THE DISTRIBUTION WITHIN THE BRAIN OF FERRITIN INJECTED INTO CEREBROSPINAL FLUID COMPARTMENTS

I. Ependymal Distribution

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ABSTRACT

From 10 minutes to 3½ hours after the intraventricular injection into rats of 15 to 100 mg of ferritin, an appreciable fraction of the protein, visualized electron microscopically, traverses the ependymal epithelium by diffusing along the dense intercellular substance of the luminal open junction and thence, by circumventing discrete intercellular fusions which partition rather than seal the interspace. These partitions shunt additional protein into the cell, where ferritin is transported within pinocytotic vesicles to the lateral and basal plasmalemma and, presumably, back into the interspace again. The basal interspace is irregularly distended by pools of moderately dense "filler" within which ferritin accumulates. The larger fraction of protein enters the ependyma by pinocytosis and is eventually segregated within membrane-enclosed organelles such as vacuoles, multivesicular bodies, and dense bodies, where the molecules may assume a crystalline packing. As a result of the accumulation of ferritin within these inclusions and within filler substance, only a small amount of protein remains to enter the underlying parenchyma. Presentation of ferritin to prefixed cells leads to a random dispersion of free cytoplasmic ferritin. This artifactual distribution in both prefixed and postfixed cells is concurrent with disruption of cell membranes.

INTRODUCTION

Substances injected into the ventricles of the brain become dispersed within the cerebrospinal fluid and are removed ultimately into the blood stream by taking one or more of several pathways. They may leave the ventricles via foramina to enter the subarachnoid space or they may move directly across the limiting epithelium or ependyma into the underlying glioneural parenchyma. It is with the second route of exit that the present account is concerned. The pharmacological (22, 63) and behavioral (33) consequences of the introduction of drugs into the ventricles have led Feldberg and co-workers (22) to conclude that the profound sensorimotor effects of unrelated substances depends, at the outset, on the fact that they can cross the ependymal lining of the ventricles to reach the same group of neurons. The mechanisms and routes by which substances pass from ventricle to parenchyma have been the subject of recent studies (45, 48, 49), and have been variously interpreted as due to metabolically active processes (16, 21) or, in the case of extracellularly

confined substances such as inulin, to a simple diffusion between ependymal, glial, and neural cells (49).

Histological investigations have long demonstrated that particulate matter (35) and, more recently, fluorescein-tagged compounds (24) including protein (34) can be taken up directly by ependymal cells as are substances of small dimensions. Dyes such as bromophenol blue (21) not only are incorporated by ependymal cells, but subsequently penetrate deeply into the underlying neural parenchyma within certain regions. The histological methods have the virtue of visually demonstrating the distribution of substances within tissues, but their efficacy is limited by the optical definition of the method used. Thus, the demonstration by fluorescence microscopy of appropriately labeled protein within the ependymal epithelium (34) has been considerably more precise than that afforded by autoradiography of isotopically labeled substances (7, 58). The fine details of the penetration of protein molecules into the epithelium and their disposition within or outside the constituent cells, however, can best be resolved by electron microscopy (8). For the purpose of following this penetration, the protein-iron complex ferritin, a form of stored iron naturally occurring within the brain (14), was used as the electron-opaque tracer in the present study.

METHODS

Adult male and female Osborne-Mendel rats, weighing 150 to 350 grams, were anesthetized with an intraperitoneal injection of 400 mg of chloral hydrate[1] per kg of body weight. For the purpose of injecting solutions into the ventricles, an indwelling catheter having a bore of 0.017 mm and an outside diameter of 0.30 mm was fixed to the skull by screws. The shank extended 4.5 mm from the surface of the skull through a hole drilled 1.5 mm lateral to the dorsal sagittal suture and directly through the coronal suture overlying a dilated portion of the lateral ventricle. The exterior portion of the shank was connected to a length of PE-10 polyethylene tubing attached to the needle of a syringe, the piston of which was pushed by a motor-driven bar.

In preparation for injection, 15 rats were anesthetized and the atlanto-occipital ligament was lanced, permitting a small amount of cerebrospinal fluid to escape. Ferritin was then injected into the ventricles at an average rate of 0.1 ml in 5½ minutes. In 5 of the animals the injection was made directly into the cisterna magna and in 2 others the catheter was held in position with a stereotaxic instrument to avoid possible brain damage incident to affixing the catheter to the skull.

In 3 additional animals two catheters, one serving as an egress tube projecting from the opposite ventricle, were implanted for injection after recovery from anesthesia. Fifteen milligrams of ferritin (in 0.15 ml of solution) were injected during a period of 5 minutes, 3 to 16 hours after anesthetization. The amounts of ferritin administered and the intervals permitted for survival are summarized in Table I. During the longer intervals, which lasted 1 to 3½ hours between the start of the injection and the fixation, an attempt was made to keep the animals well oxygenated by delivering a mixture of 95 per cent oxygen and 5 per cent carbon dioxide by means of a polyethylene tent fitted loosely over the animals' heads.

TABLE I

Amount of Ferritin and Time Permitted for Survival

No. of animals	Ferritin	Postinjection period
	mg	*min.*
4	10–90	10–12
5*	10–35	25
3‡	15–100	85
2	100	120
4	50–80	180–210

* Two of this group were not anesthetized during ferritin injection.

‡ One of this group was not anesthetized during ferritin injection.

As the ferritin (Pentex Corp., Kankakee, Illinois) had been crystallized with cadmium sulfate, this toxic salt was removed by dialysis at 10°C for 3 days against a 3.7 per cent solution of sodium versenate followed by an additional dialysis for 3 days against Elliott's (17) balanced salt solution at pH 7.4 (69). Qualitative tests for the presence of cadmium (25) were performed on each batch of solution, which was then sterilized by passing it through a 0.45-μ mesh Millipore filter into sterile ampules that were immediately sealed and refrigerated. The pH of the solutions was about 8.0 in the initial experiments but was subsequently adjusted to 7.4 by bubbling carbon dioxide into the solution just before injection.

At the end of the survival periods the brains were

[1] This dosage usually provides 60 to 90 minutes of general anesthesia in rats weighing 150 to 300 gm. The aqueous stock solutions remain bacteriostatic and chemically stable for about 6 months at room temperature.

fixed by perfusion either through the aorta or through the cerebral ventricles with 1 per cent osmium tetroxide buffered to pH 7.4 with Veronal-acetate (42), White's balanced salt solution (67), Elliott's solution (17), or Millonig's phosphate solution (39). The total time of fixation, performed by a modification of the perfusion method of Palay *et al.* (43), was 2½ to 4 hours. An additional brain was perfused through the aorta with 4.5 per cent glutaraldehyde buffered with 0.1 M sodium cacodylate (60). This specimen was thus fixed for 3 hours, then washed in several changes of this buffer for a total of 60 minutes. Small fragments were then postfixed for 60 minutes in 1 per cent osmium tetroxide buffered with cacodylate to pH 7.4.

In an attempt to determine the factors responsible for the artifactual occurrence of free cytoplasmic ferritin in some animals, the ependyma and subjacent neuropil of two rats were fixed prior to the introduction of protein. One milliliter of 1 per cent osmium tetroxide in Elliott's solution, pH 7.4, was perfused at room temperature over a 15-minute period. Two hours later, 60 mg of ferritin in a 1-ml volume of solution was injected via the same catheter over a 1-hour period. The brains were then prepared for electron microscopic examination.

After fixation, the pieces of tissue were rapidly dehydrated in ascending concentrations of cold methanol and embedded in Araldite (52). Thick sections were stained with toluidine blue (52), thin sections with a 2 per cent solution of uranyl acetate in 50 per cent methanol for 15 minutes to 3 hours at 45°C. Sections of the glutaraldehyde-fixed material were stained with lead (30). All thin sections were examined with an RCA EMU 3D or 3E microscope.

OBSERVATIONS

During the intervals of the experiment only a fraction of the ferritin left the ventricles by passing between and through the ependymal cells. For convenience of description these two routes will be considered separately as intercellular and intracellular. It is emphasized at the outset that passage occurred simultaneously along both routes but that the greater amount appeared to move through the cells. Moreover, the distribution of ferritin was the same in tissues of anesthetized and unanesthetized animals and in tissues fixed in osmium tetroxide and glutaraldehyde solutions.

In all the electron micrographs only the approximately 60-A-wide ferric micelles of the ferritin molecule (about 100 A in diameter) were discernible. The apoferritin, protein shell was invisible. On the basis of the micellar arrangement within certain organelles, it is considered likely that the apoferritin shell is retained. The terms ferritin molecule, micelle, and particle are thus used interchangeably.

Intercellular Passage

The dense interspace of the luminal junctions between ependymal cells permitted the passage of ferritin into the remainder of the extracellular space. The patency of the intercellular route is determined by the types and disposition of the specialized junctions comprising the terminal bars between the cell membranes lining the spaces. The ependymal terminal bars are exceptional in that the order of the component junctions is the reverse of that in other epithelia (*cf.* 19). The luminal junction between ependymal cells is most commonly a *fascia adhaerens* rather than a *zonula occludens* (11). In places, the apical interspace forms a loop, one end of which is a luminal *fascia adhaerens*, and the other a luminal *macula occludens* (Fig. 2). Usually, however, the luminal *fascia adhaerens* is followed immediately or at varying intervals by one or more *maculae occludentes* which probably represent focal intercellular fusions. The only complete *zonulae occludentes* occur about the processes which occasionally invaginate other ependymal cells (Fig. 9).

The extracellular spaces of the ependyma thus open into the ventricular lumen via the dense interspace of the adhering fasciae. Ferritin particles were able to leave the ventricle and enter this dense interspace within all the time intervals and at all concentrations of protein used. The micelles were scattered or aligned in tandem near the center of the interspace (Figs. 1 and 3) and were able to migrate as far as the first fusion or *macula occludens*. Micelles never occurred within any part of the fusion, but, significantly, a few reached the interspace immediately basal to a fusion (Figs. 3 and 4). Molecules further penetrated the deeper reaches of the interspace within 12 minutes after the introduction of 15 mg of ferritin. Ferritin-containing pinocytotic vesicles, coated or uncoated (11), lay adjacent to the lateral cell membrane and opened into these deeper regions of the interspace (Fig. 5).

