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CHANGES IN SPECTRAL REFLEXIONS FROM THE IRIDOPHORES OF THE NEON TETRA

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SUMMARY

1. The iridescent stripe of the freshwater teleost, the neon tetra, changes from green in the daytime to violet-blue at night.

2. Spectral reflectance measurements were used to follow these colour changes.

3. Light causes a shift in reflectance to longer wavelengths in living fish and in isolated tissue from the lateral stripe. The change is reversed in darkness.

4. The spectral reflectance shifts to longer wavelengths when the fish is disturbed in darkness. No such colour changes were seen in fishes kept alive in 10^{-4} M-reserpine.

5. Hypotonic Ringer solution causes a reflectance shift to longer wavelengths and hypertonic solution causes a shift to shorter wavelengths.

6. The iridescent reflexions from the lateral stripe which is continued across the iris originate from iridophores in the dermis. These iridophores contain regular stacks of broad, double-sided hexagonal plates that are about 10 nm thick. Each plate is contained within a pouch in the cytoplasm and is separated from its neighbour by approximately one quarter the wavelength of light.

7. A distinction is drawn between the physiologically active iridophores in the lateral stripe and iris that have broad hexagonal crystal plates which are very thin and the physiologically inactive iridophores that are also found in the iris, but in addition are found on the flanks below the lateral stripe, and on the head. These iridophores contain hexagonal crystals that are usually narrower than the active type, but are about 60–100 nm thick.

INTRODUCTION

In both vertebrates and invertebrates there are areas of the body other than the eyes that are known to be sensitive to visible light (for reviews see Steven, 1963; Millott, 1968; Wolken & Mogus, 1979). The pineal body is photosensitive in many amphibia, fishes and reptiles. In birds and frogs the diencephalon is sensitive to light, and in birds, at least, is involved in entraining circadian rhythms to light. In neonatal rats the harderian gland, which is situated just behind the eye, may house the receptor involved in the secretion of serotonin from the pineal (Wetterberg, 1970).

Light induces electrical changes in the skin of frogs (Becker & Cone, 1966), and in Pigeon squabs illumination of the body skin results in a characteristic head-wagging movement (Harth & Heaton, 1973).

Dermal photosensitivity in fishes has long been suspected (Steven, 1963), but only rarely is it known for certain that the photoreceptor is actually located in the skin. One such example is provided by the male of the Killiefish *Fundulus heteroclitus*. Foster (1933, 1937) showed that the iridocytes in isolated strips of skin changed reversibly from blue to red within a few seconds of illumination. Foster also showed that pituitrin and adrenaline caused a reddening of the iridocytes. When the bathing medium was made hypotonic the iridocytes became red, and in hypertonic media they became blue, which suggested to Foster that it was the swelling and shrinking of the cytoplasm layer separating guanine crystals that caused the colour change. Due to the more recent work of Denton (1970, 1971); Denton & Land (1967, 1971); Denton & Nicol (1965*a*, *b*, *c*) and Land (1972), there is now little doubt that the iridescent reflexions from the iridophores of fishes are produced by the constructive interference of light reflected from multilayer stacks of alternating cytoplasm stacks and guanine crystals.

The neon tetra *Paracheirodon innesi* also shows pronounced colour differences between day and night. In the daytime there is a brilliant blue-green lateral stripe that runs from the iris along the flank of the fish nearly to the tail. In the day time the upper part of the body, including the lateral stripe, has numerous expanded melanophores, and below the lateral stripe, posterior to the origin of the dorsal fin, there is a conspicuous area of expanded erythrophores. At night the lateral stripe changes to a dull violet-blue colour, as in the cardinal tetra, and both the melanophores and erythrophores contract. The over-all effect is that of a dull coloured fish, which is very difficult to detect against the bottom of the aquarium where it rests motionless.

The purpose of the investigation that is described here was to study the morphology of the structures responsible for the light-induced changes in iridescent colour and to chart the time course of the response. It became clear during the investigation that when a living fish was stressed it also changed colour, even in darkness, and we have also attempted to describe these changes, which appear to involve an adrenergic mechanism.

METHODS

The neon tetras (*Paracheirodon innesi*) used in these experiments were purchased from local aquarists who had in turn imported them from fish breeders in S.E. Asia. They were maintained under a regime of 12 hr light and 12 hr darkness, and the aquarium water was maintained at 25 °C. For some of the experiments 10^{-4} M-reserpine (Sigma) was added to the aquarium water and the fish maintained in reserpine water for at least 24 hr.

Electron microscopy

Fish were decapitated and a small section of the lateral stripe removed under fresh water (f.w.) teleost Ringer solution (Pantin, 1946). Initial fixation was in 5% glutaraldehyde in 0·05 M-phosphate–phosphate buffer. The tissue blocks were washed in 0·05 M-phosphate buffer and postfixed in 1% osmium tetroxide. The tissue blocks were dehydrated in alcohol and embedded in Araldite in the conventional way. Using this method the colour of the iridescence remained apparently unchanged until osmium fixation, but even after that the characteristic colour could still be seen, despite the black osmium stain in the Araldite block. Sections were then stained in 0·2% lead citrate and uranyl acetate.

Bone & Denton (1971) found that the iridescent colour of scales of sprat (a marine teleost) was best preserved in a solution isomotic with 60% marine teleost Ringer. The solution we found best

had an osmolarity of 86 M-osmole/kg compared to 138 M-osmole/kg of the f.w. teleost Ringer which is hypotonic by 62 %. Where it was necessary to preserve the violet-blue colour of the dark-induced state, fishes were kept for 24 hr in 10^{-4} M-reserpine and killed by decapitation and fixed in darkness. Reserpine was used because it leads to a depletion of the adrenaline stores in the tissues, and thus has a tranquilizing action and reduces colour changes due to stress in handling, a phenomenon that is described in more detail below.

The osmolarity of solutions was measured in a Wescor 5100 vapour pressure osmometer.

Effects of disturbance

Fish were kept either in fresh water or in water containing 10^{-4} M-reserpine (see above). After at least 24 hr, individual fish were transferred to 500 ml. beakers containing the water, either with or without reserpine, in which they had been living. The room temperature was 25 °C.

The fishes were allowed to settle down for 1 hr in a room brightly lit by daylight of 5×10^{-6} W/cm^2. nm at 550 nm. The room was then darkened for 30 min. Fish were either left completely undisturbed, or their water was briskly stirred for 2 sec at 60 sec intervals. When daylight was re-admitted to the room, the colour of the lateral stripe in the living fish was matched using the calibrated spectrum of a direct vision spectroscope as a standard.

