

Evidence for Two Physiologically Distinct Gap Junctions Expressed by the Chick Lens Epithelial Cell

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Abstract. Lens epithelial cells communicate with two different cell types. They communicate with other epithelial cells via gap junctions on their lateral membranes, and with fiber cells via junctions on their apices. We tested independently these two routes of cell-cell communication to determine if treatment with a 90% CO₂-equilibrated medium caused a decrease in

junctional permeability; the transfer of fluorescent dye was used as the assay. We found that the high-CO₂ treatment blocked intraepithelial dye transfer but not fiber-to-epithelium dye transfer. The lens epithelial cell thus forms at least two physiologically distinct classes of gap junctions.

GAP junctions are probably the intercellular junctions responsible for low-resistance pathways between cells (2, 12, 32). They are structures that can be found in numerous tissues and organs and in nearly all animal species. Gap junctions from diverse sources might be a single class of intercellular junction, composed of a single protein species or a set of proteins that have been conserved throughout the evolution of the different phyla. However, a number of lines of evidence indicate that there may be classes of gap junctions that are distinct from one another.

Gap junctions from different sources are sometimes anatomically different from each other, particularly as viewed by freeze-fracture electron microscopy (23). Biochemical comparisons of junctions isolated from different organs have demonstrated nonhomology between the component peptides (14, 17, 26). Functional differences between junction types are evidenced by different sieving properties of junctions that join cells in culture, depending on the cell type used (10); and a gap junction that connects two cell lines that normally make junctions with different permeability characteristics can be asymmetrically permeable (9). Where experimental systems have been developed to perfuse the inner surfaces of gap junctions with test solutions, junctional permeability is found to be sensitive to hydrogen ions in one case (35) and insensitive in another (19). In addition, a differentiating cell may express anatomically and physiologically distinct gap junctions at different developmental stages (33).

These observations can be taken to suggest that gap junctions are more than a single class of intercellular junction. However, in each case the junctions compared faced the cytoplasm of different cell types, were isolated via different methods, or were subjected to different experimental conditions. Some of the evidence strongly indicates that gap junctions can be nonidentical, particularly the biochemical evidence (14, 17, 26) and the data gathered on perfused junctions (19, 35). But the most effective means of comparing gap

junctions is to do so in a system in which a single cell type expresses more than one junction type.

A cell at the boundary of two groups of cells that are unlike and functionally coupled to each other must make gap junctions to communicate with its own cell type (homotypic junctions) and the other cell type (heterotypic junctions). These two routes of communication are not necessarily mediated by different classes of gap junctions. There are examples of electrical coupling between embryonic tissues (30, 34), between developmental compartments (5, 38), and between unlike cells in a single tissue (28, 31). In several cases gap junctions connecting two different cell types in a single tissue have been observed anatomically (1, 8, 15, 18, 20, 25, 27). In culture, some cell lines will form heterotypic junctions whereas others will not (6, 7, 11).

Freeze-fracture observations have demonstrated anatomical differences between homotypic and heterotypic junctions in chick lens (13) and goldfish saccular macula (15). There is also a report of morphologically distinct homotypic junctions between rat intestinal epithelial cells (36). An example of a functional difference between two gap junctions comes from insect epidermis, where fluorescent dye does not pass the intersegmental border even though the segments are electrically coupled (39). There is an example of a difference in regulatory properties from a culture system in which rat neonatal ventricular cells are coupled to each other and to fibroblasts. The junctional permeability of the homotypic junctions is not sensitive to the uncoupling properties of the calcium ionophore A23187, whereas the heterotypic junctions are sensitive to the treatment (4).

In this paper we report the first evidence for a difference in regulatory properties between two naturally occurring gap junctions from a single cell type. Embryonic lens epithelial cells of the chick communicate with other epithelial cells via gap junctions on their lateral membranes (homotypic junctions), and they also communicate with lens fiber cells via

gap junctions on their apices (heterotypic junctions). We have studied the passage of fluorescent dye through these two junction types under normal in vitro conditions and after incubating the lens in medium equilibrated with 10% O₂/90% CO₂.

