) also retain their configuration in spite of marked dilation of adjacent intercellular spaces. Aldehydes. Fig. 34. \times 185,000. Fig. 35, \times 255,000. Fig. 36, \times 230,000.

length of time allotted before fixation. If the concentration (5 mg/ml) and rate (0.5 ml/hr) were held constant, then 5-10 min of perfusion resulted in a penetration of peroxidase as far as 0.2 mm

from the ependyma of the iter (Fig. 37) whereas after 90 min of perfusion the protein had moved as far as 1.0 mm (Fig. 38). Since electron microscopical examination of these specimens perfused



FIGURES 37 and 38 Dependence of depth peroxidase penetrates on length of time it was perfused through ventricular system. Within 10 min (Fig. 37) it has penetrated up to 0.2 mm into the periaqueductal grey, and within 90 min (Fig. 38) in a second mouse it has penetrated up to 1.0 mm. Both figures, $\times 30$.

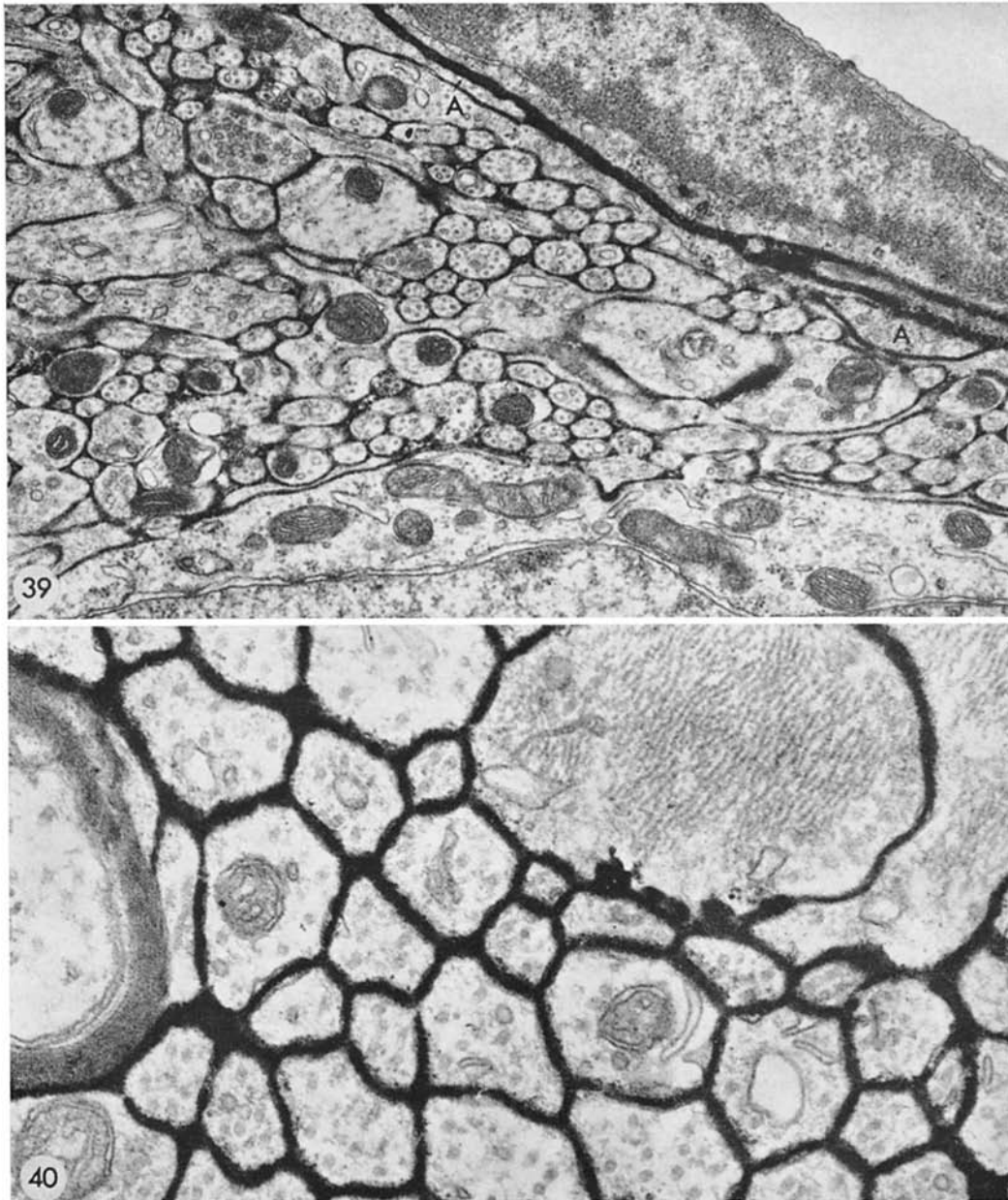
with peroxidase before fixation showed that the protein was confined to intercellular spaces, it is evident that in living tissue, under the conditions of our experiment, some regions of this space are open channels through which peroxidase can move. That diffusion of peroxidase through the neuropil after fixation did not contribute significantly to this distribution was shown by perfusing the ventricles with peroxidase following fixation. In these specimens, the protein indiscriminately entered cells as well as the clefts between them for a distance limited to 0.05 mm. The conclusion that in living tissue peroxidase moves primarily through the intercellular spaces is further supported by the occasional finding, deep within gliocytes or neurons, of vesicles filled with peroxidase which presumably was taken up by the cell prior to fixation. Therefore, the ubiquitous apposition of cell membranes in brain parenchyma (29) does not appear to be representative of the relationships of these membranes in living tissue.