Spectral reflectance measurements

A spectroradiometer was connected by way of a fibre optic light guide to the exposure meter outlet of a camera fitted to a conventional light microscope. Spectral reflectance curves were measured from fish illuminated by white light. Changes in reflectance were measured from fishes illuminated with monochromatic (543 nm) light, obtained using a Balzer B40 Interference filter of 10 nm bandwidth. An Olympus BHA microscope was fitted with a × 10 glass objective and a × 3·3 glass eyepiece. The area measured for reflectance was a circular spot approximately 0·5 mm in diameter and included about 100 reflecting cells. Light was conducted from the exposure meter port of an Olympus PM 6 camera through a Barr and Stroud glass fibre light guide to the entry slit of a Macam SR 1011 spectroradiometer. This instrument has a holographic grating monochromator and a PM 1059 Ga.As. photomultiplier tube.

The light used in the measurement of spectral reflectance was a 6 V tungsten lamp with a collimated beam and a colour temperature of 2400 °K. The light was measured by inserting the cosine corrected collecting head of the Macam spectroradiometer. At the level of the specimen this gave an intensity, corrected for losses at the air–Ringer interface, of $2{\cdot}0 \times 10^{-6}$ W/cm^2. nm at 543 nm and $3{\cdot}0 \times 10^{-6}$ W/cm^2. nm at 604 nm. The reference surface for the measurements of relative spectral reflectance was clean brushed aluminium foil.

The fish were maintained alive, and apparently healthy, in 10^{-4} M-reserpine for at least 24 hr before they were decapitated and pinned through the caudal peduncle and pectoral girdle to a flat bed of black wax lining the bottom of a shallow dish. Each fish was immersed in f.w. teleost Ringer (Pantin, 1946) and positioned on the microscope stage so that it was illuminated from a dorsal direction normal to the anterior posterior axis and at 47° to the dorsal–ventral axis; this position was chosen because it gave near-maximum reflexion. Room temperature was held at 23–25 °C. Each spectral reflectance curve took about 150 sec to complete. Readings were taken at 20 nm intervals, beginning at 420 nm travelling from short to long wavelength and returning at the alternate 20 nm intervals.

Light-induced reflectance changes

Experimental conditions were similar to those described in the previous section except that two lamps were used: one to induce changes in reflectance, and the other to measure the reflectance changes that occurred.

The light used to induce spectral reflectance changes was fitted with a 543 nm Balzer B40 interference filter of 10 nm bandwidth at 50 % transmission. The intensity was modulated both by means of neutral density, acetate filters, and by adjusting the voltage to the lamp. Adjustment of intensity by voltage control is legitimate in this instance because monochromatic light was used and changes in spectral distribution due to slight changes in colour temperature are negligible.

A second tungsten lamp was used to provide the measuring light. This was filtered to give the longer wave-length light that pilot experiments had already suggested has a relatively small effect on the initiation of light-induced spectral reflexion changes. The interference filter used was a K5

Balzer interference filter of maximum transmission at 604 nm and a bandwidth at 50 % transmission of 42 nm. The intensity at 604 nm was $2{\cdot}6 \times 10^{-6}$ W/cm^2. nm.

The monochromator of the spectroradiometer was set to accept light at 575 nm and had a band width of 10 nm, thus excluding the 543 nm experimental light.

In this series of experiments the measuring light remained switched on for the duration of the experiment. With the measuring light alone, the relative reflectance of the iridescent stripe fell to a steady constant low level 20–30 min after the room lights were switched off. When this constant low level had been maintained for 4–6 min, the experimental light was switched on and the resulting reflectance increase was measured at 60 sec intervals.

Osmotic effects on spectral reflectance

Fishes were prepared in the way described above except that they were exposed to daylight throughout. The skin was made permeable by gentle rubbing with the blunt side of a scalpel blade. The force used was too little to dislodge scales or to alter the outward appearance of the skin.

The rubbed areas showed marked colour changes when bathing solutions of different osmolarity were used. The solutions were f.w. teleost Ringer (see previous section) double-strength Ringer and distilled water. Spectral reflectance measurements were made in the same way as described above.

RESULTS

Morphology of the reflecting cell

By reflected light at near normal incidence the iridophores of the lateral stripe show up as a brilliantly reflecting mosaic of spectral colours, and it is these iridophores that are physiologically active, changing colour according to changes in illumination. In fishes that have the dull violet-blue night-time colours, there are chiefly violet and blue reflectors. In fishes showing daytime colours, the reflectors are mainly green-blue and yellow-green. Fishes that have been induced to turn to a yellow-green or even orange appearance by osmotic manipulation have reflectors that are chiefly green, yellow green and orange-red. The actual reflectance measurements include a hundred or so cells and therefore are the sum of many reflecting sources, each having a different individual spectral reflectance curve. Similarly, measurement of reflectance changes show the combined changes of many cells rather than of a single cell or part of a cell. The appearance of the iridocytes in the lateral stripe under vertical illumination is shown in Pl. 1. They contain one or more iridophores that consist of stacks of hexagonal reflecting plates. This type of iridophore is also found in the iris and is the type that changes colour when illuminated. When the tissue is teased apart in water, thin hexagonal crystal-like structures float free (Pl. 2). Also shown in Pl. 1 are the more slender type of iridophore which are found in the silvery dermis ventral to the lateral stripe and in the iris. These iridophores do not appear to change colour when illuminated and only occur with the physiologically active iridophores at the ventral margin of the lateral stripe (Pl. 1) and in the iris. When teased apart these physiologically inactive cigar-shaped iridophores yield slender needle-shaped reflecting crystals (Pl. 2).

The two types of reflecting crystal in the iridophores of the neon tetra have been illustrated by Denton & Land (1971) and Land (1972). Using the high power Huxley interference microscope, Denton & Land found that the broader crystals are substantially thinner than the needle shaped type. The two types are shown together in Pl. 2.

Under the electron microscope (e.m.) the two types of iridocyte appear different. The physiologically inactive iridocytes contain arrays of crystals that are arranged

roughly parallel to each other (Pl. 4). In Pl. 4 some of the guanine crystals have remained in place whilst others have apparently been lost during the e.m. preparation. Similar crystals have been reported in the iridophores of the Japanese porgy (Kawaguti & Kamishima, 1966*a*) and in the tree frog (Setoguti, 1967). Using interference microscopy Denton & Land (1971) consider the thickness of these needles in the iris of the neon tetra to be 86–104 nm. Under the e.m. we measure them to be 43–108 nm with a mean of 78 nm.

