FINE STRUCTURAL DEMONSTRATION OF ORDERED ARRAYS OF CYTOPLASMIC FILAMENTS IN VERTEBRATE IRIDOPHORES

A Comparative Survey

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ABSTRACT

Thin and thick sections of both physiologically active and physiologically passive iridophores from a range of vertebrate species have been examined by electron microscopy at 60 kV and at 1,000 kV. All iridophores studied have been found to contain 65-Å filaments linking successive crystals in their parallel stacks; their orientation in the cell is shown in stereo pairs of 0.25-μm sections obtained from high voltage microscopy. In addition, several of the physiologically passive iridophores contain 100-Å filaments in varying numbers. It is suggested that the thin filaments might be iridophore actin and play a role in the movement of iridophore components, and that the 100-Å filaments might play a cytoskeletal role in the iridophores in which they occur.

INTRODUCTION

It has been known for a long time that iridophores, the iridescent cells almost ubiquitous in the animal kingdom, are unique among chromatophores in their production of structural color. Their colors arise solely from the interference of light scattered from precisely ordered crystalline deposits in their cytoplasm. This ordering is generally manifested as precise parallel stacking of alternating crystalline and cytoplasmic sheets of highly uniform thickness. The reflecting crystalline sheets, generally on the order of 1000 Å thick, range in form from the single broad platelets of the fish iridophore to the mosaics of crystal tablets of the *Anolis* iridophore. Whatever their form, however, their precise parallel stacking in the cell suggests that multiple thin-film interference is the primary mode of color production in most iridophores (2, 12, 16).[1]

The importance of filaments in the control of iridophore color production has come under investigation only recently. Extensive filament networks were demonstrated in the iridophores of *Anolis*

[1] Only in one particular dermal iridophore, that of the frog *Agalychnis dachnicolor* (20, 21), does the array of crystals in the cell appear to be random and the form of the small crystals almost round. In this case, color production probably results entirely from Tyndall scattering, which would be preferentially blue for these small crystal scatterers. Tyndall blues lack the iridescence seen in thin-film interference colors.

carolinesis in 1972 (16). It was suggested that these filaments played a cytoskeletal role, at least in these passive iridophores, serving to hold the iridophores' crystalline sheets in their strict parallel array. It was further suggested that dynamic iridophores, those that had been reported to undergo active physiological changes, might contain motile filament systems responsible for mediating cellular changes by altering the array, spacing, or tilt of cellular crystals. Some support for this hypothesis has come from the demonstration that, even in *Anolis*, the filaments are at least partially composed of actin (17), suggesting that a potentially active actin-myosin system might be present in the iridophores of this and other species.

The purpose of this limited survey is to investigate the ubiquity of filament occurrence in iridophores generally, and in dynamic iridophores, particularly. Iridophores examined range from the dermal iridophores of lizard skin, fish scales (both marine and freshwater species), and frog skin to the tapetal iridophores of the fish eye.

MATERIALS AND METHODS

The species used were cardinal tetra and goldfish, both freshwater teleosts (obtained from the Golden Leash Pet Shop, Boulder, Colo.), *Holocentrus ascensionis*, a marine teleost (obtained from The Fish Tank, Marathon Shores, Fla.), *Rana pipens*, the leopard frog (obtained from Blue Spruce Biological Supply, Castle Rock, Colo.), and *Anolis carolinensis*, a lizard (obtained from Carolina Biological Supply Co., Elon College, N. C.).

The frog, lizard, and cardinal tetra were killed by decapitation. Small squares of frog and lizard dorsal skin and the entire lateral blue stripe and eyes of the cardinal tetra were cut out with a clean, sharp razor blade, and fixed immediately. The eyes were cut in half after ½ h of fixation for better penetration of the fixative during further fixation.

Goldfish and *Holocentrus* scales were simply plucked from the sides of the living fishes with flat forceps and fixed whole, immediately.

All species except *Holocentrus* were fixed in 3% glutaraldehyde in either 0.1 M Sorenson's phosphate buffer, pH 7.3, containing 8.6% sucrose and 0.005% of $CaCl_2$, or 0.1 M sodium cacodylate buffer, pH 6.8, containing 8.6% sucrose. After fixation for 2 h, the specimens were washed in the same buffer overnight at 4°C, postfixed for ½ h with 1% osmium tetroxide in the same buffer without sucrose, and washed briefly in water. En bloc staining of some specimens was done at this stage after a 2-h wash in 15% acetone: the specimens were left in 0.5% uranyl acetate in 0.1 M s-collidine buffer, pH 4.6, in the cold and dark overnight, then rinsed briefly with water. All specimens were then dehydrated through an acetone series and embedded in Epon-Araldite.