In the two animals the tissues of which had been fixed before the injection of ferritin, very few if any micelles entered the luminal end of the interspace, *i.e.* the interspace of the *fascia adhaerens*, even though considerable numbers of free particles lay within the subjacent cytoplasm (Figs. 6 and 7). Where the more basal part of the intercellular

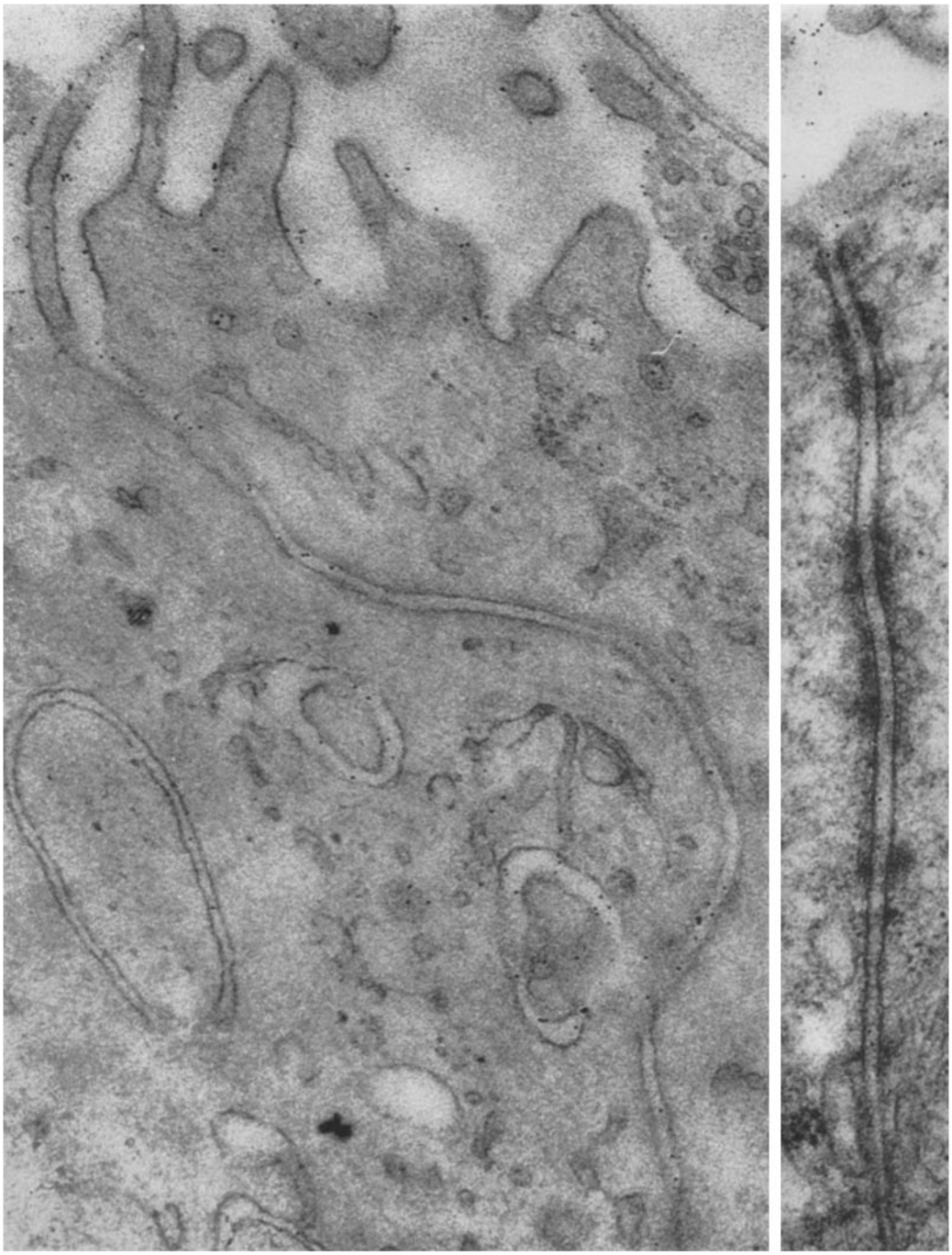


FIGURE 1 Ferritin lies close to or in contact with the irregularly evaginated plasmalemma, within pinocytotic vesicles, and has penetrated deep into the interspace of a luminal *fascia adhaerens*. × 73,000.

Inset. Ferritin has entered the moderately dense "open" interspace of the luminal *fascia adhaerens* between two ependymal cells. Anterior iter. 18 mg ferritin for 26 minutes. Osmium tetroxide fixed. × 93,000.

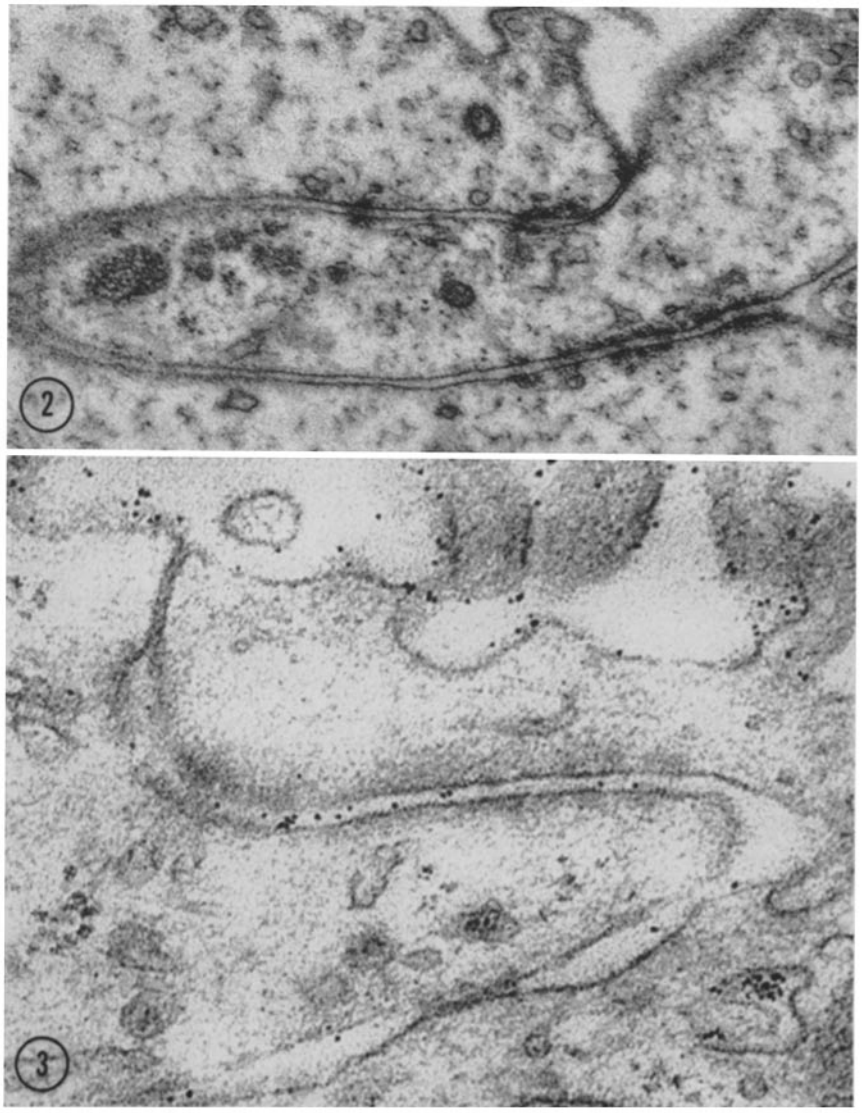
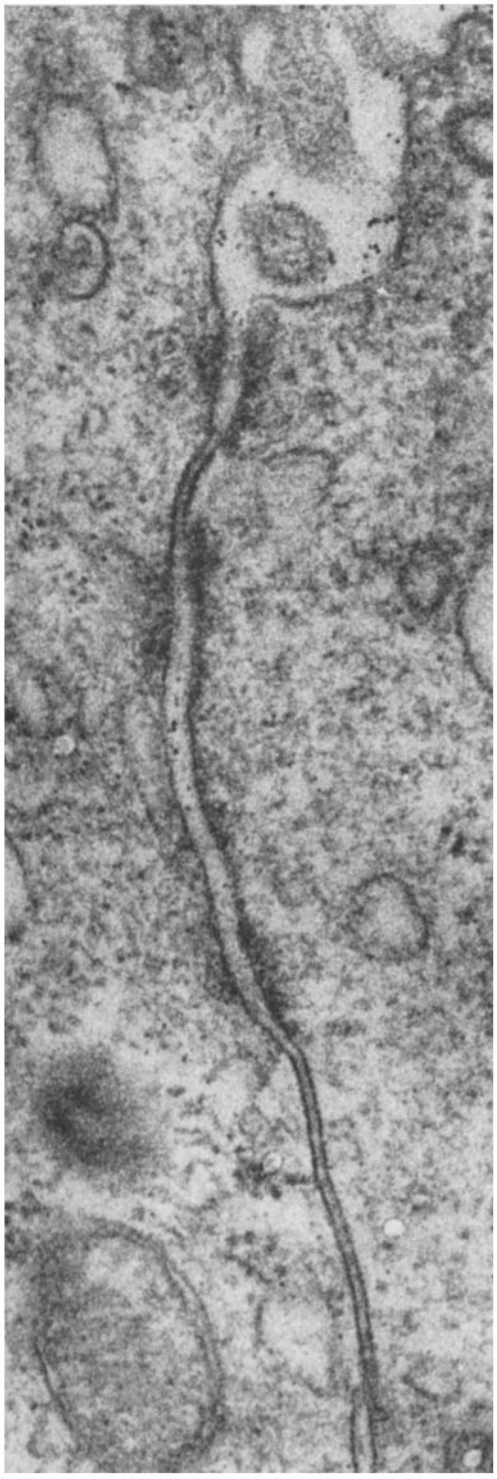


FIGURE 2 The apical interspace between two ependymal cells describes a shallow loop open on one end (lower) at a luminal *fascia adhaerens* and closed on the other end (upper) at a luminal *macula occludens*. Syrian hamster. Ventricle III. No ferritin was injected. × 59,000.

FIGURE 3 The space between two adjacent cells is sealed at this point by a luminal fusion or *macula occuldens*. Ferritin lies within the interspace of the *fascia adhaerens* immediately basal to the fusion. See general explanation of the figures. × 100,000.

FIGURES 1 TO 25 The source of the tissue, the amount of ferritin injected, and the time interval between injection and fixation are stated in the legends except those of Figs. 1, 3, 5, 12, and 13. These five figures represent sections from ventricle IV of an animal receiving 15 mg of ferritin permitted to circulate for 12 minutes. The specimen was fixed with glutaraldehyde and sections were stained with lead salts. All other material was fixed with osmium tetroxide and sections were stained with uranyl acetate. The insets to Figs. 1 and 18 illustrate material from a rat that received ferritin (pH 8.0) before anesthesia.



space was focally dilated, however, micelles were randomly scattered within it and a few particles came to rest upon the cell membrane.

INTERCELLULAR POOLS: The basal interspace of the ependyma lining the iter and ventricle IV undergoes abrupt, localized variations in width and content which influence the diffusion of ferritin. Although a continuous basement membrane was not interposed between the ependyma and the underlying glioneural parenchyma (11), the interspace between the basal part of the plasmalemma of adjacent cells was irregularly distended to form pools of moderately dense, afibrillar, amorphous "filler" corresponding to the basement membrane of other organs (47).

The distentions, about 0.1 to 0.6 μ wide, were formed by confluences between cells, the basal plasmalemmas of which were often deeply invaginated to mure canalicular extensions of the filled extracellular space (Fig. 8). These extracellular canals, sectioned transversely, resembled cytoplasmic vacuoles. However, the occurrence of dense filamentous patches of subsurface cytoplasm and of dense extracellular filler distinguished the basal pools or lacunae and their canalicular extensions (Fig. 9 to 11) from the apical pools of ventricular fluid (Figs. 13 and 18) and from pinocytotic vacuoles. In some lacunae, the dense filler and plasmalemma were separated by a light zone corresponding to that between cell membrane and basement membrane elsewhere.