The physiologically active iridocytes, as they appear in our electron micrographs, show no crystals of comparable thickness and we see only very thin densely-staining lamellae (Pl. 3). We estimate these plates to be 5–10 nm thick. In reality we think that it is more likely that plates of the order of 60–70 nm are present (see Discussion), despite our failure to see them in our e.m. preparations. Other investigators get similar results to ourselves. Kawaguti & Kamishima (1966*b*) report 0·6 nm lamellae in *Harengula* and Harris & Hunt (1973) report 10–15 nm lamellae in the salmon.

We were unable to find microfilaments in our material like those reported by Rohrlich (1974) in the cardinal tetra.

Effects of disturbance

When disturbed or alarmed the colour of the lateral stripe shifts to longer wave-lengths. This colour shift is usually seen when the fish are transferred from one tank to another or one of the other fish in the tank is netted. Undisturbed fish kept in darkness reflect mostly blue light. However, fishes that are kept in darkness during their normal night period achieve a more violet colour than those kept in darkness during their normal daytime period. The change from green to blue or blue-violet can be prevented if the water is periodically stirred. Fishes that have been kept in water containing reserpine do revert to the blue coloration typical of undisturbed fishes kept in darkness.

Fig. 1 shows the results of one such experiment. The lateral stripe of fishes kept in darkness for 30 min in individual 500 ml. beakers had a predominantly blue lateral stripe when the room was again illuminated, but regained their normal daytime colour after 7–15 min in the light. Fishes that had been disturbed by periodic stirring during the dark period were yellow-green in colour when the lights came on, and indeed became slightly more blue when left undisturbed in the light. In contrast those fishes that were living in water containing reserpine reverted to the blue colour of undisturbed fish in darkness.

Preliminary work using decapitated fishes in Ringer to which 5×10^{-5} M adrenaline was added showed that adrenaline causes a marked shift in spectral reflectance towards longer wavelengths confirming an earlier observation that Foster (1937) made on *Fundulus*.

Light-induced reflectance changes

Fig. 2 shows the effect of illumination by white light on the spectral reflectance of the lateral stripe. This series of experiments was begun at 23·15 hr, 2 hr 45 min into the fishes' normal night period. Curve (0) was measured within about 9 sec of the light coming on and shows a typical curve for a night time fish. It will be noted that the peak reflexion is likely to be in the U.V. and explains why the fish, when

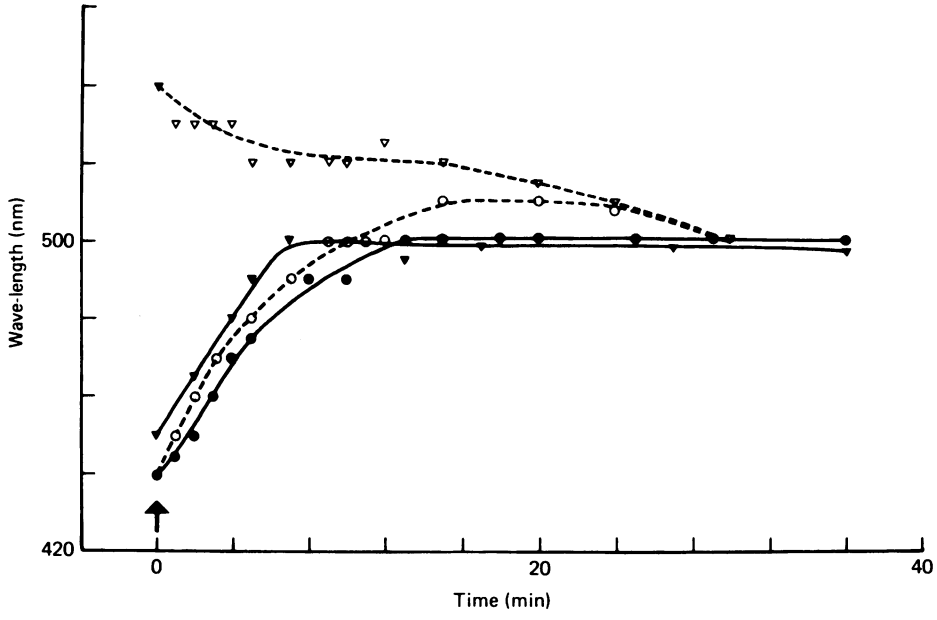

Fig. 1. Change in colour of lateral stripe of living, free-swimming neon tetras. The wavelength is that of the spectral band to which the apparent colour was most closely matched. Fish were exposed to 30 min of darkness, then illuminated (vertical arrow). Open symbols, aquarium water only; filled symbols, aquarium water with 10^{-4} M-reserpine. Triangles, fish disturbed during dark period; circles, fish undisturbed during dark period.

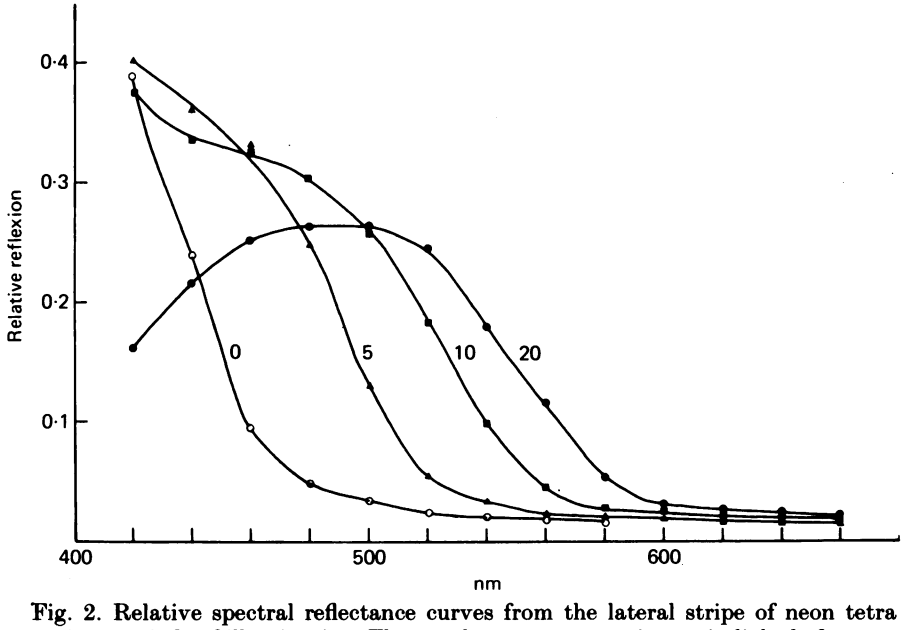

Fig. 2. Relative spectral reflectance curves from the lateral stripe of neon tetra after various periods of illumination. The numbers represent minutes in light before measurements began. Each run complete within 2 min.