Thin (silver-gold) and thick (0.25 μm) sections were cut with a diamond knife on a Porter-Blum MT-1 ultramicrotome (Ivan Sorvall, Inc., Newtown, Conn.). Thin sections were stained for 5 min each with alcoholic uranyl acetate and Reynolds' lead citrate (15), or only with lead citrate for en bloc stained specimens, and viewed on a Philips 300 electron microscope. Thick sections were stained at 60°C for ½ h each in alcoholic uranyl acetate and Reynolds' lead citrate and viewed on a JEM-1,000 high voltage electron microscope at 1,000 kV. Stereo pairs were obtained by photographing the specimen at 0° tilt, then tilting the specimen through 12° by means of a goniometer built into the microscope, and again photographing the same area.

The micrographs of *Holocentrus* included in this paper (Figs. 4 *a* and 4 *b*) were kindly loaned to the author for this purpose by Professor K. R. Porter, Molecular, Cellular, and Developmental Biology Department, University of Colorado, Boulder, Colo. The *Holocentrus* scales they depict were fixed in 6% glutaraldehyde in 0.088 M Millonig's phosphate buffer for 2 h, washed in 0.1 M phosphate buffer briefly or overnight in the cold, and postfixed in 1% osmium tetroxide in 0.1 M phosphate buffer, for an hour. The preparations were then washed, dehydrated, embedded, and sectioned as described above. Some specimens were en bloc stained as described above. Poststaining and observation were as above.

Calibration of the microscope was carried out by photographing a calibration grid (54,864 lines/inch) at each magnification setting of the microscope, measuring the spacings of lines on the negatives, and using an average of these values to calculate the true magnification at each setting. Measurements from the electron microscope film negatives both for calibration of the microscope and for determining filament thicknesses were made with the aid of a Nikon profile projector, Model 6C (Nikon, Inc., Div. of EPOI, Garden City, N. Y.), equipped with a biaxial stage micrometer. Standard deviations were calculated on the basis of 70 or more measurements of each type of filament pooled from all the cell types studied.

RESULTS

Physiologically Passive Iridophores

The iridophores in this group are not all necessarily known to be physiologically passive, but none of them has been observed to undergo physiological changes in the laboratory. Included here are the dermal iridophores of the lizard, *A. carolinesis*, the scale iridophores of the goldfish (a freshwater fish) and of *H. ascensionis* (a marine fish), and the tapetal iridophores of cardinal tetra

(a freshwater fish). Except for the *Anolis* iridophore, which is generally blue by reflected light, the iridophores described in this section are multicolored and silvery in combination, providing the animal with iridescent reflectivity rather than color.

As was shown previously (16), the bulk of the cytoplasm of *Anolis* iridophores is comprised of alternating sheets of crystal mosaics and filamentous cytoplasm, both on the order of 1000 Å thick, stacked in parallel in the cell with amazing regularity. The crystals are tablet shaped, such that they appear brick shaped in vertical section and irregularly rounded, 2000 Å in diameter, in horizontal section. They are bounded by complex membranes, "crystal membranes," and are juxtaposed closely side by side to form essentially continuous mosaic sheets a single crystal deep. Complex filament networks exist in the cytoplasm between crystalline sheets. On either side of each crystalline sheet, close by, lies a lattice of filaments. Connecting adjacent lattices to one another and to the crystal membranes are numerous filament links. In *Anolis* and all other species, the iridophore crystals are dissolved by alkaline lead staining, leaving only the crystal spaces seen in all the figures in this paper. For the sake of brevity, these will be referred to as "crystals" from here on. Microscopy of unstained specimens shows that the crystal space retains the shape of the intact crystal.

Fig. 1 shows a horizontal thin section through a dermal iridophore of *A. carolinensis*, tangential to a crystalline sheet. The filament lattice is clearly visible here. It consists of a rectangular lattice of two parallel filament arrays. These filaments appear rather coarse in cross-section (Fig. 2), whereas the cross-connecting filament links, visible only in vertical section (Fig. 2), appear considerably finer. The existence of the filaments has been documented already (16), but careful remeasurements of the filament diameters make further discussion of their significance interesting. It appears that the filaments fall into two distinct size classes: the lattice filaments are coarse, 103 Å ± 17 Å thick, resembling "intermediate" filaments found in other cell types (6, 7, 11), whereas the cross-linking filaments are only 65 Å ± 13 Å thick, in the size class of actin (7).

In the iridophores of other species, as shown below, the coarse filaments are either absent or confined almost exclusively to the cell margins outside the main area occupied by crystal stacks. They are not arranged as lattices though they frequently run in parallel bundles. Thin filaments are abundant between the crystals, but they connect directly with the crystal membranes rather than indirectly through filament lattices.