The dense pools were the first subventricular sites in which extracellular ferritin accumulated. The protein apparently reached these lacunae by diffusion and by vesicular transport. Portions of the limiting plasmalemma formed coated indentations (57) which communicated with the contents of the pools. It is emphasized, however, that after short or long intervals, only a few molecules ever reached the basal interstices (Figs. 8 and 9). When large amounts of protein were injected directly into ventricle IV and damage to the membrane of lining cells occurred, a considerable amount of ferritin leaked into the interspace and eventually

FIGURE 4 A short luminal portion and a long deeper segment of a *fascia adhaerens* is interrupted by an intercellular fusion. Note that ferritin molecules have entered the interspace of the deeper segment immediately basal to the fusion. Ventricle IV. 35 mg of ferritin for 18 minutes. × 100,000.

diffused between cells that were apparently intact. In such tissue, large numbers of micelles accumulated within the dense pools (Figs. 10 and 11). But whether the ferritin reached the pools by diffusing throughout the interspace or by leaking across disrupted cell membranes, the molecules accumulated within the lacunae in concentrations much greater than those in the usual 200-A-wide interspace and apical pools of ventricular fluid (Figs. 13 and 18), though less than those within dense bodies and vacuoles (Fig. 11)

Intracellular Passage

MEMBRANE-ENCLOSED FERRITIN: The pinocytotic uptake of ferritin by ependymal cells began within a few minutes after the introduction of the protein into the ventricles. In optimally preserved tissue, the cilia contained no ferritin. In the brain fixed with glutal, however, a curious and so far unique uptake occurred. In some ciliary shafts the matrix between the peripheral fibers and the ciliary membrane on one side was evaginated in the form of a large protuberance, the diameter of which was about equal to that of the remainder of the shaft. The ciliary membrane covering the bleb was invaginated to form the wall of short tunnels or caveolae coursing among irregularly shaped vesicles of varying diameter. Ferritin particles were adsorbed to the ciliary membrane and were contained within the tunnels and vesicles of the protuberance (Fig. 12).

Within 12 minutes, ferritin molecules came to lie in contact with or near to the irregularly evaginated luminal cell membrane and had accumulated within the apical pools (Figs. 13 and 18) formed by these evaginations and within periciliary moats. At this time, vesicles lying at random directly beneath the free plasmalemma contained micelles (Figs. 1 and 13), indicating that ferritin uptake had begun. Indeed, the membranes of some pinocytotic vesicles, empty or ferritin-laden, were still continuous with the plasmalemma (Fig. 13). A few of the vesicles were densely rimmed (11) or coated (68); but even after injection of the highest concentrations of protein, only a small fraction of the vesicles near the cell membrane contained ferritin at any one time.

In some cells which had imbibed fluid either before or during fixation, as indicated by distention of the cisternae of the endoplasmic reticulum, some pinocytotic vesicles were also swollen and no longer spherical. When such a vesicle abutted against the membrane of another vesicle or of the cell itself, the two membranes made close contact (Figs. 14 to 16). In the latter situation, a tiny localized bulge into the intercellular space was produced. The segment of contact consisted of a very dense single membrane, about 50 A thick, and thus thinner than the remainder of the plasmalemma, which was about 70 A thick.

During the phase of pinocytosis the ferritin, irrespective of its concentration, soon began to be segregated within large inclusions bounded by a single membrane, or in a few instances by a double membrane. The segregating organelles limited by a single membrane were of three kinds: vacuoles, multivesicular bodies, and dense bodies. The least numerous of these inclusions were simple vacuoles containing ferritin particles dispersed within an internum having no appreciable density. Far more common inclusions situated in the apical region of each cell were the multivesicular bodies, about 250 mμ in diameter and characterized by their content of a few vesicles 300 to 600 A in diameter. Within 20 to 40 minutes after the administration of 20 mg of ferritin, an appreciable number of micelles were segregated in these bodies among, but apparently not within, the enclosed vesicles (Figs. 17 and 18).

The greatest number of ferritin molecules accumulated within the third and most numerous type of inclusion, the dense body, which corresponds spatially to structures identified histochemically as lysosomes within ependymal cells (1). The dense bodies varied in size and were either simple or complex in structure. The simple type was recognizable as a circular or oblate profile containing a dense, finely granular matrix limited by a single or, occasionally, a double membrane (Figs. 19 to 22). The complex type contained, in addition, droplets of diverse size and density. The droplets partially protruded from the edge of the body or were embedded within it so that a thin rim of matrix intervened between the membrane of the dense body and the surface of the droplet (Figs. 23 and 24). The droplets never contained ferritin. This complex body corresponds structurally to the vacuolated body within cells of the proximal convoluted tubule of the mouse kidney (38) and to the dense bodies in the glial cells of the rat (9).

In untreated rats it was common to find what appeared to be endogenous ferritin within the matrix of the dense bodies (11). The particles,

always distributed randomly, were fewer and more variable in number than those within the dense bodies of animals which had received ferritin. Within 2 hours after the injection of exogenous ferritin, the numerous micelles within some of the dense bodies of both ependymal and glial cells were no longer randomly arranged but assumed a strikingly linear order (Figs. 20, 21, 22, and 24). This linearity involved only adjacent molecules which appeared to be in contact, whereas the remainder of the micelles were separated by varying amounts of matrix (Fig. 21). In other bodies, the entire content of ferritin molecules abutted against one another to form a crystalloid linear or arcuate array (Fig. 22). The apparent contact between micelles may be an artifact of focus or the effect of overlapping of superimposed molecules.

The tightest packing accentuated a submembranous rim of matrix which was always interposed between the packed ferritin particles and the membrane of the dense body (Fig. 21). This morphological feature has been attributed to some organelles classified as microbodies (2), and according to published micrographs, to organelles designated as dense bodies (38, 41). The rim did not contain micelles, was about 150 to 200 A wide, and could thus have accounted for seemingly membraneless dense bodies which had been merely sectioned tangentially through their peripheral rim (Fig. 21). On the other hand, some of the dense bodies were limited by a double membrane (Figs. 19 and 20).

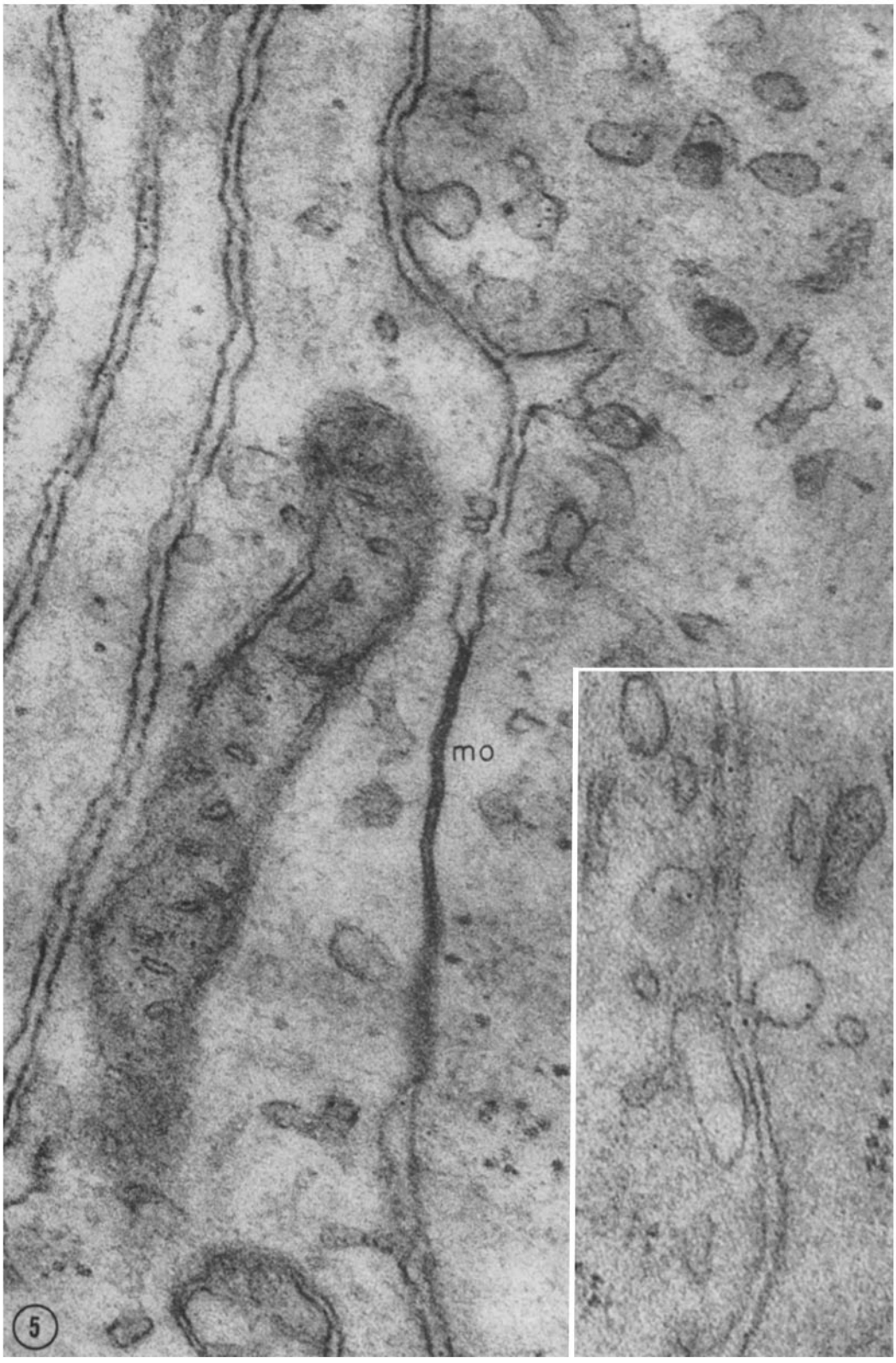
FREE FERRITIN: Both in ependymal cells which had been well fixed, and in those which were poorly fixed as judged by clumping of the granular cytoplasmic ground substance, ferritin molecules were entirely enclosed within membrane-limited inclusions. But in other epithelia which appeared to be well fixed some of the ependymal cells contained few or numerous micelles scattered randomly throughout the cytoplasmic ground substance between or upon cytoplasmic filaments and, occasionally, within a few mitochondria (Figs. 17, 19, and 23). Even where the ground substance was found to be packed with free micelles, however, none entered the cisternae of the endoplasmic reticulum, including those of the Golgi apparatus and perinuclear envelope. This non-bounded or free ferritin was invariably concurrent with membrane-bounded protein irrespective of the amount injected.

On the assumption that free cytoplasmic ferritin was an artifact arising from the rupture of the plasmalemma (56), it was of interest to ascertain whether fixation of the ependyma prior to the injection of protein would lead to a similar distribution. In these brains, numerous ferritin particles were adsorbed to the surface of both ependymal cells and their ciliary processes (Figs. 6 and 25) and within neural processes lying inside the ventricles. Within the ependymal cytoplasm, the dispersion pattern resembled that of a diffusion gradient; the concentration of freely dispersed ferritin was highest in the apical part of the cell but diminished beyond the level of the nucleus (Fig. 7).