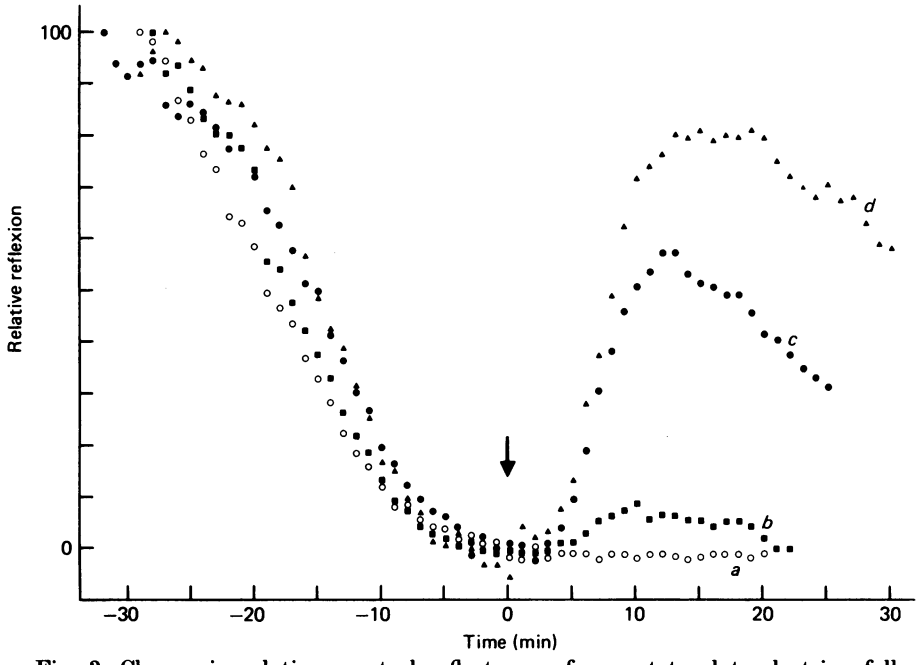

Fig. 3. Change in relative spectral reflectance of neon tetra lateral stripe following illumination of 543 nm light of various intensities. Time at 0 min represents the point when the experimental light was switched on. The measurement began immediately the period of dark adaptation began. Reflexions measured at 575 nm. All results are presented on a 0–100 scale. *a*, $0{\cdot}03 \times 10^{-7}$ W/cm^2.nm; *b*, $0{\cdot}17 \times 10^{-7}$ W/cm^2.nm; *c*, $0{\cdot}21 \times 10^{-7}$ W/cm^2.nm; *d*, $0{\cdot}85 \times 10^{-7}$ W/cm^2.nm.

surprised, when the aquarium light is switched on in the middle of the night appear to be a dull violet-blue colour.

The reflexion maxima in these experiments where light alone is varied do not extend to wave-lengths longer than 520 nm. There is some indication that stress can push the peak to slightly longer wave-lengths (Fig. 1), but this point was not specifically investigated.

The time course of reflectance changes following the onset of the stimulus illumination is shown in Fig. 3. Measurements were made at 575 nm because changes of reflectance at this wave-length are reasonably large and we think it likely that the threshold for light-induced reflectance changes is higher at 575 nm than at shorter wave-lengths. The wave-length of the stimulus light was 543 nm.

After the room lights were switched off the lateral stripe showed a decrease in reflectance at 575 nm (the stripe became more blue). The change was completed within 25–35 min. When the 543 nm experimental light was switched on, no change was noted for 2–5 min, whereupon the lateral stripe reflexions altered in the red-going direction, a change that is completed within 10–15 min. Following this initial reddening there followed a further, less marked, decrease in reflexion at 575 nm denoting a partial reversal back to the blue colour.

Osmotically-induced changes

The colour of the lateral stripe varies only slightly in response to osmotic changes in the surrounding medium provided that the fish is alive or has not been wounded or handled. However, gentle rubbing of the lateral stripe resulted in clear changes in colour when the medium was osmotically different to the tissues. These colour changes are similar to those described by Foster (1937) and Denton & Land (1971). Hypertonic solutions resulted in a shift in spectral reflectance towards the short wavelength and hypotonic solutions had the reverse effect (Fig. 4).

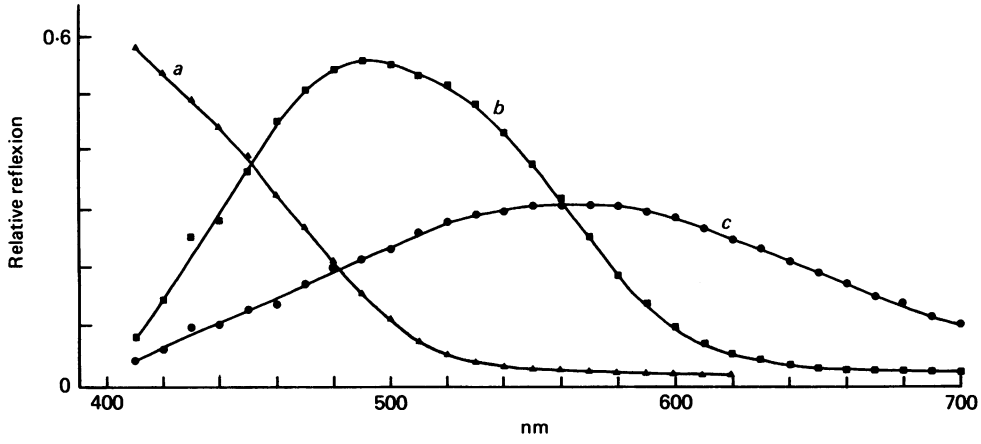

Fig. 4. Effect of osmolarity of the bathing medium on the lateral stripe of neon tetras. *a*, double strength f.w. teleost Ringer (305 M-osmole/kg); *b*, f.w. teleost Ringer (138 M-osmole/kg) *c*, distilled water.