Materials and Methods

Fertilized Leghorn chicken eggs (Spafas, Inc., Norwich, CT) were incubated at 37°C for 6 d, and embryos between developmental stages 23 and 28 were obtained (16). Corneas were removed and the lenses were dissected free of the surrounding optic cups. The dissections were performed in Eagle's minimal essential medium with Earle's salts (Gibco Laboratories Inc., Grand Island, NY), equilibrated with 95% O₂/5% CO₂ at 37°C.

Electron Microscopy

Lenses were placed directly from control medium into a fixative solution composed of 1% paraformaldehyde, 2% glutaraldehyde, 90 mM NaCl, 30 mM Hepes (Sigma Chemical Co., St. Louis, MO), and 5 mM CaCl₂. All lenses were fixed overnight.

For thin sectioning, lenses were postfixed in 2% OsO₄ in water for 2 h and then block stained in 1% uranyl acetate in water for an additional 2 h. Lenses were dehydrated in ethanol and embedded in Epon-araldite. Thin sections were cut using an MT-5000 ultramicrotome (Sorvall Instruments, Wilmington, DE) and were stained on the grid with 1% Pb citrate in 0.1 N NaOH.

For freeze-fracture, aldehyde fixed lenses were equilibrated for 1 h with 30% glycerol in the same buffered salts used for fixation. They were then frozen in Freon 22 and fractured in a Balzers BAF 301 freeze-fracture machine (Balzers Corp., Hudson, NH) according to standard procedures. The lenses were mounted with the epithelium facing upward and were fractured at the depth appropriate for viewing the epithelium-fiber interface.

Dye Transfer Experiments

Properties of dye transfer between epithelial cells were examined by injecting the fluorescent dye Lucifer yellow CH (37) into single epithelial cells and using fluorescence microscopy to monitor its passage into adjacent cells. The lenses were mounted on edge in a Lucite chamber, with the anterior (epithelial) surface of the lens facing the microelectrode. Temperature was maintained at 37°C by a peltier thermionic heater (Midland-Ross Corp., Cambridge, MA) built into the stage of the Zeiss IM-35 inverted microscope. After a control injection was made in each lens, the medium was changed to one equilibrated with 10% O₂/90% CO₂. After 10 min one or more experimental injections were made, and then the lens was returned to control medium for 20 min before being injected again to assay for recovery from any block of dye transfer that had occurred in the high-CO₂ medium. Fluorescence and bright-field photographs were taken at each stage of the experiment. Details of the mounting procedure, and of enzymatic digestion of the lens capsule, iontophoretic injection of fluorescent dye, and photographic techniques have been previously published (24, 33).

The whole-mount preparations were not sufficient for assessing dye transfer properties at the epithelium-fiber interface. The transfer of dye from epithelium to fibers was difficult to detect because of the large sink for any dye transferred into the fibers, and the properties of dye transfer from the fibers to the epithelium could not be unequivocally determined in whole-mounts because of the thinness and curvature of the epithelium. The experiments were therefore performed by the mounting of lenses as above but with the posterior surface of the lens facing the microelectrode. The lens was then incubated in control medium or in the high-CO₂ medium. In each lens a single lens fiber was impaled and fluorescent dye was iontophoresed into it for ~10 min. After the injection, the lens was quickly placed into a fixative solution composed of 2% paraformaldehyde in 0.1 M cacodylate buffer for 1 h. Each lens was embedded in JB-4 (33), and serial sections were cut on a dry glass knife and individually flattened on tiny droplets of distilled water. After the sections were surveyed by fluorescence microscopy to locate the cloud of dye, phase-contrast and fluorescence photographs were taken of the epithelium-fiber interface.