As in cells fixed after the injection of protein, a few molecules entered an occasional mitochondrion but did not penetrate into the cisternae of the endoplasmic reticulum. An important difference, however, was that in the prefixed cells, few or no ferritin particles entered membrane-bounded structures such as pinocytotic vesicles, vacuoles, or multivesicular bodies (Figs. 7 and 25). The dense bodies were the only organelles containing appreciable amounts of ferritin, which, presumably, had been segregated during the life of the animal. In both prefixed and postfixed groups, where the cytoplasmic contents were reasonably well preserved, large or very small disruptions of the plasmalemma and of a few dense bodies were the only

FIGURE 5 Ferritin has penetrated the deeper reaches of the lateral interspaces between ependymal cell processes. Although the membranes are disrupted, micelles do not lie free in the cytoplasm but are confined to vesicles (upper right), the membranes of which are continuous with the plasmalemma. Note the ferritin within the interspaces at the left and the few micelles on either side of the *macula occludens* (*mo*) or fusion. See general explanation of the figures. × 108,000.

Inset. A ferritin micelle occupies a vesicle, the interior of which communicates with the ferritin-containing lateral interspace. × 108,000.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obvious, consistent artifact coincident with free ferritin.

DISCUSSION

Intercellular Passage

The direct entry of ferritin from the ventricle into the luminal interspace is an unusual route for the transepithelial passage of a large colloidal particle. In other epithelia, the first luminal junction is a "closed" one consisting of a belt of membrane fusion or *zonula occludens* (19). Neither bulk nor particulate tracers can pass from the lumen to the intercellular spaces. But in the ependyma of the rat (11) and Syrian hamster (10), the luminal junction is commonly an "open" adhering zonula or fascia lying in direct continuity with the ventricular lumen. The designation *fascia adhaerens* implies adhesion between cell membranes. Although the interspace of this junction has a density which may be associated with a cementing substance, it permits diffusion of ferritin along the entire interspace as far basally as the first *macula occludens*. Where the apical interspace forms a loop open at one end by a luminal *fascia adhaerens* and closed at the other end by a luminal fusion, ferritin can reach the interspace immediately basal to the fusion by passing through the open junction (Figs. 2 and 3).

Usually, the maculae occur at various levels along the lateral plasmalemma and so probably act as a series of discrete partitions rather than as continuous belts of fusion. The interspace, instead of being completely occluded at its luminal end, is merely partitioned at varying distances along the lateral surface of contiguous cells. A ferritin molecule, once having gained access to the interspace via the *fascia adhaerens*, is then free to diffuse along a labyrinthine, subdivided interspace. Diffusion is thus impeded, not prevented. This partitioning of the ependymal interspace is a condition intermediate to the complete luminal occlusion in some epithelia (46, 19) and the entirely open interspace in an epithelium where there are no specialized intercellular junctions (32).

In addition to circumventing discontinuous partitions, ferritin could reach the interspace on either side of a deeply situated fusion in two possible ways. There is, first, some evidence that the *macula occludens* may be a transient structure. Five-layered junctions, apparently identical with *maculae occludentes*, form between Schwann cell channels surrounding the squid's giant axon, in response to immersion of the nerve in hypotonic salt solution (Fig. 9 of reference 66). Conversely, the median dense lamina of the *zonula occludens* can be reversibly separated into its component leaflets by withdrawal of calcium from the extracellular fluid (61). If the ependymal fusions are labile, then ferritin could be squeezed into the interspace at either end of a newly formed occlusion. Secondly, fusions may be bypassed by pinocytotic vesicles which empty their contents into the interspace at either end of a fusion (Fig. 5).

The observed intercellular passage of ferritin is in keeping with the recently expressed view that the ependyma is less of a barrier to the egress of ventricular contents than was hitherto supposed. The extracellularly confined substance inulin, when perfused through the ventricles of the dog, diffuses freely between ependymal cells into the interstices of the neural parenchyma below (49). In dead dogs, a like perfusion resulted in the transependymal diffusion of less inulin because, it was concluded, the cells had swelled and the interspace

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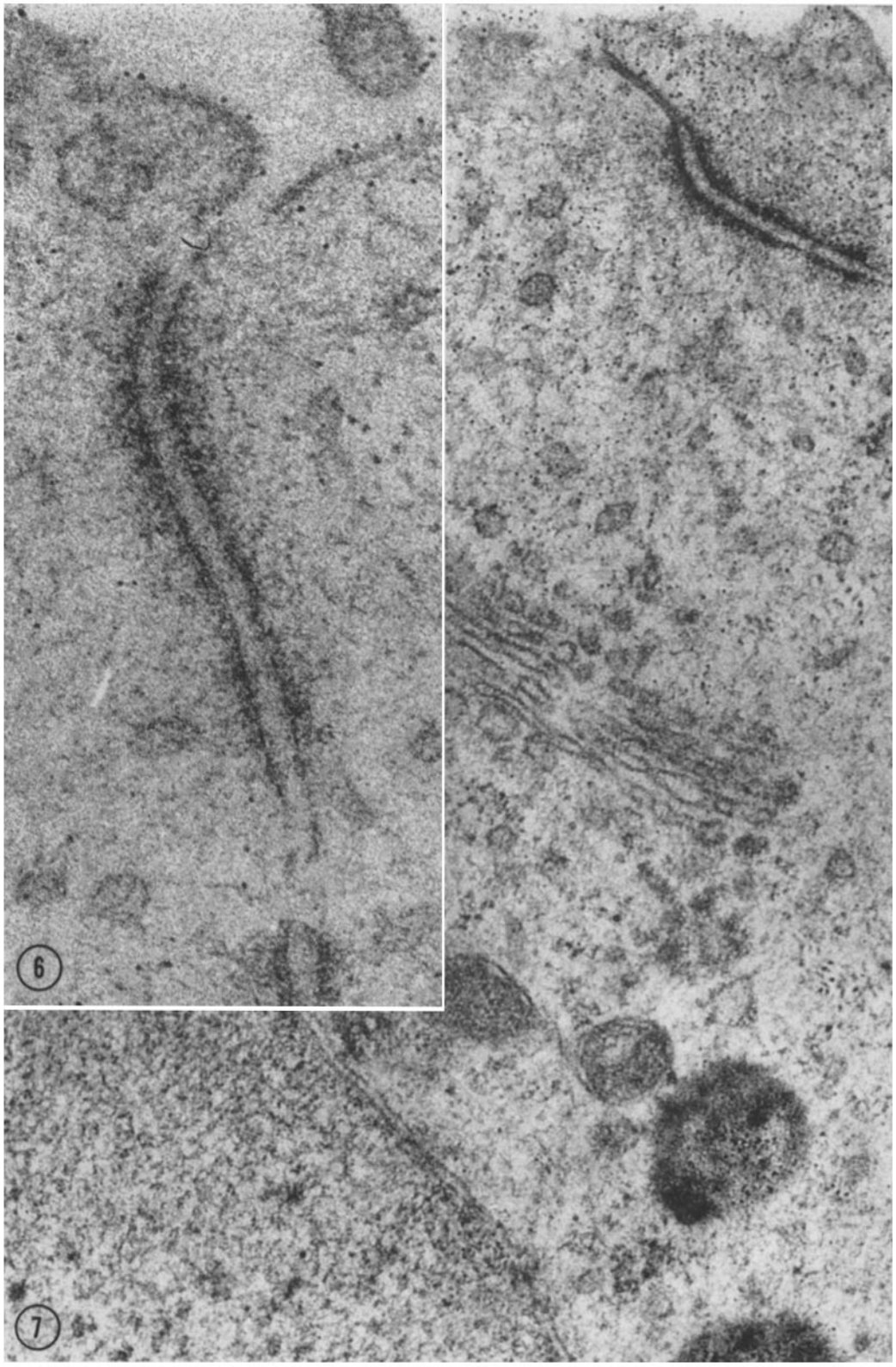
FIGURES 6 AND 7 Ependymal cells fixed for 2 hours preceding the injection of 60 mg of ferritin. Anterior ventricle IV.

FIGURE 6 Ferritin molecules lie close to or in contact with a disrupted cell membrane but have not entered the interspace of the luminal *fascia adhaerens*. Free micelles lie scattered within the cytoplasm. × 137,000.

FIGURE 7 In these cells the concentration of free ferritin is greatest in the apical region and diminishes toward the level of the nucleus (lower left). Membrane-limited organelles do not contain micelles except for two dense bodies (lower right). No molecules have entered the interspace of the *fascia adhaerens*. The luminal junction is a *macula occludens*. × 75,000.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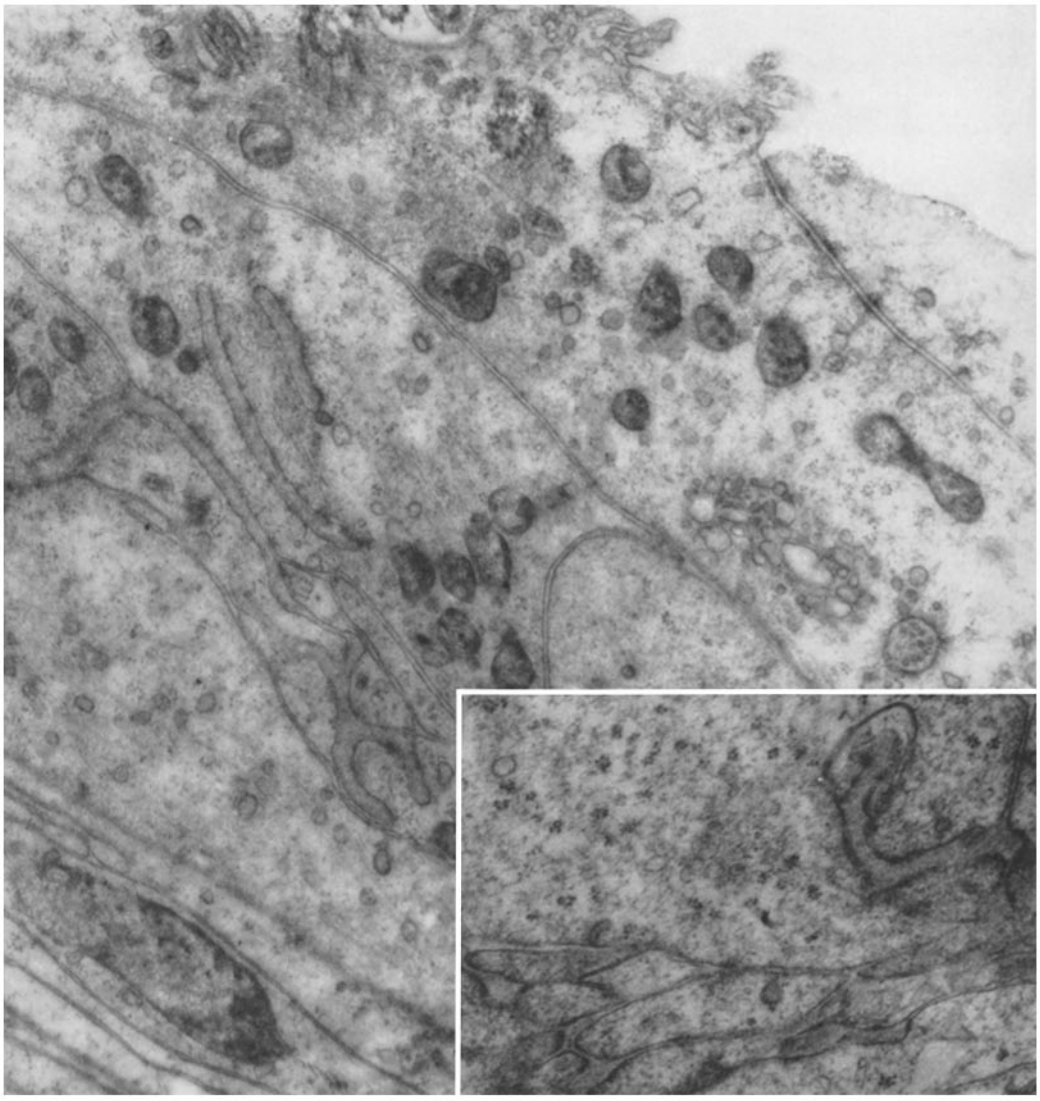


FIGURE 8 The bases of five ependymal processes meet to form canalicular confluences which contain a moderately dense filler and which are delimited by plasmalemmas bearing patches of dense cytoplasm. Little or no ferritin has entered this region of ventricle IV (upper right). 100 mg of ferritin for 1½ hours. × 23,000.