We noticed little difference in fine structure between light and osmotically changed iridophores of the same in-block colour except that in hypotonic solutions (less than 138 M-osmole/kg) where the iridophores changed to orange, the electron-dense cytoplasm lamellae were disrupted to form rounded globules (Pl. 5). It is interesting that the thin 'crystal' lamellae remain straight and apparently intact after such osmotic manipulation.

Relation between fine structure and colour

Electron micrographs of the lateral stripe of fishes killed and fixed in their night-time blue and violet livery and fishes in their green daytime livery show differences in the thickness of the multilayers (Fig. 5). The apparance and thickness of the layers was similar to that shown by fishes subject to mild osmotic manipulation. Strong illumination did not result in either the pronounced reddening characteristic of exposure of the dead fish to hypotonic solutions or of the disruption of the electron dense cytoplasm layers characterized by that treatment.

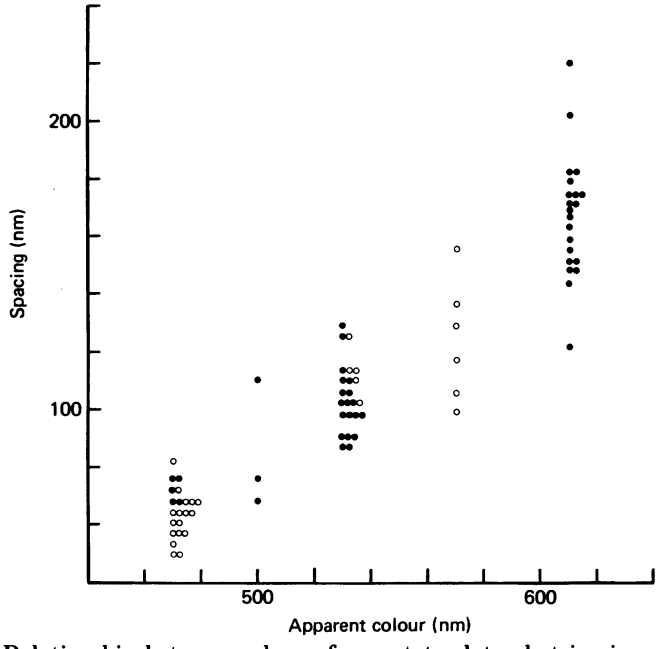

Fig. 5. Relationship between colour of neon tetra lateral stripe in resin block prior to sectioning for e.m. and the average spacing between crystal plates. The colours, which are approximate only, are designated by the corresponding spectrum wavelength. Each point represents one iridophore. Filled circles, tissue fixed in light, but subject to different osmotic treatments; open circles, tissues all fixed in isosmotic fixatives but colours manipulated by different light treatments.

DISCUSSION

In *Fundulus*, Foster (1937) showed that the control in the iridescent colours of the male was partly through the direct effects of light on the skin, and partly through a hormonal route, possibly an adrenergic mechanism. Both types of agent result in a change in thickness of the cytoplasm layers that separate the guanine crystals in the reflecting multilayer stack. The neon tetra appears to respond in a similar way to *Fundulus*.

Light microscope observations of the iridocytes *in situ* and of tissue that has been teased apart show that the broad crystals described by Denton & Land (1971) are present in the physiologically active iridocytes. Using interference microscopy Denton and Land estimate that these crystals are 62–66 nm thick. Crystals of this thickness are not visible in our e.m. preparations although very thin plates of 5–10 nm thick are clearly visible. These plates are similar in appearance and thickness to those seen in electron micrographs of *Harengula zunasi* iridocytes (Kawaguti & Kamishima 1966*b*); the Atlantic salmon *Salmo salar* (Harris & Hunt, 1973), *Lebistes reticulatus* (Takeuchi, 1976) and possibly *Halichoeres poecilopterus* (Kawaguti, 1965). We think it most likely that the crystals are either lost during preparation for the e.m., or do not accept the osmium, uranyl acetate or lead citrate stains.

Denton & Land (1971) believe that the reflecting system in the neon tetra, as for many other fishes is 'ideal' which means that the optical thicknesses of the crystal

plates and the intervening cytoplasm plates are approximately equal. The optical thickness is nt where n is the refractive index of the plate and t is its actual thickness. The wavelength reflected most strongly is 4 nt. If the crystal plates are guanine ($n = 1{\cdot}83$) of average thickness 64 nm, the value of 4 nt is 468 nm and the thickness of the cytoplasm plate ($n = 1{\cdot}33$) is about 88 nm. The combined thickness of crystal plate and cytoplasm plate becomes 152 nm. In fact the measured spacing between corresponding points in the multilayer stack reflecting blue light (at least in Araldite) is 50–90 nm (Fig. 5). There are several uncertainties in measurements of this kind, for example the colour of the reflected light depends upon the angle of incidence of the light on the multilayer stack; embedment in Araldite will itself alter the refractive index of the reflecting plates and shrinkage or swelling of the tissue is possible despite the care taken to prevent it. Everything considered it seems likely that crystal plates about 60–70 nm thick are present, but that they do not show up on the e.m. preparations made by us or by other investigators.

Until we are sure that we can identify the reflecting surfaces there will be uncertainty about the mechanisms that cause the colour changes in the living fish. Rohrlich (1974) has suggested that the spacing of the crystal plates in the cardinal tetra may be controlled by the contraction of filaments, perhaps of actin, running through the cytoplasm between the crystal plates. We have seen no such filaments in our preparations, but this may simply be due to failures in our technique. It may be significant that in our experiments the separation of the crystal plates is similarly related to the colour of the reflexions in both the light and osmotically manipulated material (Fig. 5). It is also true that the shapes of the spectral reflectance curves are similar in both light and osmotically treated tissue (Figs. 2 and 4 respectively). Possibly the system involves changes in membrane permeability or an active pump that results in the swelling and shrinking of the cytoplasm layers in the physiologically active iridophores.

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EXPLANATION OF PLATES

PLATE 1

The ventral margin of the iridescent lateral stripe under vertical illumination. The stacking arrangement of the broad hexagonal reflecting plates in the physiologically active iridophores is seen (*a*). Also visible are the cigar-shaped physiologically inactive iridophores (*i*). The inactive iridophores tend to be arranged with the long axis arranged dorso-ventrally. The physiologically active iridophores are less well ordered than the physiologically inactive type and lie external to them. The top of the picture is dorsal, the bottom is ventral.