A hitherto unrecognized functional leak may occur around the system of tight junctions comprising the blood-brain and blood-cerebrospinal fluid barriers; where this leak might be situated is shown in Fig. 41, at the large arrow. The leak becomes apparent when peroxidase or lanthanum,

introduced into the bloodstream, is able to cross the endothelium of the choroid plexus and to permeate the basement membrane shared by both plexus and neuropil. From this membrane, the tracers are able to penetrate, more or less completely, the interspaces of the neuropil. The completeness of filling is better appreciated with lanthanum (Fig. 40) because it has great electron opacity and allows recognition of structures in longitudinal, oblique, and normal planes of section. This widespread, complete filling supports the conclusion that the labile appositions are no barrier to the intercellular passage of tracers introduced before or during fixation and, therefore, are probably not present in life.

DISCUSSION

Junctions between glial cells (45), ependymal cells (5), cerebral vascular endothelial cells (42), and between certain neuronal processes at electrical synapses (3, 15, 26, 56) have heretofore been thought to have in common a five-layered configuration characteristic of tight junctions. The present survey, though limited to only a few selected regions of the brains from a few vertebrates, indicates that some of these junctions are indeed pentalaminar tight junctions whereas



FIGURES 39 and 40 Extracellular passage of colloids in the neuropil. In Fig. 39, peroxidase has moved from the cerebral ventricles into clefts between neurons and glial cells, including those between several perivascular astrocytic end-feet (*A*), and thence into the basement membrane around a capillary, the lumen of which is at the upper right. In Fig. 40 lanthanum has passed the functional leak at the base of the choroid plexus (see Fig. 41) to fill the interspaces of the subjacent neuropil, including the clefts around a myelinated axon (left). Most of the processes are neuronal; the two processes containing filaments (upper right) are astrocytic. Mouse. Fig. 39, $\times 23,000$. Fig. 40, $\times 60,000$.