Inset. A focal dense confluence formed by four basal ependymal processes communicates with a network of narrower, filled distentions of the extracellular space. Very dense patches of subjacent cytoplasm occur along the plasmalemmas. Posterior ventricle III. No ferritin was injected. × 22,000.

had correspondingly diminished. In the present study, there were no appreciable changes in the width of the cells or interspaces, and yet ferritin injected *after fixation* was largely excluded from the luminal interspace. It is conceivable that after fixation, the properties of the dense substance within the interspace could have been altered so as to prevent diffusion into and through the junctional filler.

After entering the ependymal interspace of the living rat, ferritin accumulates in the filled distentions of the basal interspace. This region of the

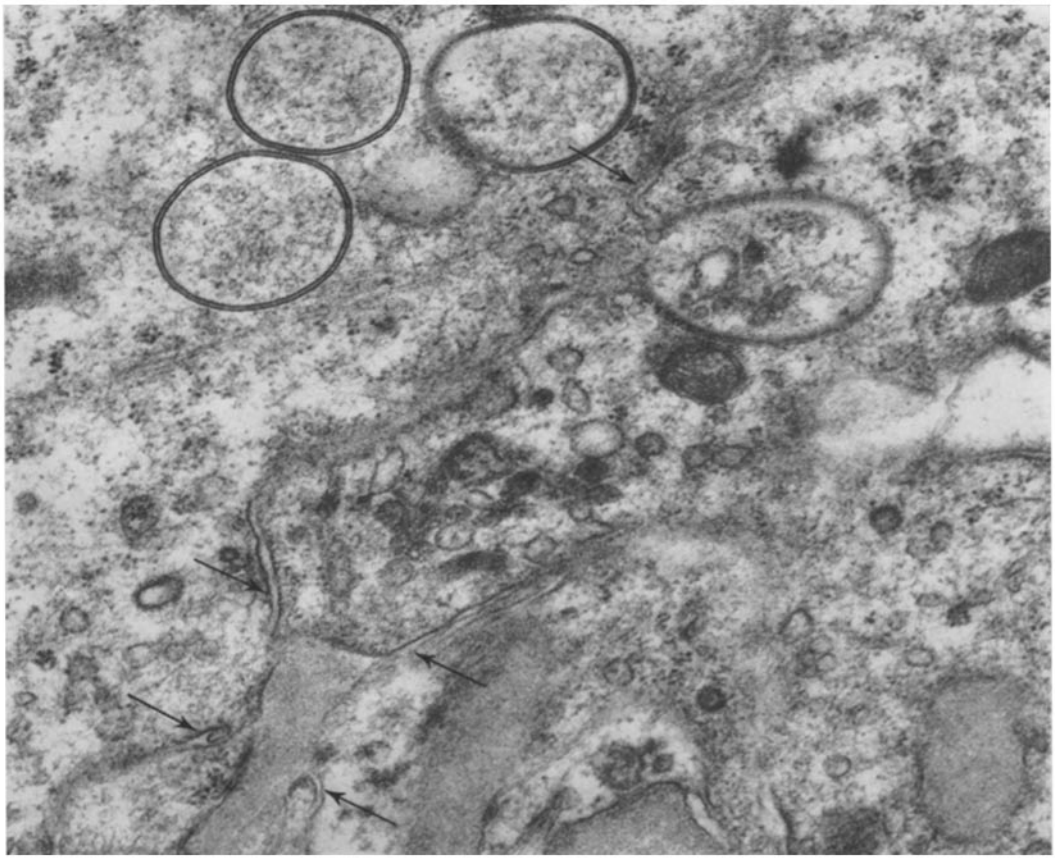


FIGURE 9 The usual interspace (arrows) which is abruptly distended into dense pools (bottom) is often as abruptly closed, within a fraction of a micron, by circular or oblate regions of membrane fusion; four of these intercellular fusions appear in this section. Neither basal interspaces nor dense pools contain ferritin. Ventricle IV. 35 mg of ferritin for 18 minutes. × 43,000.

interspace undergoes striking variations in dimensions within the compass of less than 600 A (Figs. 8 to 11). The regular 200-A-wide space may be abruptly widened at one site and obliterated by an intercellular fusion at an adjacent locus. The focal enlargements of the interspace contain a dense material similar in appearance, except for the absence of fibrils, to the basement membrane of renal epithelium (20, 36). These basal pools and their canalicular extensions are analogous to the pools of extracellular ground substance within the synaptic bed of Mauthner's cell (55) and between the packet glial cells of the leech's central nervous system (12). This dense filler, demonstrable after either formalin (55) or osmium tetroxide fixation, appears, therefore, to be a fundamental component of the cerebral extracellular space of different species. The dense filler of the ependymal pools may serve to filter out ferritin and other large particles in the same way that the renal (20) and cerebral (13, 9) perivascular basement membrane does.

Intracellular Passage

The intracellular movement of an appreciable amount of ferritin is coincident with the pinocytosis of a still larger fraction. Although the intercellular route has not been hitherto demonstrated, cellular uptake or phagocytosis of large particles has long been recognized (35). The pinocytosis of the ferritin molecule appears identical with the rapid ingestion of thorium dioxide by the epithelial cells of the ciliary body (44) and choroid plexus (64), and of ferritin by tumor cells grown in tissue cul-

ture (59) and by ganglia and their satellite cells (56).

However, the incorporation of protein within caveolae and vesicles of ciliary evaginations is unique. Vesicles occur within cilia (50) and modified cilia (15, 65) of sensory cells but have not been previously described in cilia of a non-sensory epithelium. The ependymal cilia, numbering about 40 per cell (11) and approximating 10 μ in length, may provide a considerably extended surface area for pinocytosis. Since, however, this process was observed in only one animal, its importance is still questionable.

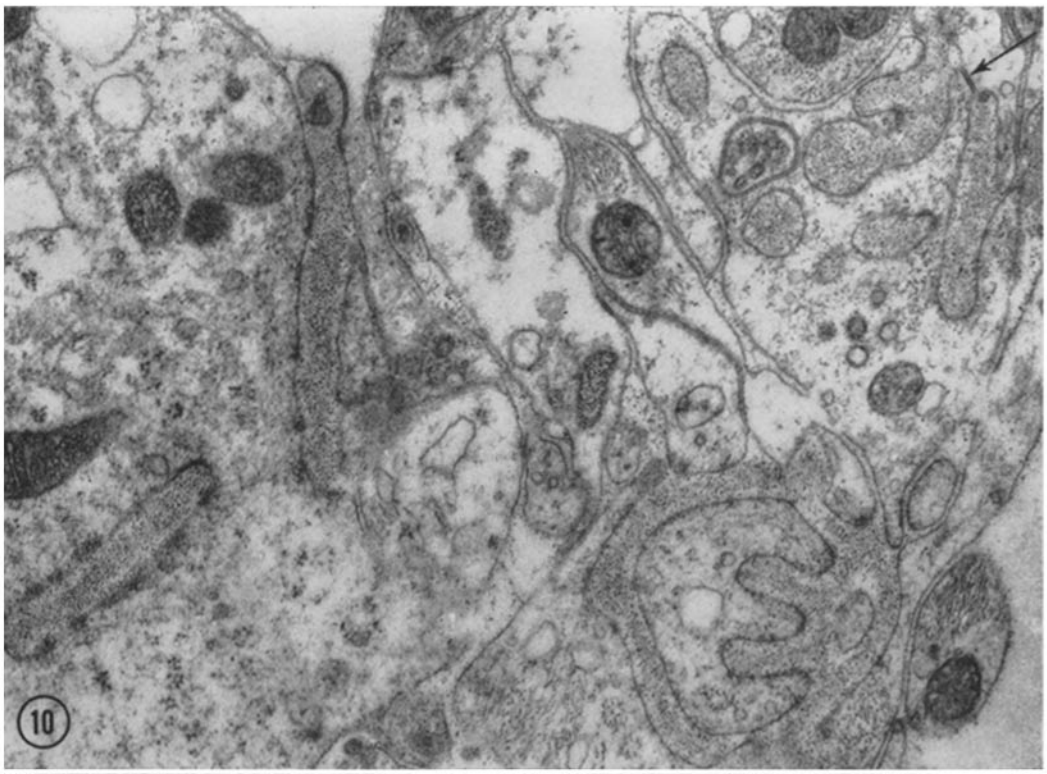
Admittedly, even the smallest amount of protein used in this study presented a highly abnormal environment for the ependyma, which is bathed by a fluid of exceptionally low protein content (see, *e.g.*, 4). The consequences of injecting this iron-containing protein might be comparable to those following intraventricular or subarachnoid (29) hemorrhage. Indeed, after the administration of hemoglobin, ferritin eventually appears within cytoplasmic membrane-limited organelles of various organs (53).

The organelles which segregate particles in ependymal cells are, primarily, the dense body and the multivesicular body. The multivesicular body amasses fewer micelles, which are presumably carried to this body in pinocytotic vesicles (9). The membranes of vesicle and multivesicular body probably make contact and coalesce, as suggested by formation of the thin, very dense shared membrane of contiguous vesicles (Figs. 14 and 15). The unusually narrow width of the common membrane may be due, in part, to an intermixing of membrane lipoprotein which may then be degraded and reallocated (3) so as to bring the contents of vesicle and multivesicular body into communication. Even if such a degradation did not occur, the solutes within the vesicle would have but one exceptionally thin membrane to cross in order to reach the internum of the multivesicular body or the extracellular space.