PLATE 2

The two types of reflecting plate from the iris. Superior illumination. The broader hexagonal plates are from the physiologically active cells. Their stacking arrangement is shown in Pl. 1. The indistinct outline of the long edges of these plates is characteristic. The needle-shaped hexagonal plates have come from the physiologically inactive iridocytes. The corresponding cells in the skin are approximately the same length but are about twice the width.

PLATE 3

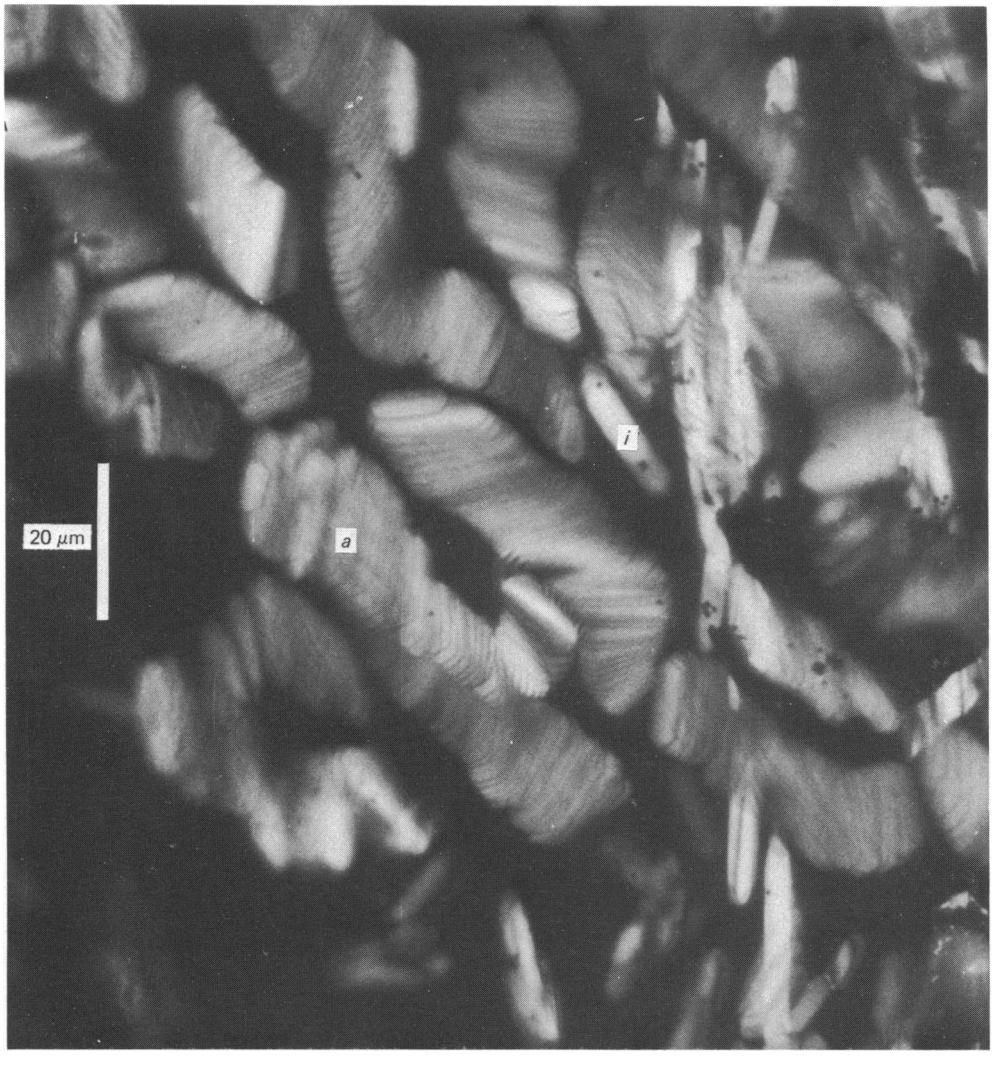
Physiologically active iridophores in the lateral stripe. The double-sided crystal plates (arrow) are contained in membrane-bounded envelopes in the cytoplasm. The clear areas may either be the results of splitting under the electron beam, or may be areas where larger guanine plates have formed, but have been lost during sectioning and staining procedures. The spacing distances that are represented in Fig. 5 are shown by the double-headed arrow.