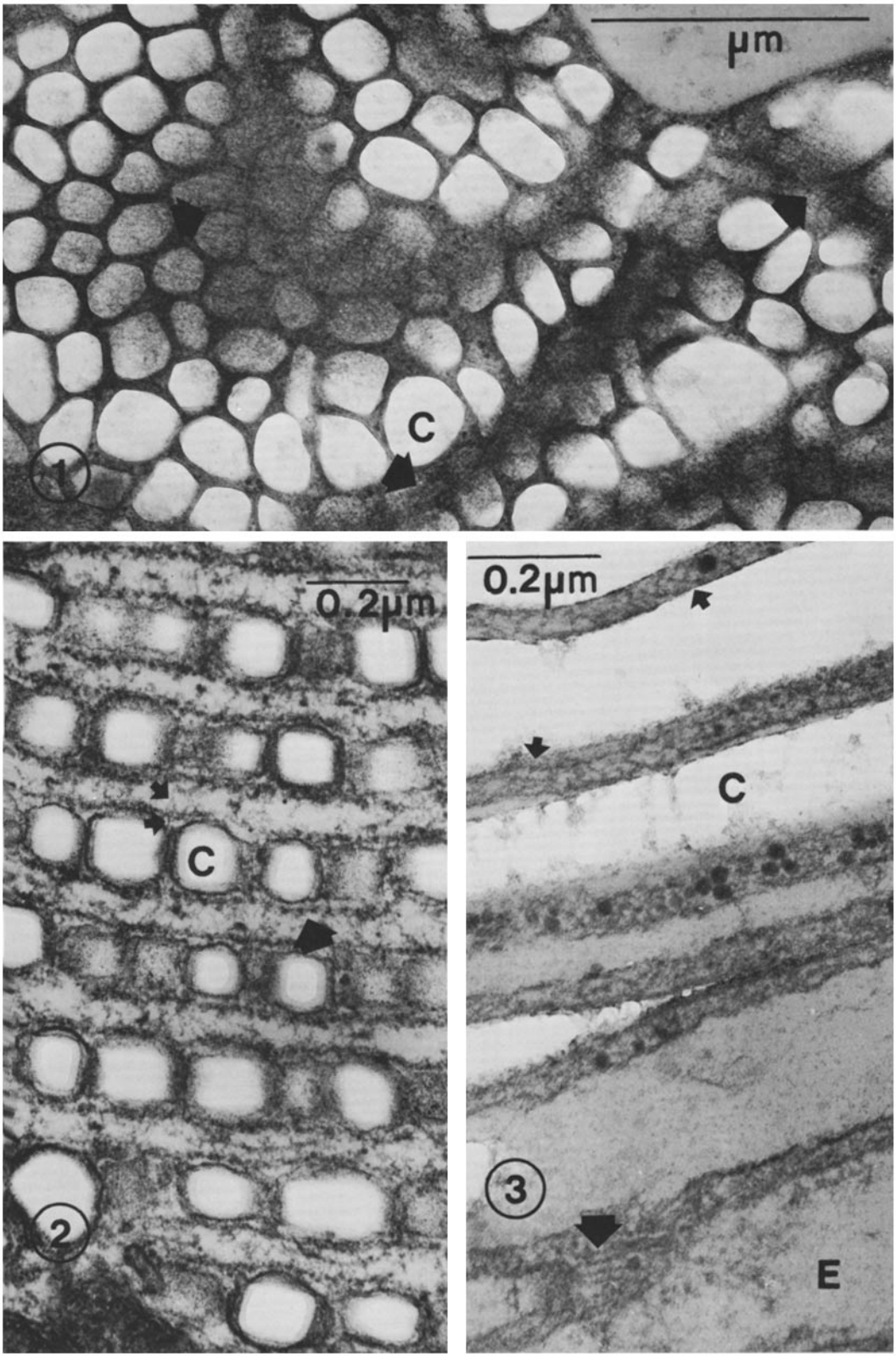
The goldfish iridophore is depicted in vertical thin section in Fig. 3. As is typical in fish iridophores, the crystals here occur as thin, broad platelets rather than as the tablets seen in *Anolis* iridophores. Crystal sheets are therefore single platelets rather than crystal mosaics. Parallel layering of platelets and cytoplasmic sheets still occurs with very regular spacing. The large arrows in Fig. 3 point to areas where filaments run in crisscross patterns, connecting membranes of successive crystal platelets. These cross-connections are the thin 65-Å diameter filaments. Occasionally, arrays of the thicker (100 Å) filaments are seen to run parallel to the crystal platelets in the cell margins (extracellular areas, *E*, are marked in Fig. 3 to indicate cell margins).