Results

Electron Microscopy

A thin section of the epithelium-fiber interface of an embryonic lens is presented in Fig. 1A. The epithelial cells and the

fiber cells make close membrane associations with both cell types. Not all of these membrane appositions are necessarily gap junctions, but freeze-fracture electron microscopy demonstrates the presence of epithelial-epithelial gap junctions (Fig. 1B) (3, 13, 22, 24, 29), epithelium-fiber gap junctions (Fig. 1C) (13), and fiber-fiber gap junctions (Fig. 1D) (3, 21, 22, 29, 33). The postfixation morphologies of these three types of gap junctions are not identical. The most striking difference is that the epithelial-epithelial junctions have tightly grouped or crystallized connexons (24, 29), whereas the epithelium-fiber junctions and the fiber-fiber junctions display a more random distribution of connexons.

Intraepithelial Dye Transfer

Fig. 2 and Table I demonstrate that intraepithelial junctional permeability is sensitive to high-CO₂ treatment. Fig. 2A shows a bright-field image of the lens viewed with fluorescence in Fig. 2, B-D. The fluorescence micrograph in Fig. 2B shows that a control injection created a cloud of fluorescent dye that filled a group of epithelial cells. After a change to medium equilibrated with 10% O₂/90% CO₂, a second injection filled a single cell (Fig. 2C, Table I); the dye was restricted from moving into adjacent epithelial cells because of a decrease in junctional permeability that occurred in the high-CO₂ medium. Due to the small size of the cells, direct intracellular pH measurements were not attempted. After a return to control medium, a final injection of fluorescent dye demonstrated a recovery from the block of dye transfer (Fig. 2D and Table I). This reversible block of intraepithelial coupling was similar to that previously found in younger lenses that had not yet established epithelium-fiber coupling (24). In general, it was not possible to observe dye transfer in our experimental cell after return to control conditions. This may be due to binding of the dye to cytoplasmic components, damage to the cell concomitant with removal of the microelectrode, or both.

Fiber-to-Epithelium Dye Transfer

Fig. 3, A and B, respectively, show phase-contrast and fluorescence micrographs of an embryonic lens that was injected posteriorly with fluorescent dye. This lens was incubated in the high-CO₂ medium before the injection. It is evident from Fig. 3B that the dye was not restricted from entering the epithelium; dye transfer via epithelium-fiber gap junctions was not blocked in the high-CO₂ medium (Table I). When lenses were incubated in control medium before injection, the fluorescent dye moved from the fibers to the epithelium in every case (Table I). There is evidence that this pathway is also open to the diffusion of low molecular weight metabolites (13) and ions (31). It is also clear from Fig. 3B that fiber-fiber coupling is insensitive to the CO₂ block, a finding similar to those obtained in younger lenses (33).

Table I. Physiological Data (Number of Embryos)

Experiment	Dye transfer not blocked	Dye transfer blocked	No. recovered (attempted)
Intraepithelial coupling, 90% CO ₂	0	13	10 (11)
Epithelium-fiber coupling, 90% CO ₂	17	0	—
Epithelium-fiber coupling, controls	17	0	—