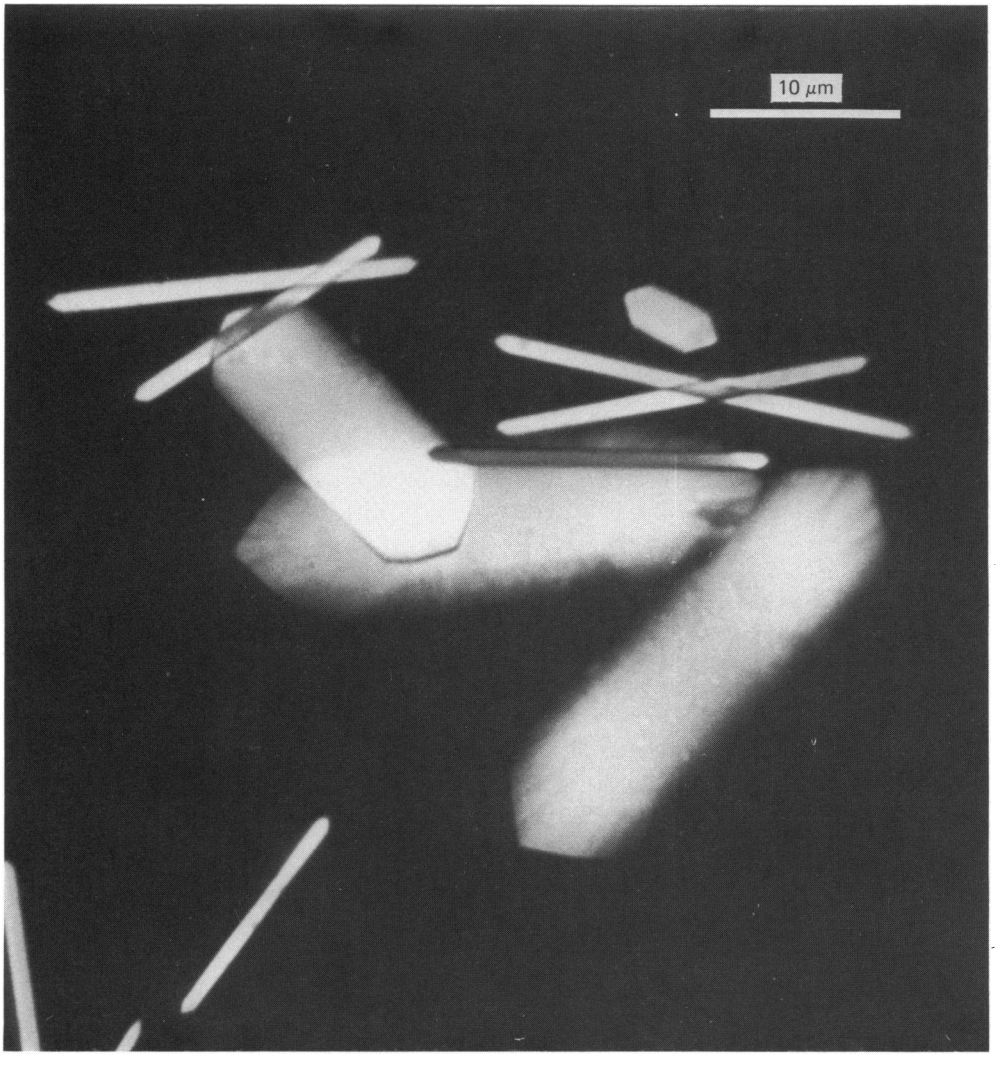
PLATE 4

Section of a physiologically inactive iridophore in the iris showing the broad spaces from which the guanine crystals have been lost (*s*). There is also an area where guanine crystals have remained in place (*g*).

PLATE 5

Section of a physiologically active iridocyte in the lateral stripe that has changed to an orange-red colour in distilled water. The double-sided crystal plates have retained their shape, but the cytoplasm layer has in places broken down into smaller globules. At the top of the picture are regular orthogonally arranged collagen fibrils of the *stratum compactum*. Note that splitting occurs along the plane of the crystal plate. There are indications that occasional thin plates may be forming thicker crystals (arrow).