The *Holocentrus* iridophore, depicted in vertical thin section in Fig. 4, is almost identical morphologically to the goldfish iridophore. Here, too, 65-Å filaments link successive crystal platelet membranes to one another. Thick, 100-Å filaments are more numerous in the *Holocentrus* iridophore than in the goldfish iridophore, but again, they are restricted for the most part to the cell margins outside of the stacked platelet area of the cell.

Figs. 5 *a* and 5 *b* show vertical thin sections of a tapetal iridophore from the eye of cardinal tetra. This iridophore, like those of the *Holocentrus* and goldfish scales, has 65-Å filaments crisscrossing between pairs of crystals in parallel stacks, linking these crystal platelets at regular intervals. The filament types and their positions in the cell are thus the same in these iridophores from the tapetum of the fish eye and in iridophores from fish scales, indicating that there is no obvious difference in iridophore morphology correlated with differences in location or biological function of the iridophore in the animal.

Physiologically Active Iridophores

Included here are the dermal iridophores of *R. pipiens*, the leopard frog, and the scale iridophores of cardinal tetra, a freshwater teleost. Hormonally-induced changes in *R. pipiens* iridophores have been described by Hadley (5) and Taylor and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Bagnara (22). They involve gross changes in cell shape and therefore in effective reflectivity. Diurnal changes in the gross color of scale iridophores of cardinal tetra have been observed by this author (unpublished observations). During the day, in light, the iridophores of cardinal tetra are green, with iridescence ranging from blue to yellow. At night, in the dark, the same iridophores are very dark bluish-violet and show almost no iridescence. In both of these species, but especially in cardinal tetra, the iridophore crystal platelets are flattened and fairly broad. No 100-Å filaments are evident in the iridophores of either species, but 65-Å filaments are abundant.

Fig. 6 shows a low-magnification view of a vertical thin section through a scale iridophore of cardinal tetra. The regular parallel stacking of broad, thin crystal platelets is clearly evident, as are the great numbers of thin filaments connecting successive platelets (arrows). A better idea of the three-dimensional structure of the filamentous cytoplasmic layers can be obtained from stereo pairs of thick sections, as shown in Fig. 7. Crisscrossing of thin filaments linking successive crystals is so dense and regular that it appears as a diagonal lattice in some areas of the picture (arrows).

Thin-filament cross-links like those of cardinal tetra iridophores occur between stacked crystals of the *Rana* iridophore, as shown in Figs. 8 *a* and 8 *b*. Crisscrossing analogous to that found in fish iridophores is seen best in Fig. 8 *b*. Fig. 8 *a* shows parallel arrays of thin filaments running among the crystals without any obvious regular orientation with respect to these crystals. It is possible that they surround groups of stacked platelets (these occur in several groups with different orientations in the iridophores of this species), supplementing the intercrystalline links between pairs of adjacent crystals as observed in all the iridophores described so far. Similar parallel arrays of very numerous thin filaments are seen to be abundant in the cell cortex (Fig. 8 *b*).

Two stereo pairs of thick sections, Figs. 9 and 10, show three-dimensional images of the parallel filament arrays among groups of crystals and the crisscrossing filament links between crystal pairs of the *Rana* iridophore. The image in Fig. 10, especially, resembles that seen for the cardinal tetra iridophore in Fig. 7. Again, the intercrystal links are so dense and regularly positioned that they appear to occur in latticelike arrays (arrows).

DISCUSSION

Many authors have recognized the high level of ordering of iridophore crystals and the necessity for the precise maintenance and control of this ordering for the production of iridescent colors. Some have asked how this order is maintained in the cell (12, 16), but the question has remained essentially unanswered. A suggestion that maintenance of the cell's architecture is achieved by means of cytoskeletal filaments (16) seems a likely possibility, and will be discussed below in light of results presented in this paper.

A second major problem in iridophore biology has been that of how physiologically active iridophores control their activity (5, 20–22). Even the nature of the activity is poorly understood: whether or not the iridophore platelets undergo changes of tilt, spacing, or form, and whether they migrate actively in and out of stable cell extensions or are passively carried during shape changes of

FIGURE 1 Horizontal thin section through an *Anolis* dermal iridophore showing rounded membrane-bound crystals, *C*, and filamentous cytoplasm. The 100-Å filaments so prominent in this view consist of two arrays of parallel filaments arranged in a rectangular lattice (arrows). × 43,000.

FIGURE 2 Vertical thin section through an *Anolis* dermal iridophore showing rows of brick-shaped, membrane-bound crystals, *C*, and two types of filaments in the cystoplasm. Cross sections of 100-Å filaments (lattice filaments) lining the crystal rows on either side are shown with a large arrow. Short 65-Å filament links connecting lattices to one another and to the crystal membranes are shown with small arrows. × 78,750.