The chief repositories of pinocytosed ferritin are the dense bodies, some of which, because of their apical position, are presumed to be lysosomes (1). The apical distribution of fluorescent protein within ependymal cells, after injection of the labeled protein into the ventricles of the cat (34), may be accounted for by incorporation of the protein into dense bodies. The disposition of ferritin within a given dense body depends on its heterogeneous structure. In the "simple" body, the matrix or internum may be completely filled with micelles which are excluded from the light peripheral zone. Protein molecules are likewise absent from the presumably hydrophobic lipoidal droplets of the "complex" dense bodies. However, in both types of dense bodies, the ferritin can assume a crystalline arrangement. Crystals of ferritin occur not only in dense bodies of ependymal and glial cells, but also within a variety of cells in different species (5, 26, 53, 54, 62).

This crystalline arrangement provides a clue to the fate of the stored ferritin. An enzymatic hydrolysis of apoferritin within inclusions of amebae has been proposed on the morphological basis of aggregation of micelles into dense, amorphous masses (40). The occurrence of crystals, on the other hand, signifies that the apoferritin shell is retained (18). Conversely, a number of heavy metals, unassociated with protein, are segregated in random patterns within dense bodies (see, *e.g.*, 27). Apoferritin, furthermore, requires a prior drastic denaturation before it can be digested en-

FIGURES 10 AND 11 Two serial sections of the basal regions of ependymal cells illustrate the accumulation of ferritin within the filler and, to a lesser extent, the light zone of the intercellular pools. The remainder of the interspace contains micelles but in lower concentrations. Fig. 11 demonstrates the continuity of the lower left channel of Fig. 10 with the rest of the interspace. Conversely, Fig. 10 shows that the three oblate structures (*c*) of Fig. 11 are not vacuoles but rather parts of the lacunar system. Their ferritin content is not as great as that of the cytoplasmic vacuoles (*v*). The process in the upper right of both figures contains a considerable amount of free ferritin. In Fig. 10 the plasmalemma of this process is broken, but at the level of Fig. 11 it appears intact. Note the *macula occludens* between two canals in Fig. 10 (arrow at the upper right corner). Ventricle IV. 100 mg of ferritin for 2 hours. Fig. 10, × 31,000; Fig. 11, × 38,000.



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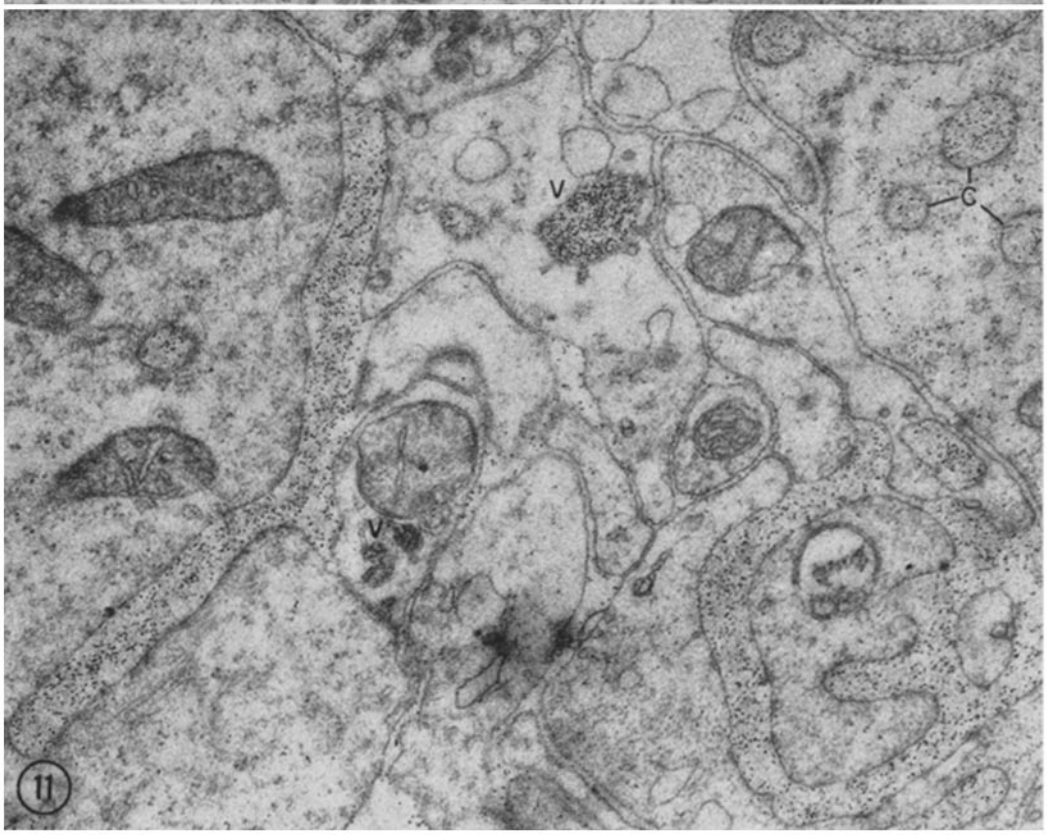

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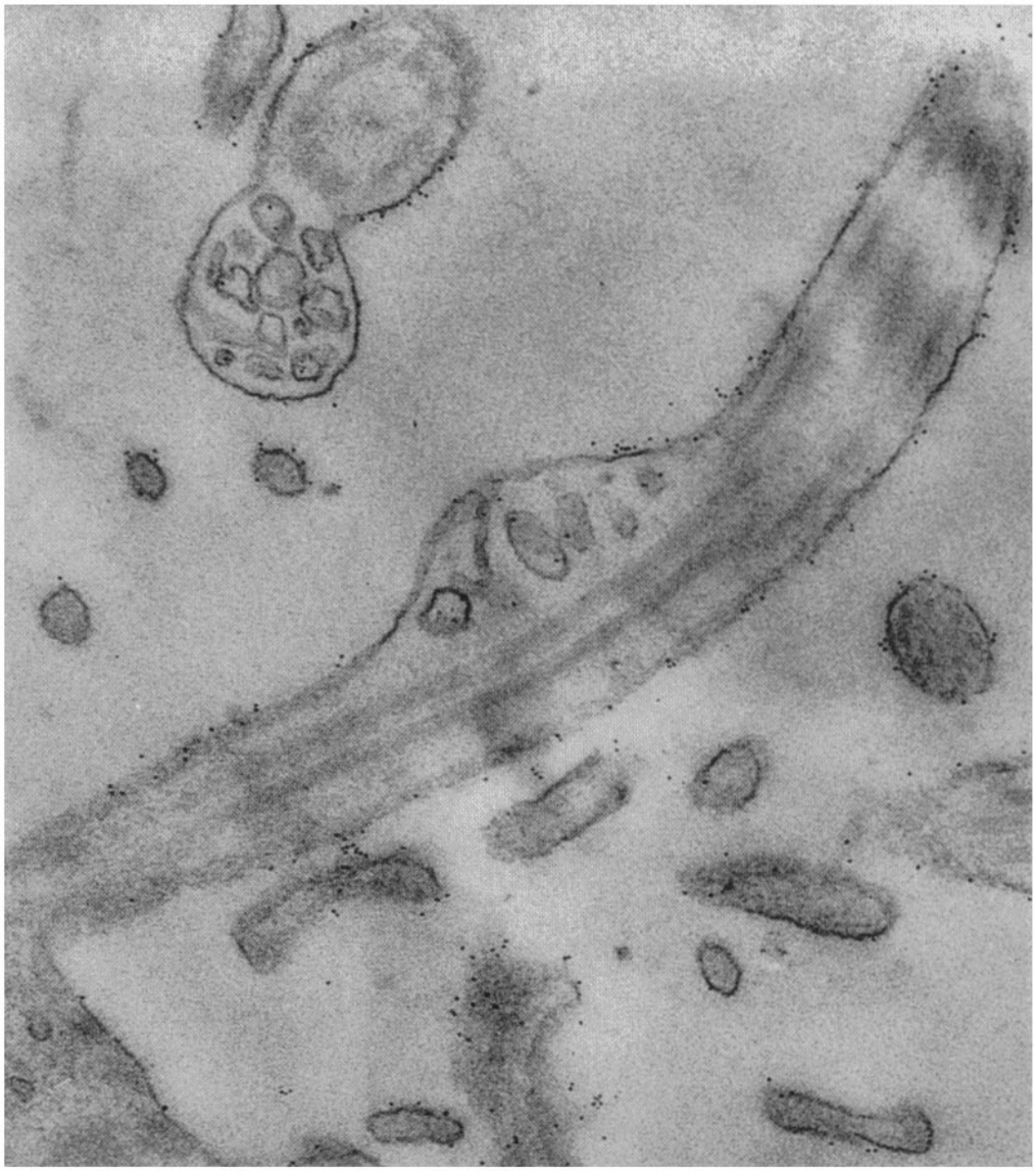


FIGURE 12 Ciliary evaginations contain ferritin-occupied tunnels and vesicles. Ferritin molecules lie immediately adjacent to or in contact with the plasmalemma and occupy a pinocytotic vesicle lying beneath the membrane. See general explanation of the figures. × 75,000.

zymatically *in vitro* (37). It would appear, therefore, that in a variety of cells over varying periods, the entire ferritin molecule, not merely its iron micelles, is sequestered undigested within the organelles.

Free Ferritin

Pinocytosed ferritin and Thorotrast are segregated in membrane-limited inclusions within cells of various tissues (5, 40, 44, 56, 64). A number of reports, however, contain descriptions of free fer-