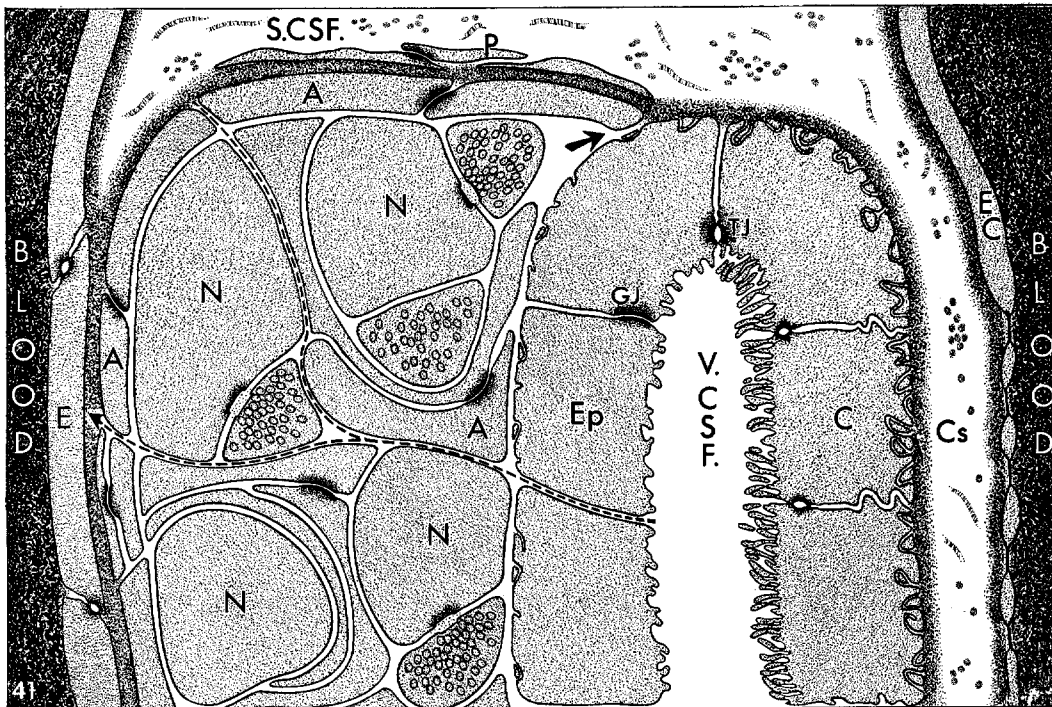


FIGURE 41 Schema of blood, brain, and cerebrospinal fluid relationships in the mouse.

- | | |
|--|---|
| <i>A</i> , astrocytic process | <i>GJ</i> , gap junction |
| <i>C</i> , choroid plexus epithelium | <i>N</i> , neuron |
| <i>Cs</i> , choroid plexus stroma | <i>P</i> , pia |
| <i>E</i> , endothelium of parenchymal vessel | <i>S.CSF.</i> , cerebrospinal fluid of subarachnoid space |
| <i>EC</i> , endothelium of choroid plexus vessel | <i>TJ</i> , tight junction |
| <i>Ep</i> , ependyma | <i>V.CSF.</i> , cerebrospinal fluid of ventricles |

The dashed line follows two typical open pathways connecting ventricular cerebrospinal fluid with the basement membrane of parenchymal blood vessels and with the basement membrane of the surface of the brain. Peroxidase is seen within these pathways in Figs. 10-12, 14, and 39. Where peroxidase cannot cross cellular layers such as the parenchymal vascular endothelium (*E*) and the epithelium of the choroid plexus (*C*), the component cells are joined by tight junctions. The thick arrow (top) indicates a "functional leak" whereby substances crossing the fenestrated endothelium of the choroid plexus can pass along the choroidal stroma to enter the parenchyma of the brain at the root of the choroid plexus. A "leak" in the opposite direction could also occur, as indicated by the arrow point.

others are seven-layered gap junctions. Since both types of junction are demonstrated simultaneously by a variety of techniques for tissue preparation, the consistency of their extent and distribution under these conditions denotes a real structural difference regardless of how their structure in life is ultimately related to that observed after chemical fixation. Because an appreciation of the functional roles of these junctions must be based on a knowledge of their extent, distribution, and configuration, these morphological attributes will be considered first.