FIGURE 3 Vertical thin section through parts of three goldfish scale iridophores showing thin, broad, membrane-bound crystal platelet cross sections, *C*, and two types of filaments in the cytoplasm. 65-Å filaments crisscross between pairs of crystals (small arrows); 100-Å filaments run in parallel bundles at the cell margins (large arrows). Extracellular areas, *E*, are marked for orientation. × 102,900.

the cell itself are still largely unanswered questions. Whatever the exact nature of the events involved, however, it would be interesting to find some structural indications of a system of motility built into the cell. In particular, the presence of actin filaments would raise the tantalizing possibility that the physiological responses observed might be mediated by actin-myosin interactions.

From two points of view, then, the examination of filament occurrence in iridophores is important in understanding iridophore biology. The existence of filaments in iridophores has been noted previously, but only by a few authors, most of whom placed little emphasis on their occurrence and offered no suggestion for their role in the cell. Taylor (20) saw filaments in the iridophores of several species of tree frogs, especially those treated with melanophore-stimulating hormone (21) and Kawaguti (8) and Kawaguti and Kamishima (9) noted them in fish iridophores. Setoguti (18) described 60-Å filaments in tree frog skin iridophores and suggested that these might be involved in crystal movement and changes in cell shape. Other authors have depicted iridophore filaments in electron micrographs without commenting on their presence (1, 3, 10, 12–14, 19).

This paper describes the occurrence of two classes of filaments in iridophores from a variety of vertebrate species. The thin, 65-Å filaments may well be actin (17), and serve the cell in motility. The thick, 100-Å filaments are probably structurally supportive, cytoskeletal elements. In his study of the developing Z-bands in myoblasts of larval newts, Kelly (11) noted huge numbers of similar 100-Å filaments running through the immature cells (and, in fact, in other immature cells of mesenchymal origin) and suggested that these filaments might be playing a cytoskeletal role in the developing cells. Fay and Cooke (4) noted 100-Å filaments remaining in smooth muscle cells which they had treated with low calcium. Such treatment removed all visible thick and thin contractile filaments and all contractile activity.

Among the iridophores described in this paper, *Anolis* iridophores have been examined most carefully. Both filament classes are obvious here, but the 100-Å filaments are far more abundant and organized than in other species. This could very reasonably be attributed to the need for a gelled layer of cytoplasm around the crystals to hold the nonplatelet crystals in essentially continuous sheets, allowing thin-film interference to operate. In other iridophores, for example in those of many teleosts, the reflecting platelets are already broad reflecting "films." No gelation of the cytoplasm is necessary for the maintenance of their integrity, and, in fact, no 100-Å filament lattices occur in the iridophores of these species. Less organized 100-Å filaments are found in the cortical regions of the fish cells, but this is not an unusual finding. It is a general phenomenon that the cortex of almost all cells is more gelled than the internal cytoplasm of the cell.

