NEW EVIDENCE SUPPORTING THE LINKAGE TO EXTRACELLULAR SPACE OF OUTER SEGMENT SACCULES OF FROG CONES BUT NOT RODS

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ABSTRACT

Previous electron microscopic examinations of outer segments of photoreceptors suggest that many flattened saccules of cones are continuous with the cell membrane and that their lumina connect with the extracellular compartment but that most saccules in rods appear to lack these connections. The saccules probably contain photolabile pigment, and certain potentials appear to result from dipole formation during pigment bleaching. The detection of dipoles from rod saccules may require that the lumina of rod saccules connect with extracellular space, and questions have been raised whether the interpretation of micrographs is correct or the isolation of rod saccules is the result of artifact. Accordingly, lanthanum and barium precipitates were produced near fixed and unfixed frog photoreceptors. Lanthanum precipitates appeared to infiltrate the saccules of fixed cones and the few surviving cones exposed prior to fixation, but no rod saccules were infiltrated except occasional, most basal saccules or saccules within narrow zones of probable damage. Barium precipitates did not infiltrate saccules of either variety of unfixed photoreceptor, but they did occasionally infiltrate around the saccules at points of damage in rod outer segments. The results thus support the view of the patency of saccules of frog cones and are consistent with, but do not prove, the isolation of saccules of frog rods.

A great deal of information has accumulated with reference to the bleaching of rod pigments in vertebrate photoreceptors (7, 24, 25, 29, 30, 46, 47, 55). However, while recent evidences of electrical signs associated with the bleaching of receptor photopigments (early receptor potential, ERP) (2–6, 8–12, 22, 42, 43) and possible later responses of receptors (late receptor potential, LRP) have begun to bring into focus some ideas of receptor responsivity (11, 54), there nevertheless remain large gaps in our knowledge of photoreceptor physiology. In particular, the molecular events linking light absorption by visual pigment and the consequent appearance of some form of signal remain unknown. It seems likely that a rod may be excited by a single quantum of light (35); this suggests that the bleaching of a single pigment molecule can be detected. What is the basis of such an exquisitely sensitive mechanism?

Abundant evidence indicates that the photolabile pigment rhodopsin is largely, if not entirely, contained in the outer segment of the photoreceptor. Estimations of its quantity per receptor (29, 46, 55), taken together with the requirement of detergents to solubilize rhodopsin (24), chromophore orientation as deduced from polarized light studies (26, 49, 55), all strongly suggest that the visual pigment is associated with transverse membranes that fill this portion of the cell (16). Presumably cone pigments are similarly associated with membranes. Examination of the rod outer segment of the frog (39–41) and other vertebrates

(16) by electron microscopy reveals that the transverse membranes are those of a pile of scalloped, flattened saccules which are enclosed in an external cell membrane as a pile of hollow discs might be enclosed in a bell jar. The paired membranes of any single saccule appear to be not continuous either with the cell membrane or with the membranes of adjoining saccules (18, 19, 41). The exceptions here are occasional saccules, at the base of some rod outer segments, which are continuous with the cell membrane as if they formed or were forming by invagination or ingrowth of this membrane. Electron microscopy suggests that only the lumina of these exceptional rod saccules are continuous with the extracellular space (15, 17, 39). This exceptional situation in the basal saccules of some rods, however, is the typical situation in almost all saccules of cones of vertebrates other than mammals (15, 17, 39). In the cones of mammals the number of saccules seen in longitudinal sections to be continuous with the cell membrane is much less than in cones of inframammalian species, but this may only mean that the connecting necks are smaller and are missed in most sections.

However, the above view of the outer segments of rods has been challenged. Recent considerations of the electrical requirements for detection of the early receptor potential (ERP) appear to some physiologists to demand either that the membrane of most rod saccules be continuous with the cell membrane (38) or that monolayers of visual pigment be oriented in the same direction in both walls of each saccule (8). This latter concept is surprising in view of the apparent symmetrical method of saccule development by ingrowth or infolding of the cell membrane. In a symmetrically formed saccule, one might expect that anything longitudinally oriented in one wall would be turned 180° from its counterpart in the other saccule wall. The reason for the concern of physiologists is that the early receptor potential can have an action spectrum compatible with that of either rhodopsin (22) or cone pigment (43). This potential appears in microseconds and resists numerous treatments (2, 4, 8, 11, 12, 22) that block conventional nervous potentials. It thus seems highly likely that the potential originates by formation of a dipole in the early stages of photopigment bleaching. Since an effect is discernible, it would appear that these charges cannot be oriented randomly or so that the dipoles would be allowed to cancel each other out (8). Lettvin (38) regards a saccule whose lumen connects with the extracellular space as permitting an adequate asymmetry because the pigment is placed in a membrane which is an extension of the cell membrane. This membrane is, then, a boundary separating the intracellular and extracellular compartments. While dipoles forming in such a membrane would possess opposing orientations on the opposite sides of the saccule, they would always possess a constant relation to the border between cytoplasm and extracellular space and might be "seen" in parallel electrically.