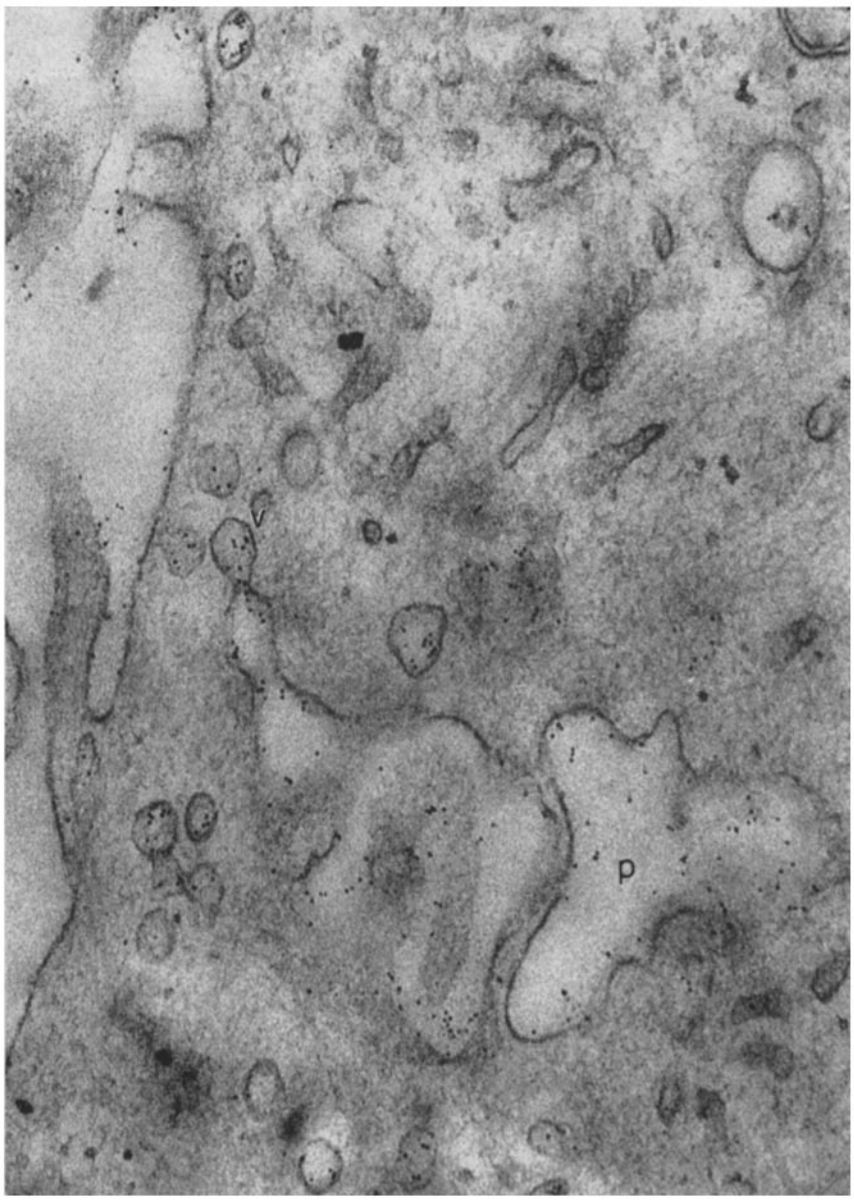
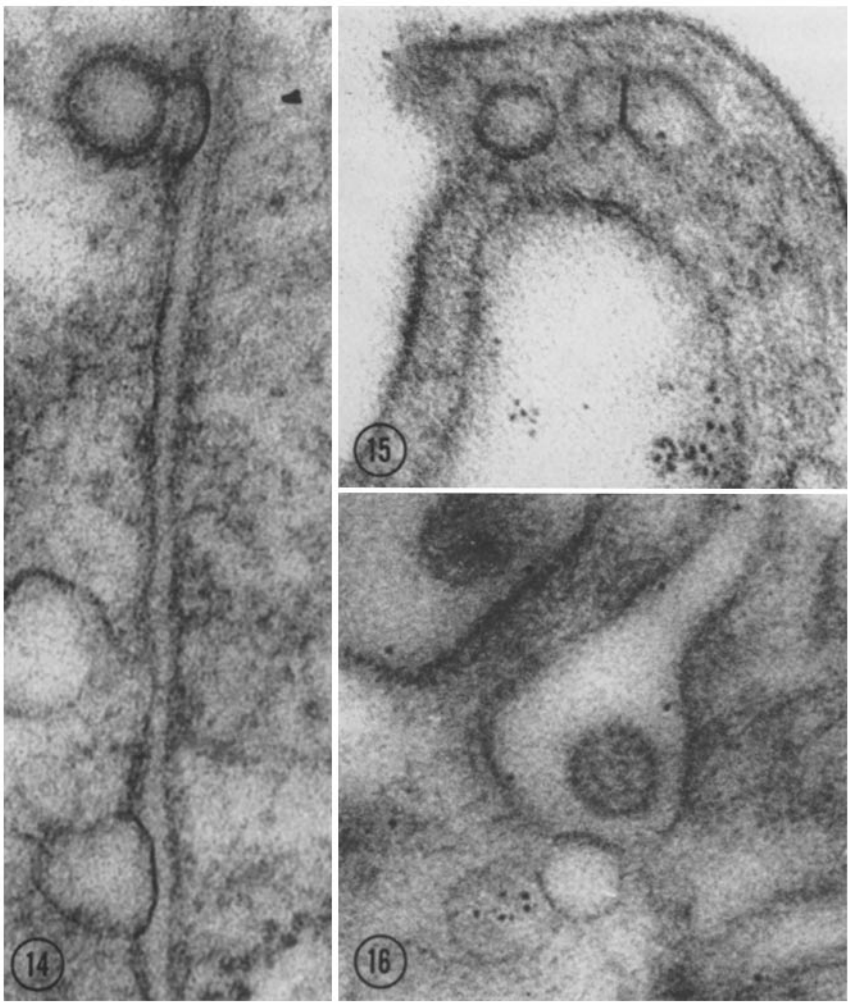


FIGURE 13 Pools (*p*) of ventricular fluid containing ferritin are mured by the irregularly folded plasmalemma, which is continuous with the membrane of a budding ferritin-laden pinocytotic vesicle (*v*). See general explanation of the figures. × 84,000.

ritin (6) together with membrane-enclosed ferritin (5, 26, 28, 31, 51, 53, 62). The occurrence of free cytoplasmic ferritin or colloidal gold has been interpreted by a few authors as the result of cell injury or death (*e.g.*, 56, 59). This conclusion is consistent with the finding that the deposition of free micelles follows multiple micropuncture of the plasmalemma for the repeated injections of ferritin into amebae (23).

The deposition of free ferritin throughout the ground matrix of well preserved cytoplasm (Fig. 17) is an artifact resulting from disruption of cell



FIGURES 14 TO 16 Eighteen milligrams of ferritin for 25 minutes.

FIGURE 14 The membranes of two swollen vesicles have apparently fused with the plasmalemma, which consequently bulges into the interspace. The coalescences are denser but not thicker than the remainder of the cell membrane. The upper fused vesicle appears to lie beneath a coated vesicle membrane. The lower fused vesicle and another vesicle are superimposed. Anterior iter. × 141,000.

FIGURE 15 Two of several ferritin-containing vesicles within this luminal ridge have come into contact. The shared dense membrane is about 50 A; the membrane of the far left vesicle is about 70 A thick. Anterior iter. × 167,000.

FIGURE 16 An empty vesicle has made contact with the luminal plasmalemma and the membrane of a ferritin-containing vesicle. The shared membrane of the two vesicles is denser and thinner (about 40 A) than the rest of the membrane. Posterior iter. × 134,000.

membranes. In a given section of a cell containing free molecules, the integrity of the plasmalemma or limiting membrane of organelles does not preclude damage some distance away (*cf.*, Figs. 10, 11, 17, and 25). The ruptures developing before or during fixation result in diffusion of micelles into and out of cells; consequently, a collection of many particles within a segment of the usual 200-A-wide

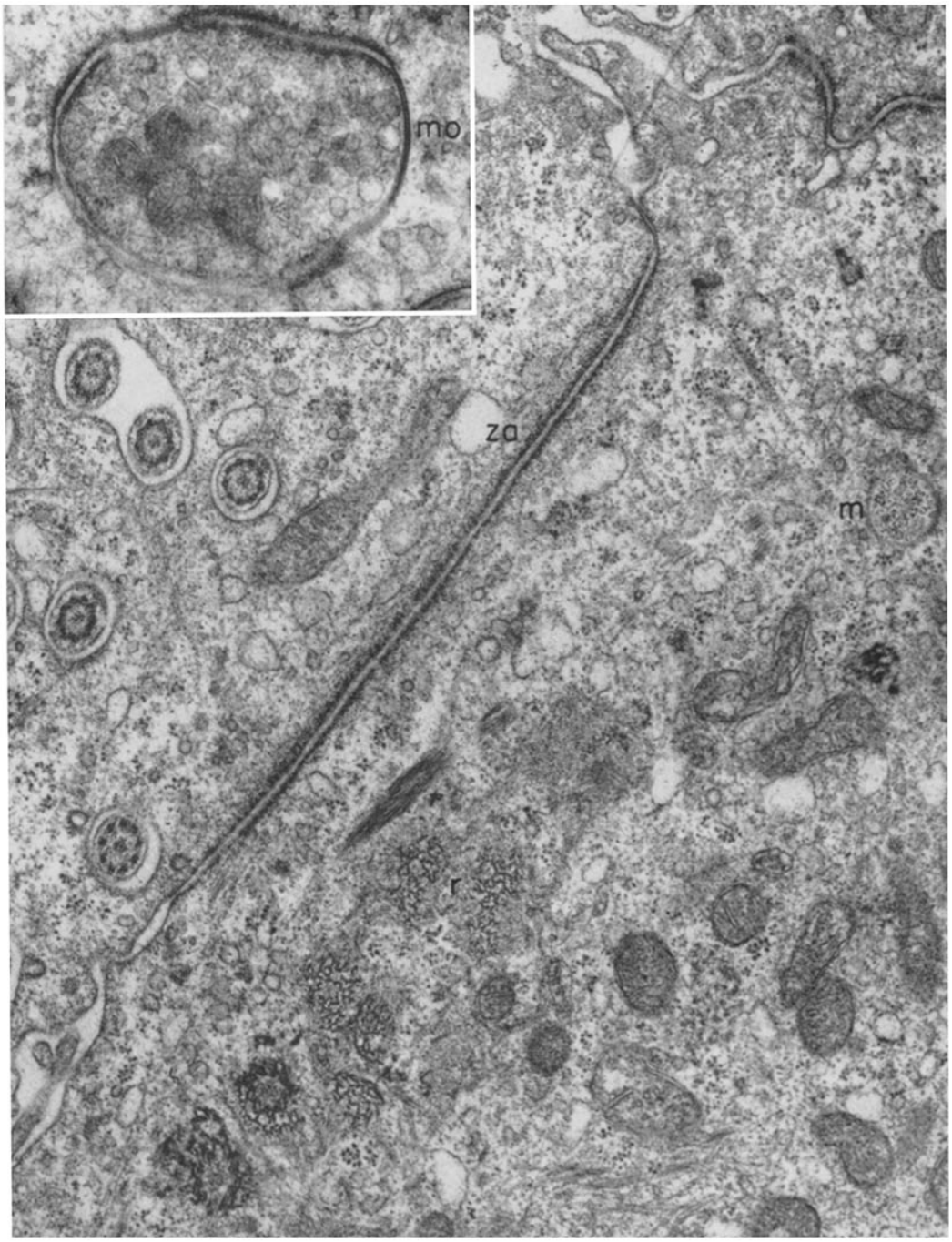


FIGURE 17 Two adjacent ependymal cells are joined by a *zonula adhaerens* (*za*) sectioned longitudinally; the upper luminal junction is a *macula occludens*. Free ferritin is dispersed within the cytoplasmic matrix of both cells despite the comparatively good state of preservation of plasmalemma and cytoplasm, including the basal bodies, their rootlets (*r*), and granular zones. A ferritin-laden multivesicular body (*m*) occurs at the right. Iter. 100 mg ferritin for 2 hours. × 30,000.

Inset. A *fascia adhaerens* alternates with a *macula occludens* (*mo*) between an ependymal cell and an invaginating cell process sectioned transversely. Anterior iter. See general explanation of the figures. × 37,000.