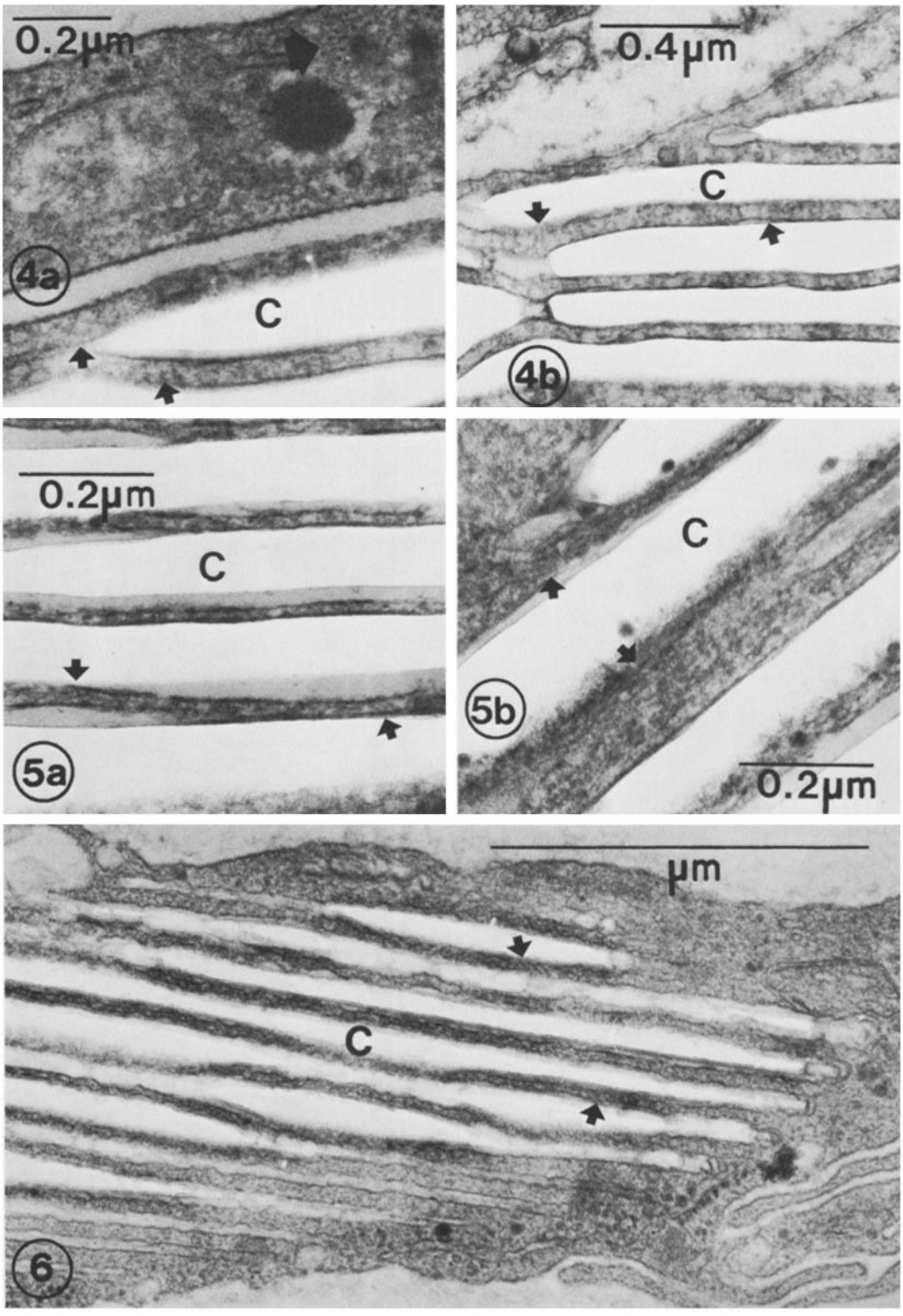
If the integrity of the reflecting films is assured, either intrinsically or by means of cytoplasmic gelation, the only further requirement for proper iridophore organization is appropriate spacing and tilt of these films.

As has been described, thin filament links crisscross at various angles and connect successive crystalline films to one another, either directly by inserting into the crystal membranes, or indirectly (in *Anolis*) by inserting into 100-Å filament lattices

FIGURE 4 Vertical thin sections through *Holocentrus* scale iridophores. Membrane-bound platelets appear as in the goldfish, as do the 65-Å filament connections between them (small arrows). In addition, Fig. 4 *a* shows cross sections of 100-Å filaments at the cell margin (large arrow). These micrographs were kindly loaned to the author for publication by Professor Keith R. Porter, Molecular, Cellular, and Developmental Biology Department, University of Colorado, Boulder, Colorado. Fig. 4 *a* × 75,000. Fig. 4 *b* × 54,000.

FIGURE 5 Vertical thin sections through a tapetal iridophore from the cardinal tetra eye. Membrane-bound crystal platelet cross sections, *C*, and 65-Å filament cross-links (arrows) are indistinguishable from the same features of the scale iridophores shown in Figs. 3 and 4. Fig. 5 *a*, 5 *b* × 102,900.