Astrocytes and those ependymal cells over most of the ventricular surface are joined by gap junctions. A closely related type of gap junction joins neurons at certain synaptic endings while another variety of gap junction, characterized by a narrower gap, joins oligodendroglial processes external to the myelin sheaths in the fish. An important feature of gap junctions is that they are discontinuous plaques rather than continuous belts or zonules around the perimeters of cells. This has been previously demonstrated in ependyma (5, 6), in perivascular astrocytic sheaths (34, 48), and

in heart muscle (51). The present study shows gap junctions discontinuously distributed between pial mesothelial cells and between contiguous astrocytes at the limiting glial surface of the brain (Fig. 13). Glial and neuronal gap junctions within the parenchyma of the brain may be assumed also to be discontinuous because of their low frequency in relation to the number of glial processes in most planes of section, although this discontinuity remains to be demonstrated.

Pentalaminar or tight junctions join vascular endothelial cells (43, 49) (Fig. 21), choroid epithelial cells (2, 10) (Fig. 24), oligodendroglial processes within some myelin sheaths (45) (Fig. 30), ependymal cells overlying the area postrema and median eminence (49), some neuronal cell bodies (27) (Fig. 28) and processes (46, 62), and neurons to supporting cells in olfactory epithelium (Fig. 31). Tight junctions, unlike gap junctions, may form continuous zonulae or belts around cells. Thus, tight junctions, studied in a variety of epithelia outside the brain, have been called *zonulae occludentes* (17). Within the brain, the tight junctions between vascular endothelial cells (48), choroid epithelial cells (2, 10), and ependymal cells overlying the area postrema and median eminence (49) also appear to be complete or nearly complete *zonulae occludentes*. Unlike true tight junctions or gap junctions, labile appositions (Fig. 32) indiscriminately connect processes of different types of cells, for example, neuronal and glial. Labile appositions are more prevalent under some conditions of tissue preparation (28) and within some regions than in others.

The gap junction, after uranyl block treatment, has seven layers: three from each of the apposed cell membranes and one from the enclosed, median, lucent gap. However, when blocks are treated with permanganate (6, 44) or when the plastic-embedded sections are stained with uranyl or lead salt, the lucent gap becomes a median dense line, but the outer leaflets of the cell membranes bordering the gap are invisible or indistinguishable from the stained material within the gap. Because this median line imparts a five-layered appearance, the gap junction has been previously interpreted as a tight junction. Use of the uranyl block stain, then, mordants or stabilizes the outer leaflets of the cell membranes and so renders them stainable with lead salt after plastic embedding; the substance within the gap, however, is not made stainable. It is emphasized that

the total width of the gap junctions is unaffected by these various preparative techniques with the exception of KMnO_4 fixation, and so a gap substance, stained or not, appears to be present between the apposed cell membranes at gap junctions. This substance bridging the gap would account for the observation that the width of the gap does not change after the application of various preparative techniques which either enlarge or obliterate adjacent intercellular clefts (Fig. 34).

A periodic structure within the gap substance can be outlined with lanthanum after osmium tetroxide or glutaraldehyde fixation. Our interpretation of this structure agrees with that of a previous study on the gap junction between cardiac muscle cells (51). The lanthanum penetrates the median gap which appears somewhat wider than the gap seen in uranyl block treatment (Figs. 8, 19), presumably because the lanthanum line includes at least part of the outer leaflet of both cell membranes. In the tangential plane, the region filled by lanthanum is a lattice surrounding lucent polygonal subunits (Figs. 8, 9, 20). Because these subunits match the background density of the sections, they are thought to contain little lanthanum and, therefore, to bridge the gap. The interpretation that the lucent polygonal subunits correspond to the substance in the gap which stains when uranyl or lead salts are applied to the section, depends on the assumption that the lanthanum in the gap is outlining structures rather than staining them. This assumption appears to be applicable to extracellular space outside of gap junctions (Fig. 40), but is not necessarily applicable to the gap. In an alternate model, which makes the gap junction analogous to the septate desmosome (11), the lanthanum would act not as a tracer but as a stain for the polygonal lattice surrounding the lucent subunits. The dense lattice of Fig. 20 would represent the impermeable walls surrounding the lucent subunits. The entire subunit, 80 Å wide, would be the diffusion channel isolated from the extracellular space by the surrounding, stained lattice or wall. The penetration of peroxidase, administered before fixation, into the slit of gap junctions would appear to rule out this second model because a large protein molecule would hardly be expected to penetrate a gap substance capable of being an effective barrier to ions. However, our present study does not rule out the possibility that some peroxidase artifactuously entered the gap during fixation. There-