20 μm
a
i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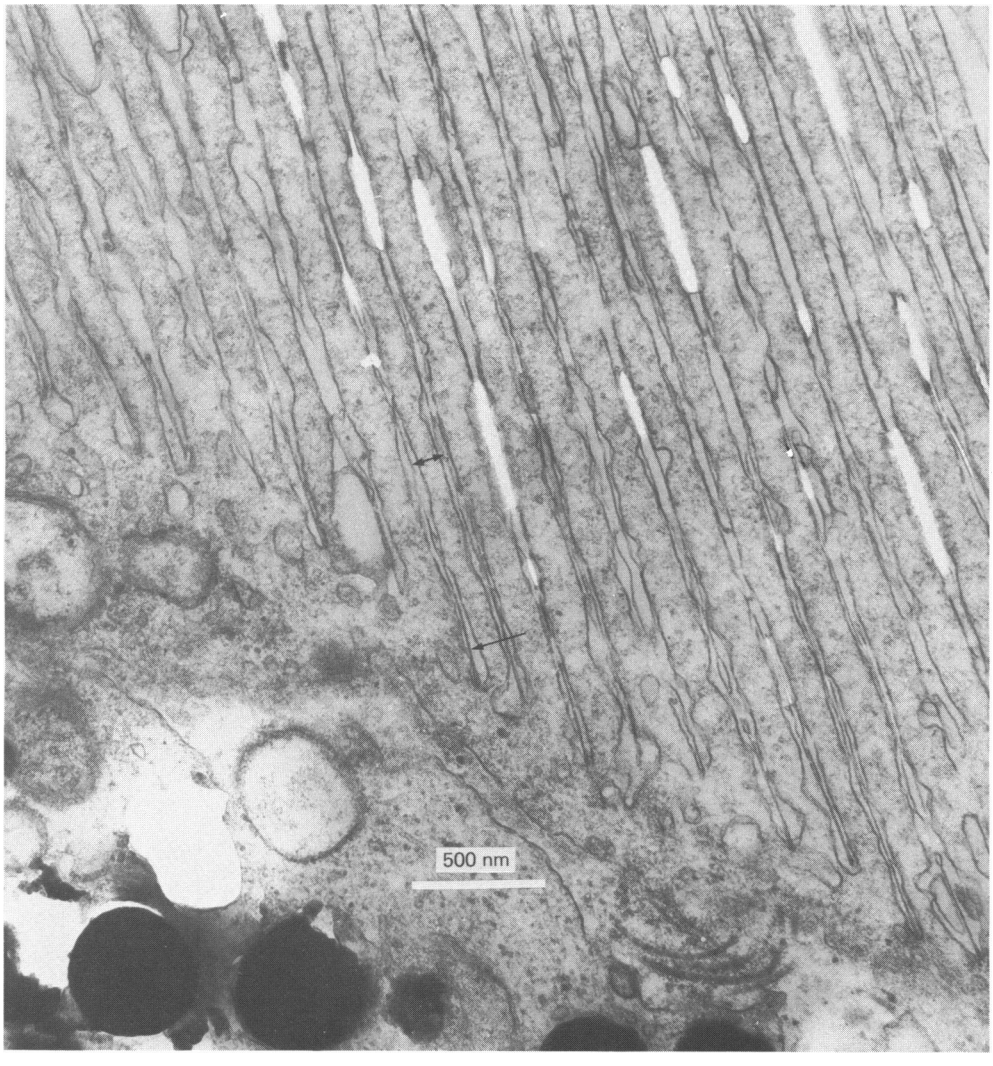
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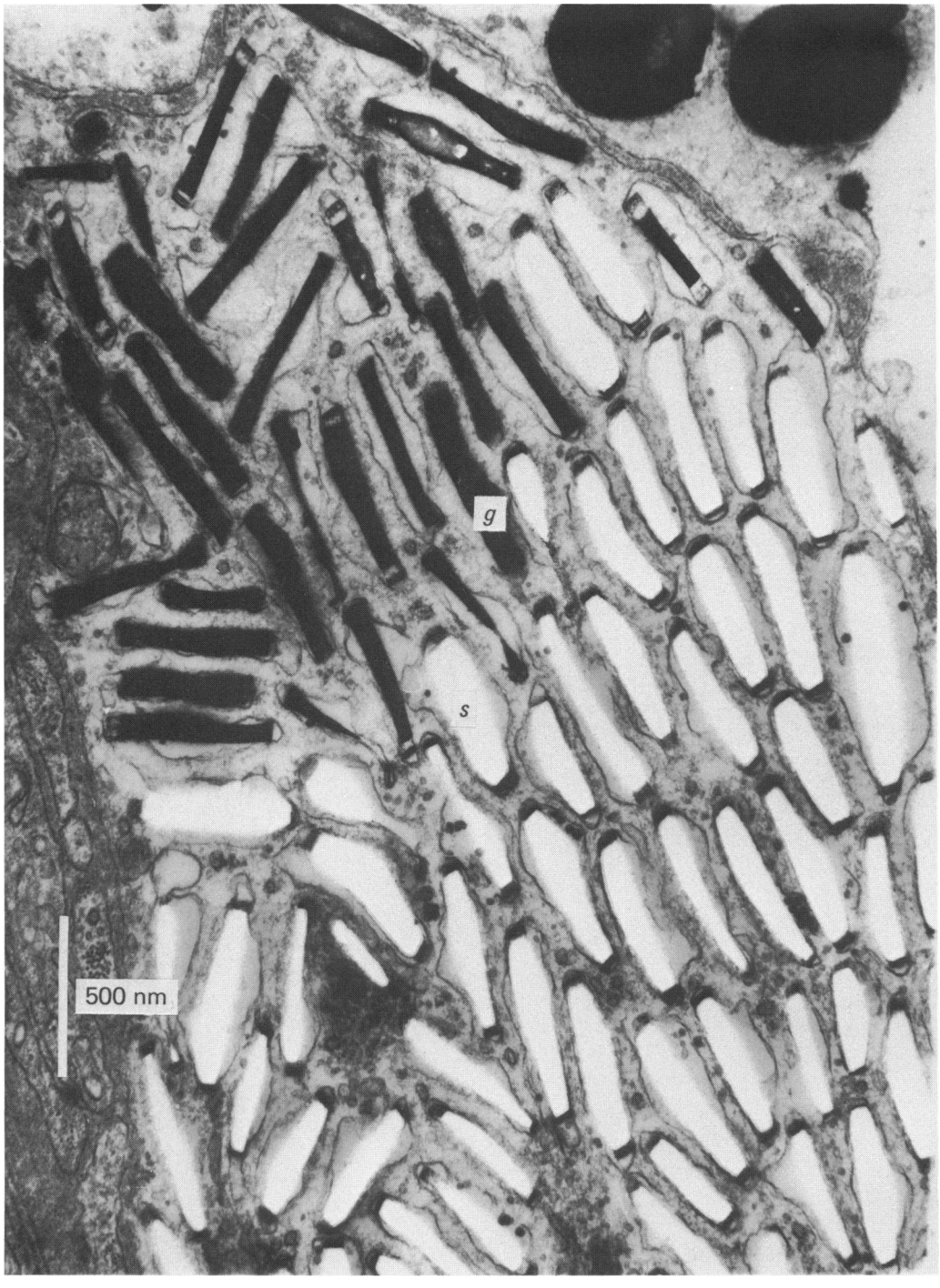
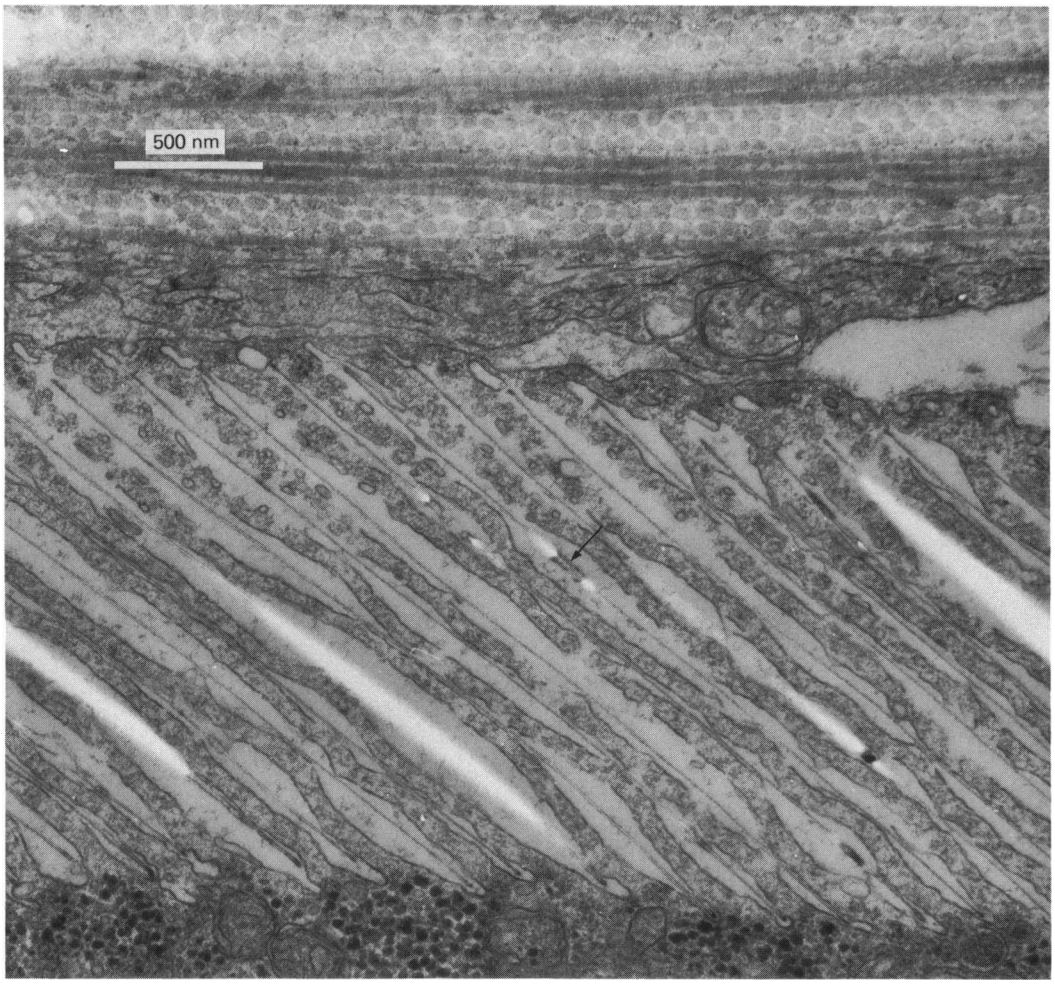

g
s
500 nm


500 nm