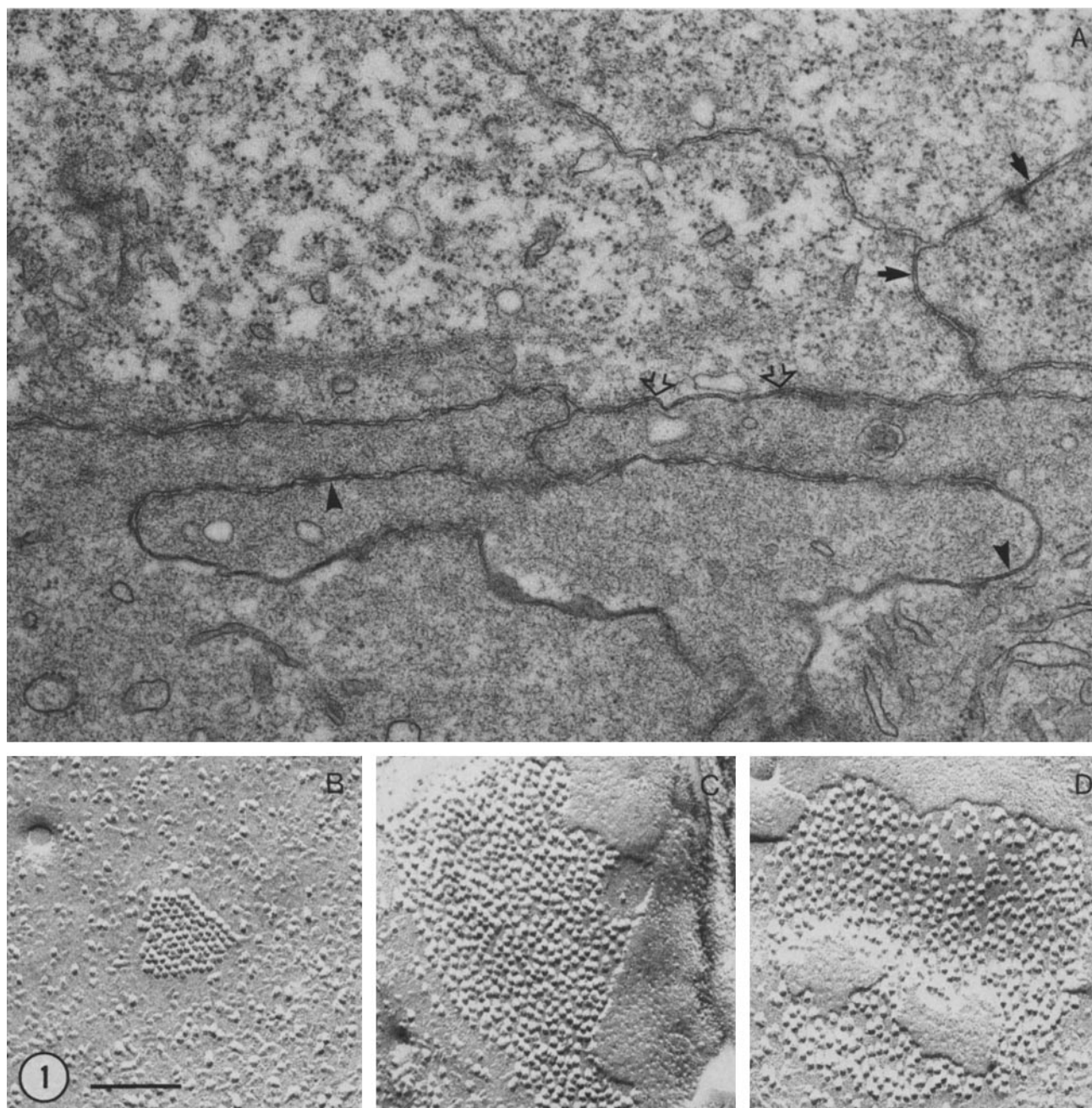


Figure 1. *A–D* are electron micrographs of embryonic lenses. The thin section in *A* shows the epithelium-fiber interface. Numerous close appositions of the cell membranes are visible; these interactions can be epithelial-epithelial (solid arrows), epithelium-fiber (open arrows), or fiber-fiber (arrowheads). *B–D* are freeze-fracture electron micrographs demonstrating the postfixation morphologies of the three junction types, epithelial-epithelial, epithelium-fiber, and fiber-fiber, respectively. Bars: (*A*) 0.15 μm and (*B–D*) 0.1 μm .

Fig. 3, *C* and *D* are micrographs of a lens that had not yet established epithelium-fiber coupling, even though the two cell types were closely apposed. The lens was injected posteriorly after incubation in control medium. This lens is presented to demonstrate that the fluorescence seen in the epithelium in Fig. 3*B* is due to dye transfer from the fibers rather than caused by some artifact of preparation. A comparison of Fig. 3, *C* with *D* reveals that even though the fiber cells filled with the dye, no dye passed from the fibers to the epithelium. This is a stage 22 lens; epithelium-fiber coupling was never observed in lenses younger than stage 21, and it was always present by stage 23.