fore, it remains conceivable that the gap substance in gap junctions could correspond to either the lucent subunits or their surrounding lanthanum lattice. A study of septate desmosomes with lanthanum and peroxidase should be done before the septate desmosome can be considered a model for the gap junction. Finally, it should not be assumed that all gap junctions are the same; variations that have already been shown, such as the width of the gap and the prominence and degree of organization of the gap substance, suggest important structural differences between gap junctions.

There is little doubt that the pentalaminar tight junctions between vascular endothelial cells (Fig. 21) and choroid epithelial cells (Fig. 24) represent actual fusions between the outer leaflets of cell membranes. Regardless of the preparative methods, their over-all width at the most constricted point is less than twice that of an immediately adjacent cell membrane (48). This dimension is not changed by exposure to hypertonic fixatives although the rest of the intercellular space is widened (Figs. 35, 36). The inability of peroxidase to penetrate these junctions (Figs. 23, 27) indicates that a considerable degree of constriction must have been present before fixation. Nevertheless, the limited accumulation of pools of lanthanum in the clefts between individual members of a row of tight junctions (Fig. 25) indicates that these junctions might best be described as a series of discontinuous, overlapping intercellular fusions around the cell perimeters. The failure of lanthanum to cross the complete series of junctions may be accounted for in two ways: one of the junctions may form an uninterrupted belt of fusion, or the discontinuities between individual junctions may be so short and infrequent that the lanthanum must travel a long distance to cross the endothelium. In either instance, the discontinuities would be large enough to admit lanthanum but too small to admit peroxidase. In this respect, the vascular endothelium in brain might be similar to that in heart (31), although the perforations or "pores" through the tight junctions of cerebral endothelium would be more narrow (19).

Other varieties of tight junctions, such as those constituting the minor period of myelin, are also relatively stable intermembranous appositions. As expected, neither lanthanum nor peroxidase could penetrate this junction in myelin. Interneuronal pentalaminar junctions unite somata in the mesen-

cephalic nucleus of the trigeminal nerve although neither their stability nor their occlusion has been tested with hypertonic fixative or tracers. Other such interneuronal junctions in vertebrates are those in the retina (e.g., 46) and lateral vestibular nucleus (62). All of these neuronal tight junctions presumably differ from those between endothelial and epithelial cells in brain in one fundamental respect: like gap junctions, they extend for limited distances rather than completely around cells. However, the interneuronal pentalaminar junctions in the retina of some invertebrates are very extensive indeed (35).

Labile appositions of cell membranes are easily differentiated from tight junctions since they form random neuronal-neuronal and neuronal-glial contacts, lack any associated cytoplasmic fuzz, and have a median lamina that is about twice as wide as a single dense leaflet of a cell membrane (Fig. 32). Some of these features are shared by the very numerous pentalaminar contacts within chemically fixed, albeit freeze-substituted, normal cerebellar cortex. This apposition may be a type of artifactual labile apposition that, judging from the published micrographs (e.g., Figs. 12 and 17 of reference 64), is widely and randomly distributed at synaptic terminals and between small axons. Like the usual labile appositions, this contact has no associated cytoplasmic web, but unlike those in our material the contact arises where two or more sides of an axon are so evaginated as to make a pentalaminar contact with several of its neighbors. The configuration of the tight and gap junctions, in the mouse brain at least, remains constant with a wide variety of preparative techniques. Yet, with these same techniques, the labile appositions in our material vary from being almost ubiquitous to virtually absent. It is apparent that labile appositions differ from tight and gap junctions in their structure and in being readily made or broken by variations in procedures of tissue processing.