FIGURE 6 Vertical thin section through a scale iridophore of cardinal tetra. This low-magnification view shows the precise parallel stacking of membrane-bound crystal platelets, *C*, and the numerous short filament links connecting them (arrows). Filaments of only one kind, 65-Å in diameter, occur in the iridophores of this species. × 58,380.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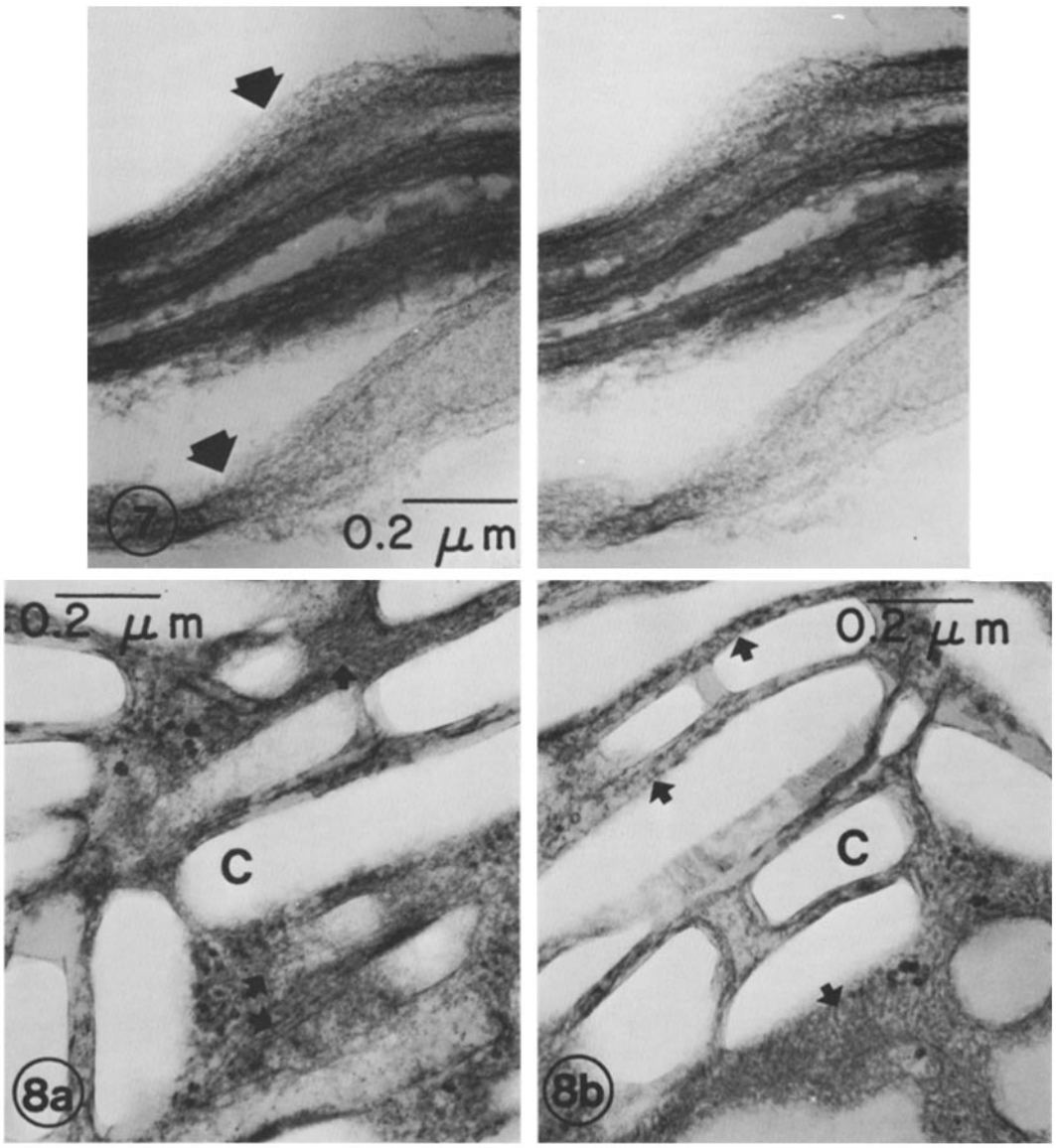

FIGURE 7 Stereo pair of a 0.25-μm oblique section of a cardinal tetra scale iridophore viewed at 0° tilt and 12° tilt at 1,000 kV. 65-Å filaments crisscrossing between crystal platelets show up very clearly in the three-dimensional view provided by this stereo pair, especially in the areas marked with arrows. × 75,000.

FIGURE 8 Vertical thin sections through two dermal iridophores of *Rana*. Again, membrane-bound crystal platelets, *C*, and 65-Å filaments (arrows) are obvious. Fig. 8 *a* shows bundles of parallel filaments seemingly surrounding groups of stacked platelets. Fig. 8 *b* shows such filament bundles in the cell margins, but shows short crisscrossing filaments linking pairs of crystals, as in the fish iridophore. Fig. 8 *a*, 8 *b* × 68,600.

and further linking these to the crystal membranes. Either way, they could determine and maintain the regular spacing between successive crystalline films. If they are composed of actin (as has been indicated in another study, 17), they are potentially capable of interacting with myosin to cause a contraction of all or part of the intercrystal cytoplasm and thus of reversibly bringing the crystalline films closer together or tilting them. While *Anolis* iridophores are physiologically pas-

sive, they are surely not evolutionarily isolated. It is not surprising, therefore, that actin should be found here even if it is not needed for motility in this species' iridophores, but rather serves only as a structural link.