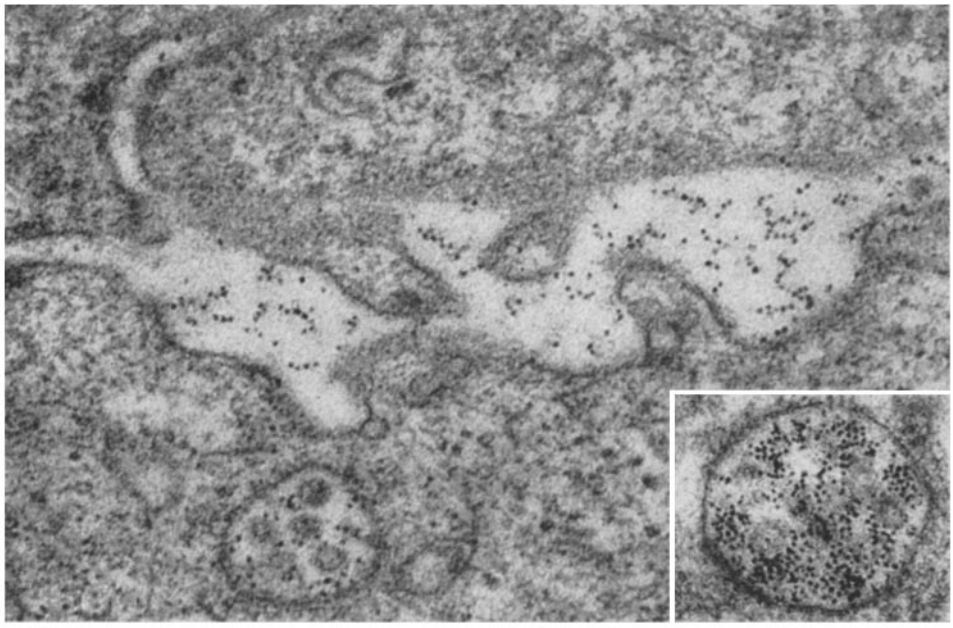


FIGURE 18 Ferritin has accumulated within a pool of ventricular fluid and within a multivesicular body. The micelles are dispersed among but not within the enclosed vesicles of this body and of the one in the inset. Anterior iter. 18 mg of ferritin for 25 minutes. × 97,000.

Inset. Posterior ventricle III. 20 mg of ferritin for 43 minutes. × 110,000.

interspace may also be suspect. As similar discontinuities arise in otherwise well fixed tissue from animals not receiving intraventricular injection, it is highly unlikely that the ruptures are caused by the hydrostatic pressure of the injection itself.

CONCLUSION

If the plasmalemma remains intact throughout the experimental procedures, the ferritin molecule traverses the ependyma only by pinocytosis and intercellular diffusion, not by intracellular diffu-

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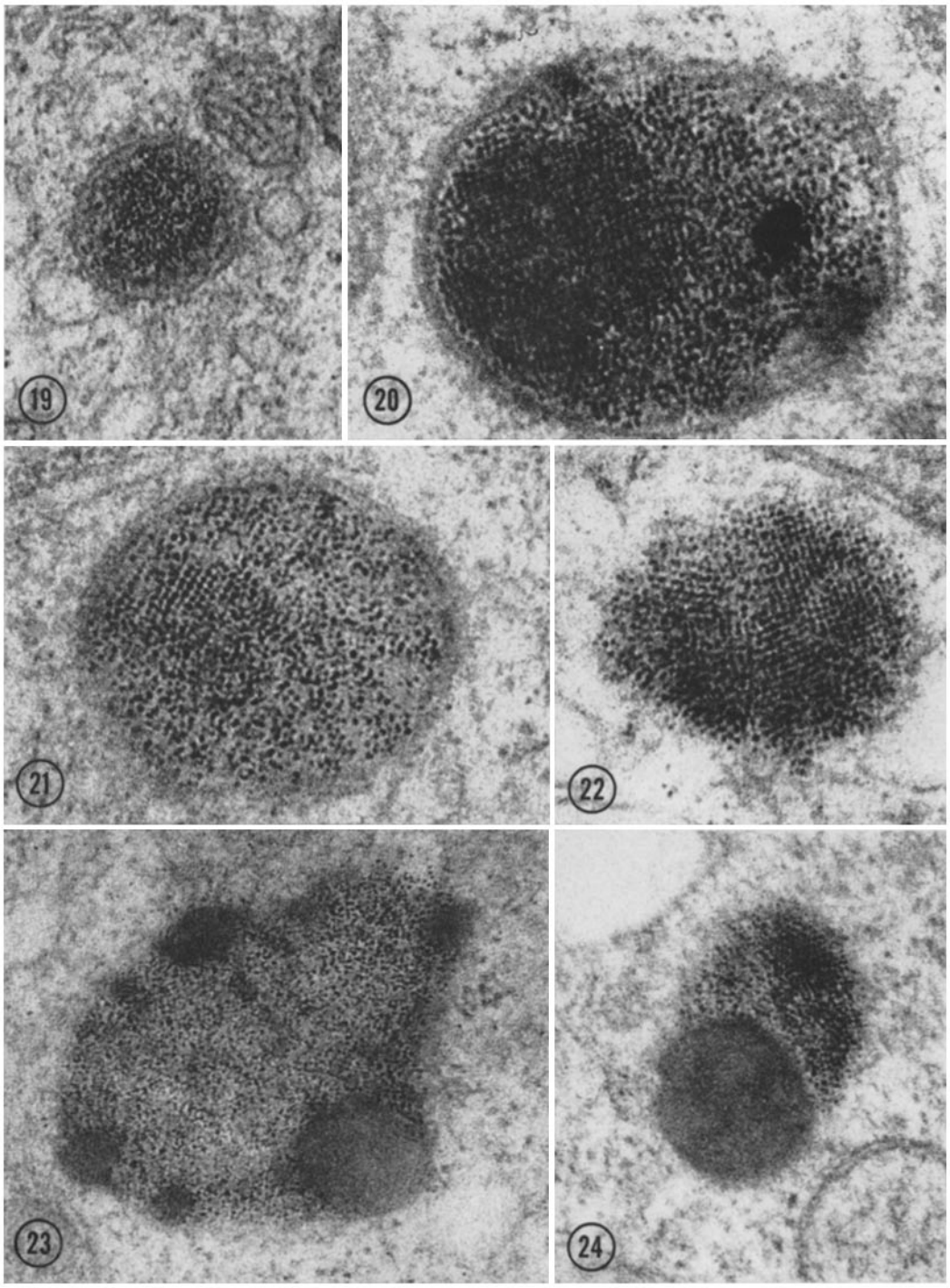
FIGURES 19 TO 24 Iter. 100 mg of ferritin for 2 hours. Free ferritin occurred in each of the cells. Fig. 20 illustrates a dense body from a subependymal glial cell.

FIGURES 19 TO 22 Four simple dense bodies illustrate various magnitudes of crystalloid packing. In Fig. 19 a dense body, about the size of a mitochondrion, contains ferritin and is delimited by a double membrane. The few free particles in the cytoplasm have not entered the adjacent mitochondrion. The dense body of Fig. 20 is also surrounded by a double membrane and contains micelles, most of which form a crystalloid array. In Fig. 21, the internum is not tightly packed with ferritin and only a part of the protein is arranged in ordered rows. In this dense body, the ferritin is excluded from a dense peripheral zone. In Fig. 22 the entire content of ferritin is packed in crystalloid fashion. This body has apparently been sectioned tangentially through its peripheral zone and thus appears membraneless. A comparatively large number of free molecules lie within the cytoplasm of this cell. Fig. 19, × 102,000; Fig. 20, × 143,000; Fig. 21, × 132,000; Fig. 22, × 143,000.

FIGURES 23 AND 24 The matrix of these complex dense bodies contains ferritin but their droplets do not. In Fig. 24 some of the micelles are aligned in ordered rows. Fig. 23, × 84,000; Fig. 24, × 100,000.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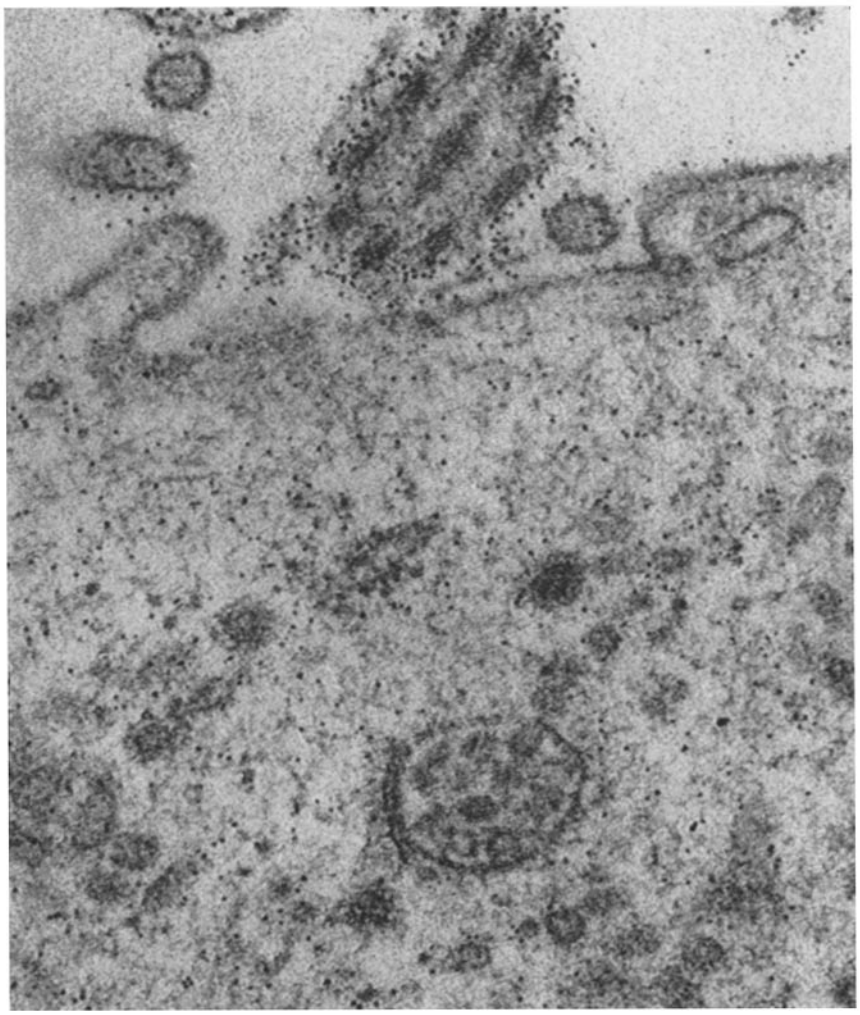

FIGURE 25 In this cell fixed prior to the introduction of ferritin, many micelles are adsorbed to the membrane and subfibers of cilia and are randomly dispersed throughout the cytoplasm. No molecules, however, have entered the multivesicular body, which is capped by a dense lamina. Note the discontinuities in the limiting membranes of this organelle and of the cilium. Anterior ventricle IV. 60 mg of ferritin. × 97,00.

sion. The protein, once having crossed the epithelium of the ventricles, accumulates within extracellular pools. The dense substance of basement membrane material and of dense bodies may thus be regarded as a system capable of trapping colloidal particles with the result that only a small fraction of the protein remains available to the underlying tissue.

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