Nevertheless, an alternative interpretation concerning the prevalence of numerous labile appositions is that they occur in life but are separated at the time of fixation, permitting the widespread entry of peroxidase perfused through the cerebral ventricles. If such a spurious entry did take place, the depth of penetration should be independent of the time permitted for the perfusion of protein solution before fixation. The fact that the extracellular movement of peroxidase is time-depend-

ent, as illustrated by Figs. 37 and 38, demonstrates that this movement had taken place before fixation. Furthermore, the pinocytosis of peroxidase by cell processes deep within the parenchyma is manifested by its accumulation within segregation bodies. Since pinocytosis cannot be performed by a fixed cell membrane, the protein must have entered the interspaces, thereby being available to the plasmalemma before fixation (7). Thus, the patency of most of the cerebral interspace is evidence that the ubiquitous labile junctions do not represent the intercellular relationships within the unfixed brain. The almost total filling of the interspace, in some areas, by the smaller lanthanum colloid suggests that the proportion of open spaces is very large (Fig. 40). The conclusion that the extracellular spaces of the brain are generally open is in accord with the interpretations of earlier studies of a similar nature using ferritin as a tracer (7). Studies on the retina using peroxidase as a tracer have shown that extracellular spaces there, as in the brain, are patent (36). The picture of the parenchyma in the vertebrate brain that now emerges is one in which neuronal and glial cells are almost everywhere separated by clefts of variable width except at gap junctions where the cleft is reduced to a slit of constant width, and at tight junctions where it is obliterated (Fig. 41). The possible functions of the tight and gap junctions, as dictated by their morphology, locations, and permeability to peroxidase and lanthanum will now be considered.

Intercellular Adhesion

Tight junctions have been implicated in the attachment of cells in a variety of epithelia (17). However, the role of an attachment device is more readily appraised when either tight junctions or gap junctions provide the sole contact between cells. The tight junctions between cells of the retinal vascular endothelium (59), the cerebral vascular endothelium, and the epithelium of the choroid plexus in specimens subjected to hypertonic media are the only junctions available for maintaining the plasmalemmas in close approximation. The outer leaflets of the contiguous cells remain in contact at the tight junctions while elsewhere along the cell membrane they become widely separated.

Between astrocytes, the only frequent intercellular link is a gap junction, and the constituent outer leaflets likewise retain a constant gap width

after hypertonic treatment. Contiguous astrocytes remain fastened together even though the clefts near their gap junctions may be distended. The firmness of both tight and gap junctions is emphasized by experiments in which either the concentration of environmental cations is greatly reduced (43, 58) or in which the osmolarity of the fixative is greatly increased (18) (Figs. 34–36). In either experiment, tight and gap junctions remain unaltered and are the last junctional elements to be separated.

A thorough consideration of the molecular forces responsible for repulsion and attraction between cell membranes suggests that once molecular contact has been made, as in the case of occluding junctions, the adhesion is stable (14). The gap junction resists disruption under at least some conditions, such as strong osmotic forces and after chelation of cations (43). Therefore, like tight junctions, it may also form a stable adhesive site, the molecular bonds being provided by the polygonal subunits contributed by both plasmalemmas. Nevertheless, the increased permeability of cerebral vessels associated with certain pathological states points to the probability that the bonds within endothelial tight junctions can be widened or broken, perhaps reversibly.

This system of gap junctions between astrocytes would link them into a spongelike network cradling the neural elements of the brain. The gap junctions, as the predominant link between astrocytes, might have much to do with maintaining the neuronal organization of the brain. The ependymal gap junctions would function to maintain ependyma as a cellular layer bounding the neuropil at cerebral ventricles, much as the glia delimits the neuropil at the pial surface.

Intercellular Communication

An intimate intercellular contact, provided by specialized junctions, may also permit ionic or electrical communication between cells. At least three varieties of junctions have been so implicated: (i) septate (39), (ii) tight (3, 18, 34, 35, 56), and (iii) gap (51, 52) including neuronal-neuronal junctions (9).