If, indeed, the 65-Å filaments are composed of actin, then it is of special interest that they should be found in such abundance in physiologically active iridophores. In the motile *R. pipiens* and cardinal tetra iridophores examined here, the filaments found were almost exclusively of the thin type. They occurred not only between pairs of

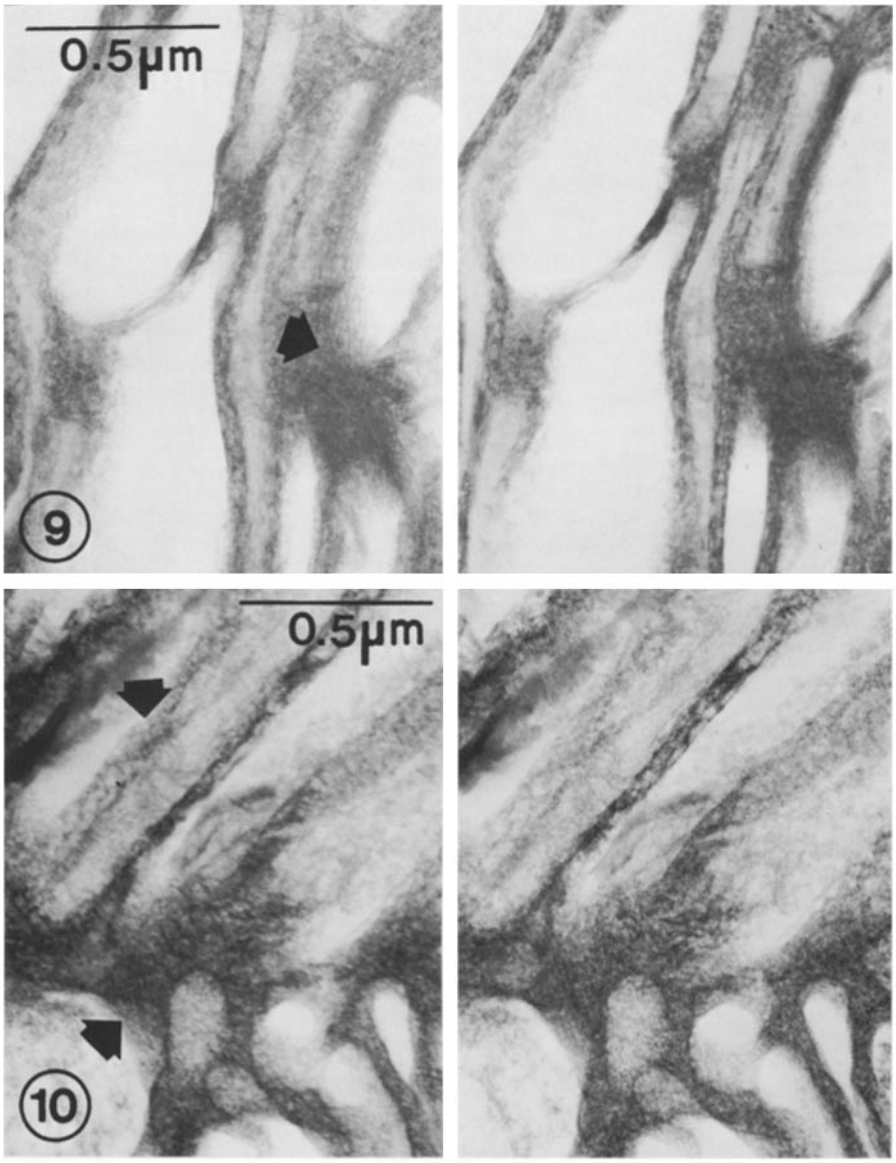

FIGURE 9 Stereo pair of a 0.25-μm vertical section through a *Rana* iridophore viewed at 0° tilt and 12° tilt at 1,000 KV. The arrow shows the thin-filament bundles apparently running along a stack of platelets, as in Fig. 8 *a*. × 50,000.

FIGURE 10 Stereo pair as in Fig. 9, but a different cell. The three-dimensional image given in stereo dramatically illustrates filaments between crystals and at the cell margins (arrows). This image very much resembles that shown for the cardinal tetra iridophore in Fig. 7. × 50,000.

adjacent crystals, potentially regulating the spacing and tilt of such crystals, but in *Rana* also occurred among groups of stacked crystals, potentially coordinating the relative tilts and spacings of these crystal groups. Further biochemical characterization of the iridophore filaments from these particular species, and experiments combining physiological and ultrastructural probes are needed to define the role and nature of these filaments further.

In conclusion, filaments are ubiquitous in iridophores. Two size classes, measuring 103 Å ± 17 Å and 65 Å ± 13 Å, probably participate in cytoskeletal and motile functions of the cell, respectively. Further definition of their nature and role in the cell awaits further experimentation.

This work was carried out in the laboratory and under the supervision of Professor K. R. Porter, whom I would like to thank heartily for his helpful guidance, for the use of his laboratory facilities, and for the *Holocentrus* micrographs that he kindly let me use. I also gratefully acknowledge the use of the JEM-1,000 high voltage microscope installation of the Department of Molecular, Cellular, and Developmental Biology, University of Colorado, Boulder, Colorado, and I wish to thank K. Takasaki and G. Wray, whose technical assistance made the high voltage work possible.

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