Discussion

The data presented in this paper demonstrate that the two morphologically distinct gap junctions through which lens epithelial cells communicate with adjacent cells are also distinct physiologically. Because the two types of gap junctions connect the epithelial cells to other epithelial cells in one case, and to fiber cells in the other case, it is possible to test them separately for a decrease in junctional permeability in response to high- CO_2 treatment. By performing two experiments, one involving the injection of fluorescent dye into epithelial cells and the other involving dye injection into fiber

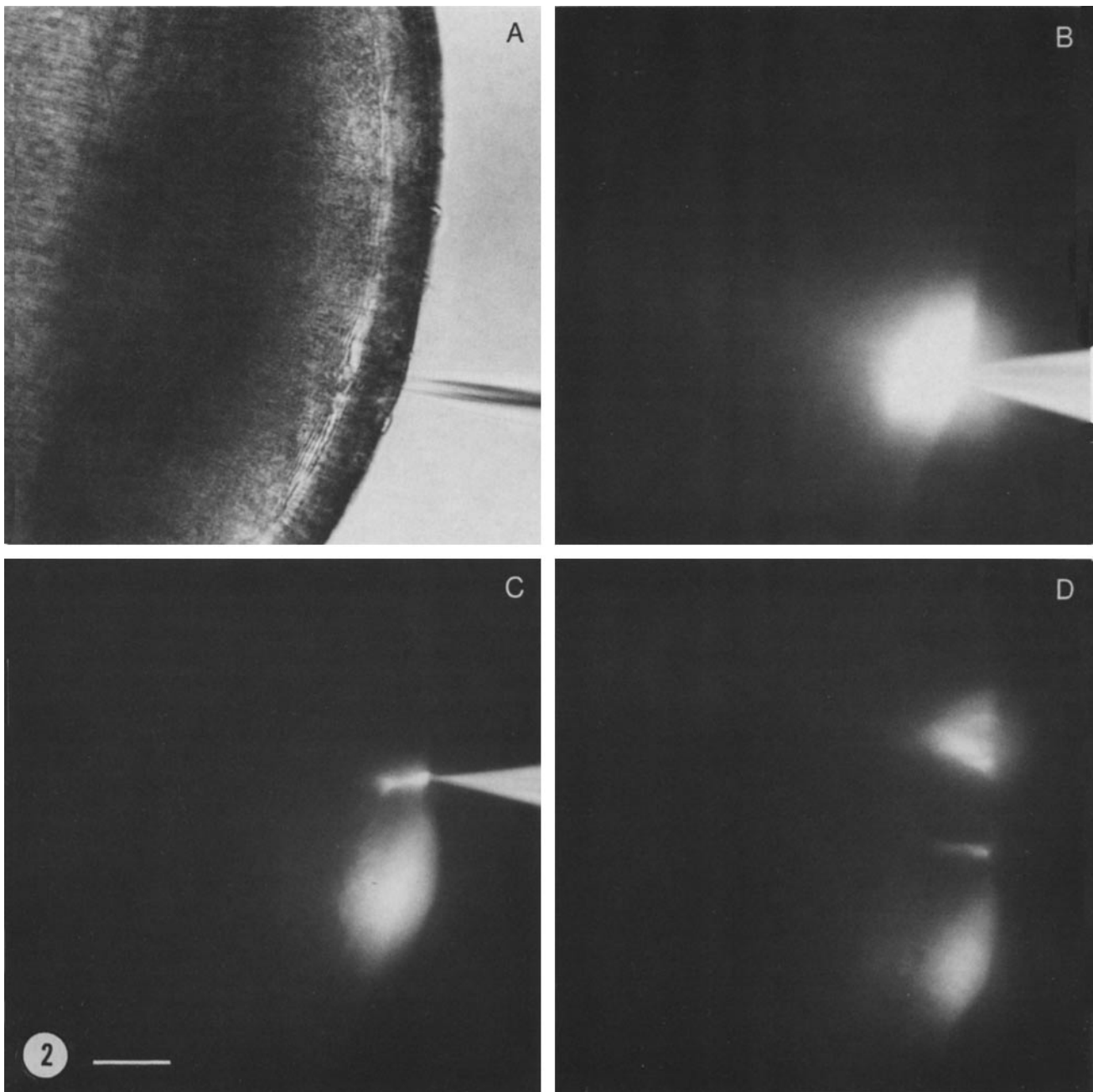


Figure 2. *A* is a bright-field micrograph of a living embryonic lens mounted for an experiment testing intraepithelial coupling. *B–D* are three consecutively taken fluorescence micrographs of the same lens. In *B* a control injection of fluorescent dye filled a group of epithelial cells. *C* shows an experimental injection made in medium equilibrated with 10% O₂/90% CO₂. The fluorescent dye filled a single epithelial cell. *D* was taken after a return to control medium and a third injection. The final dye injection again filled a group of epithelial cells; thus, it demonstrates recovery from the CO₂-induced block of dye transfer. Bar, 25 μm.

cells, we have shown that whereas intraepithelial coupling was sensitive to incubation in a high-CO₂ medium, epithelium-fiber coupling was not sensitive.

Because the two gap junction types considered reside in the membrane of the same cell, the observed distinction in control of junction permeability cannot be directly attributed to a difference in the environments of the junctions. It is true that one half of an epithelium-fiber gap junction is provided by the fiber cell and faces the cytoplasm of that cell. These junctions might therefore be heteromolecular. They may be composed of two different types of connexons, one epithelium

specific and the other fiber specific. However, we assume that if the connexons on one side of the junction are closed, then that junction is uncoupled regardless of the condition of the connexons provided by the other cell. Since epithelial-epithelial gap junctions are sensitive to the CO₂-induced block of dye transfer, the epithelium-fiber junctions would also be sensitive if the epithelial halves of the junctions were identical to the junctions connecting the epithelial cells.

It can be argued that the observed results are due to there being many more junctions connecting the epithelium to the fiber cells than there are connecting epithelial cells to each