(i) **SEPTATE DESMOSOMES:** The septate desmosomes of invertebrate epithelia are now regarded as preferential sites of cell to cell passage of ions and current (39). Although the interpretations of the underlying structural pattern vary with the investigator (38, 39), it would seem

likely that the septa or "cross-bars" represent a stack of parallel subunits bridging the intercellular cleft. Each subunit is envisioned as surrounding a core, serving as an aqueous diffusion channel, with an impermeable wall that insulates the core from the extracellular space so that ions or current cannot be shunted into this space (39).

One interpretation of the configuration of septate desmosomes which, in the light of more recent work is probably erroneous (21), is that the septa are formed by the fused extensions of the outer and middle leaflets of conjoined cell membranes (38). A comparable arrangement on a smaller scale was thought to occur at close appositions between electrically coupled smooth muscle cells where the outer leaflets of either cell membrane meet as a reflection over the common middle leaflets that bridge the gap (Fig. 22 of reference 57). If this relationship were real, then substances passing across such junctions would be confronted by only three membrane leaflets. The conjecture could then be made that cell-to-cell passage of ions takes place selectively at gap or septate junctions because the inner and middle leaflets of the entire plasmalemma are permeable whereas the entire outer leaflet is impermeable but is reflected out of the way at each bridge. However, we could not obtain convincing examples of these bridges in any gap junctions and, even where possibly present (Fig. 7), they were few and far between.

(ii) GAP JUNCTIONS: Gap junctions may have a "directional impermeability" as suggested by the extracellular confinement of lanthanum. Although lanthanum hydroxide may penetrate the minute crevices around the subunits, neither this substance nor peroxidase can enter the cytoplasm from the junctional slit. Conceivably, there are permeable channels coursing through the subunits from cell to cell (51), but the walls of the subunits must be impermeable. Regardless of the route of ion passage, the gap junctions provide, almost invariably, the sole junction between astrocytes in the brains of vertebrates. In *Necturus*, the glial cells of the optic nerve are electrically coupled (34), presumably by the gap junctions that were interpreted as pentalaminar, tight contacts. The calyces of the ciliary ganglion in the chicken are united to ciliary ganglion cells by gap junctions as well as by junctions resembling neurohumoral synapses elsewhere (63). The occurrence of the two types of junctions at a synaptic

ending mediating both neurohumoral and electrical transmission provides strong circumstantial evidence that the gap junctions are the sites of the electrical synapse (55, 40, 63).

A comparable duality of transmission may occur between the axonal club endings and the lateral dendrite of Mauthner's cell which have been shown to be electrically coupled (20). The coupling has been attributed to a specialized junction (56) which appears, in our preparation, as a gap junction with a particularly marked periodicity identical to that previously analyzed in detail by Robertson (56). This gap, like the synaptic cleft of neurohumoral junctions, communicates with the rest of the interspace (7) as manifested by the entry of lanthanum. Here too, however, one must assume that some portion of the connection between the ending and the dendrite is sealed in order to prevent leakage of current into the interspace.

(iii) TIGHT JUNCTIONS: There is no evidence that contiguous endothelial cells or contiguous choroidal epithelial cells, characteristically joined by a row of punctate tight junctions, are electrically coupled. Indeed, it is not likely that supporting cells and bipolar neuroepithelial cells within the olfactory epithelium are electrically coupled, although they are everywhere joined by junctions (47) of the type found between endothelial cells and choroid plexus epithelial cells within the brain. Nevertheless, there is at least one type of pentalaminar junction that may be related to ionic interchange.

The numerous processes of reticular cells in the eye of *Limulus* are united by extensive pentalaminar junctions that retain their configuration after uranyl acetate block treatment (35) and after immersion in hypertonic seawater.² These cells are electrically coupled (61), presumably at these junctions (35). It must likewise be assumed then, that the inner and middle leaflets and the shared median lamina are permeable to ions only in the cell-to-cell direction. A comparable unidirectional permeability might also be invoked in the instance of the pentalaminar junction between cells of the mesencephalic nucleus of nerve V (27), between the sensory endings in the lateral vestibular nucleus (62), and between processes in the inner plexiform layer of the retina (46). From the above circumstantial evidence, it may be anti-

² A. Lasansky. Personal communication.

pated that in the vertebrate nervous system electrical interaction can be subserved by both gap and tight junctions.