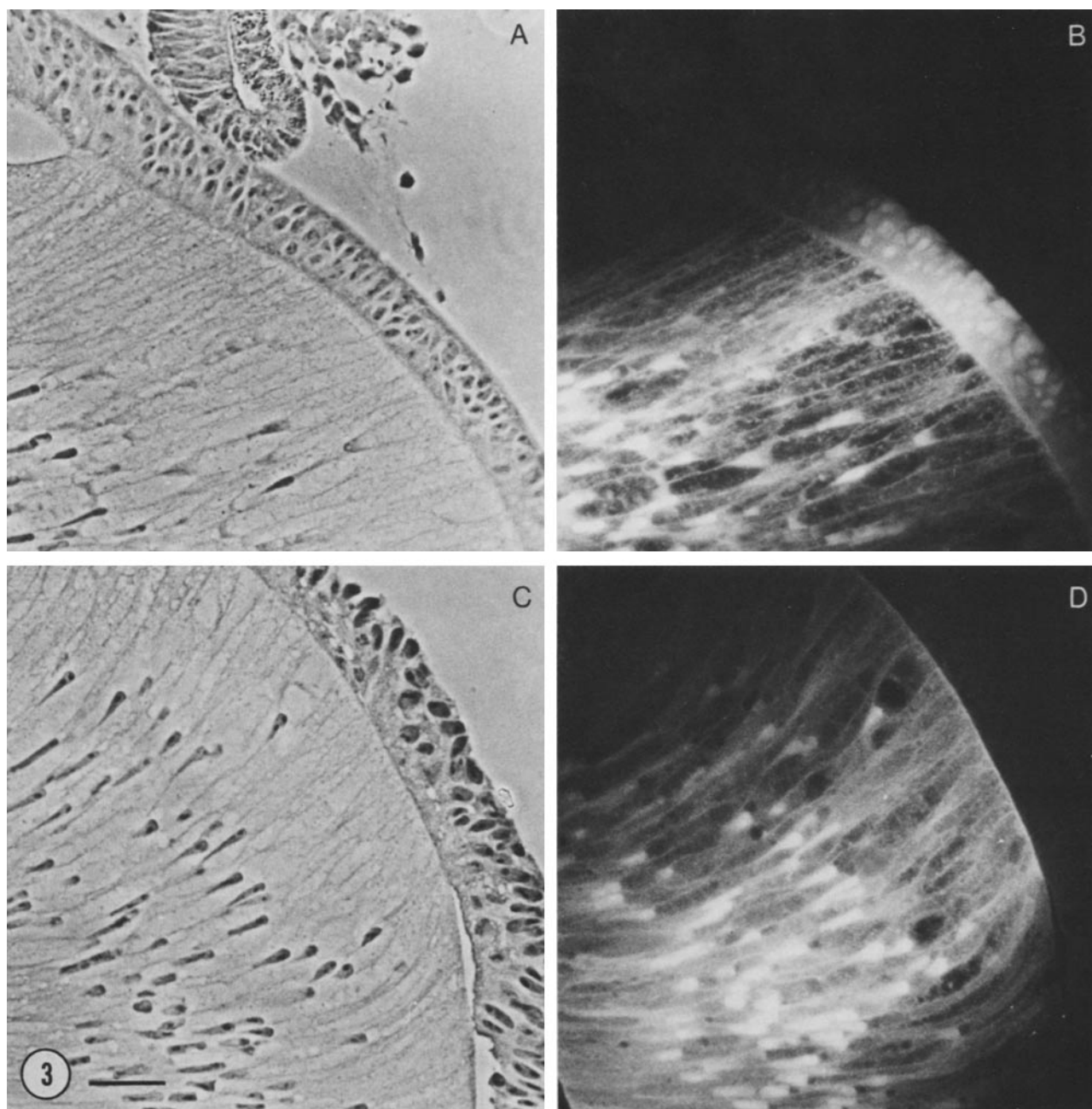


Figure 3. *A* and *B* are phase-contrast and fluorescence micrographs, respectively, of a 5- μ m section of an embryonic lens. The lens was soaked in high-CO₂ medium before fluorescent dye was injected into a fiber cell. The epithelium is filled with fluorescent dye in *B*, indicating that epithelium-fiber coupling was not blocked by the high-CO₂ treatment. Two similar micrographs of a younger lens are shown in *C* and *D*. This lens had not established epithelium-fiber communication, even though the two cell types were in contact. The absence of fluorescence in the epithelium in *D* illustrates that the positive epithelial fluorescence in *B* is not due to an artifact of preparation. Bar, 25 μ m.

other. Then, if the high-CO₂ treatment reduced the permeability of both sets of junctions equally, dye transfer might fall below detectable limits for intraepithelial coupling and still be detectable between the epithelium and fibers. This possibility cannot be answered directly, since it is not possible to calculate the relative numbers of active connexons connecting the two cell types. The geometry of the cells makes it difficult to estimate accurately the areas of close membrane appositions that are present between the different cell types. Even if such estimates were obtained, it would be impossible to know

if all of the close appositions are gap junctions. In addition, the number of connexons per unit junctional area may differ (intraepithelial gap junctions condense when fixed [24]), and there is no way of being sure what fraction of the connexons present is actually involved in communication. With these limitations in mind, it has never been our impression, from the thin sections or the freeze-fracture replicas, that either the lateral or apical surfaces of the epithelial cells have many more gap junctions than the other surface.

The study of homotypic and heterotypic gap junctions

expressed by a single cell type permits a direct comparison of the structural and physiological characteristics of gap junction-mediated cell-cell communication. Evidence has accumulated, through the use of a number of experimental approaches, that suggests that gap junctions from different sources may be nonidentical. The data presented in this paper contribute to the emerging view of gap junctions as a diverse set of intercellular junctions with different structures, biochemistries, functions, and regulatory properties, depending on what cells and tissues they connect.

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