Intercellular Passage

The role of gap and tight junctions in influencing intercellular passage of substances is determined by two factors: the membranous relationships within the junctions and the extent to which cells are encompassed by these junctions. However, there are several reasons for believing that gap junctions exert only a limited influence on extracellular passage. Because gap junctions constitute only a portion of cell perimeters, they can be circumvented by ferritin (6), which is too large to enter the junctional slit, or by peroxidase, which appears to enter junctional slits though probably far less readily than the rest of the interspace. Nevertheless, it had not been demonstrated that the peroxidase which does enter the gap junctions can diffuse out again. Because gap junctions can be circumvented, it is possible that not all the tracer within the gap between ependymal cells, for example, enters progressively from the ventricular side, some perhaps entering in a retrograde direction from the underlying clefts. The same considerations would apply to gap junctions between astrocytes. Tight junctions, on the other hand, actually prevent intercellular movements of proteinaceous tracers because these junctions not only occlude the interspace but, in endothelia and epithelia, form continuous or overlapping bands that cannot be bypassed. It has not been determined whether hydrated channels perforate the junctions as pores for the diffusion of smaller substances, although an "effective pore radius" of about 8 Å has been proposed for cerebral vascular endothelium (19). However, it is possible that the functional pores within tight junctions might be smaller than 8 Å if they are comparable to the tight junctions in frog skin which are thought to be impermeable to small ions (18).

Gap junctions rather than tight junctions connect glial cells and ependymal cells and are consequently distributed throughout the entire brain from its internal ependymal surface to its external glial surface. These surfaces can, therefore, no longer be construed as "limiting membranes" since even proteins pass unselectively through the intercellular spaces of their constituent cells.

Thus, a protein or smaller substance introduced into the cerebral ventricles could move through a labyrinthine intercellular space to reach the subarachnoid space (Figs. 10-12, 14, and 41). Whatever the "filler" in these intercellular spaces, it apparently allows the nonselective extracellular movement of materials as large as protein. Factors that might control movement of a substance through these spaces would be its diffusibility in the extracellular "filler," the configuration of the spaces, uptake by cells lining these spaces, and the direction or velocity of flow of extracellular fluid. The influence of gap junctions might be in holding together sheets of astrocytic processes at certain locations such as the surface of the brain, making the pathway between these processes more circuitous.

Some tight junctions, because of their structure and the belts they form around cells, organize these cells into limiting sheets or barriers to the movement of proteins. The only known locations, to date, of such barriers within the brain are the endothelium of leptomeningeal and parenchymal vessels in mammals (48) and amphibians (4), the epithelium of the choroid plexus (2, 10), and the ependyma overlying the area postrema and median eminence (49). Thus, in regions in which the capillaries are fenestrated and permeable to peroxidase, it is the overlying epithelium which is impermeable. In the choroid plexus, for example, the tight junctions between epithelial cells block the movement of proteins from choroidal blood to ventricular cerebrospinal fluid and in the opposite direction as well (10). The extent and nature of other secondary barriers between these specialized areas and the general parenchyma of the brain will be considered in a subsequent publication.

To what extent barriers depending on tight junctions are effective for substances smaller than protein remains to be determined. If they are effective, they would oblige substances crossing the blood-brain or blood-cerebrospinal fluid interfaces to cross cells, permitting selective and possibly active mechanisms of uptake or exclusion to come into play (48). The generally open nature of the interspaces in the parenchyma of the brain indicates that the tight junctions at the blood-brain and blood-cerebrospinal fluid interfaces define the only continuous cellular layer at which these selective or active processes could be located. Thus, tight junctions could provide for cellular

control over which substances enter or leave the brain or cerebrospinal fluid from the blood. Once within the brain, these substances could enter or circulate through a highly circuitous but open parenchymal interspace where further refinements

in fluid composition could be made on a neuronal and glial, cell-by-cell basis (Fig. 41).

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