This would be a possible explanation in the case of saccules in many cones where electron microscopy supports such continuity of saccule and plasma membrane. But what of rods where only basal saccules of occasional outer segments show such connections? If rod saccules were not continuous with the cell membrane, then electrical manifestations of dipoles in one saccule wall would be cancelled out by equal and opposite dipoles in the other wall.

Quite apart from the problem of the early potential, the question of continuity of rod saccule and plasma membrane has bearing on the later events following pigment bleaching. If the visual signal originated in bleaching rhodopsin in saccule membrane but were subsequently manifested by potential changes in the plasma membrane of the outer segment, then the discontinuity of these membranes would require these events to be linked by a diffusion step.

Experiments were therefore devised which were potentially capable of disproving (*not proving*) the apparent view as seen in electron micrographs that most rod saccules are isolated from the extracellular compartment. These experiments involved attempts to directly infiltrate the rod and cone saccules of the frog retina, fixed or unfixed, with various materials. The frog retina was selected for this study because most, if not all, of the lumina of cone saccules appear as if they connect with the extracellular space and because these saccules thus act as a control on the behavior of rod saccules where the lumina appear to be isolated. In addition, studies are available of the behavior of the ERP in the frog retina and in retinas of other animals after a variety of treatments, including aldehyde fixation (2, 8, 32).

MATERIAL AND METHODS

A total of over 250 animals were employed in the various experiments here described; generally 12

animals were used in an experiment. Frogs (*Rana pipiens*) were obtained from Wisconsin (Steinhilber) or collected (*Rana climitans*) at ponds near Woods Hole and dark-adapted at room temperature for 2–3 hr or overnight. They were then decapitated, and the eyes were removed. The eyes were submerged in ice-cold, diluted (225 milliosmols) Earle's physiological saline (Baltimore Biological, Baltimore, Md.) which had been made by adding 24 ml of distilled water to 76 ml of the commercial medium. Osmolarities were measured by freezing-point depression. The anterior portions of the eyes were dissected away, and the retinas were removed with watchmaker's forceps. Removal of the retina was attempted without the pigment epithelium, but partial success was the typical result. Dark adaptation in the cold made this separation even more difficult. Shaking the retinas in physiological solutions was briefly tried so that the pigment cell layer could be removed. However, subsequent microscopic examination very often showed that this removal was incomplete, since processes of pigment cells sometime persisted between receptors or since the anterior faces of pigment cells sometimes persisted as a fairly intact membrane. In most experiments, dissections were made under red illumination (Corning 2404 filter, Corning Glass Works, Corning, N.Y.), and room manipulations occurred under very dim white illumination. For conventional fixation, diluted Earle's saline containing 2% osmium tetroxide and 2 ml of M/15 phosphate buffer (pH 7.4) was employed, and it yielded excellent preservation.

Lanthanum Precipitates

This final procedure was related to methods of Doggenweiler and Frenk (28) and Revel and Karnovsky (44) and was based on numerous pilot studies. Retinas were generally fixed for various intervals at 20°C in 2.5% glutaraldehyde in 0.1 M phosphate buffer, final pH 7.6, final osmolarity 570 milliosmols. The retinas were given three 10-min rinses in 5 ml of the same buffer and were then exposed for varying periods to 5 ml of 1% lanthanum nitrate in 0.1 M cacodylate or tris buffer, final pH 7.2, final osmolarity 538 milliosmols. This was usually followed by dehydration but sometimes was followed by fixation in 2% osmium tetroxide before dehydration.

Unfixed retinas, following 10–40 min of exposure to 0.1 M phsophate buffer, pH 7.6, osmolarity 235 milliosmols, were exposed to the cacodylate-buffered 1% lanthanum for intervals from 5 to 60 min; then they were successively fixed in 2.5% glutaraldehyde in cacodylate or phosphate buffer for 1 hr and sometimes, following glutaraldehyde treatment, in osmium tetroxide for 1 hr.

Controls on this procedure were omission of either phosphate or lanthanum ion. Variations included pretreatment with pronase (Calbiochem, Los Angeles, Calif.) or bovine testicular hyaluronidase (Sigma Chemical Co., St. Louis, Mo.) as an attempt to weaken gels or other materials occurring about the photoreceptors.

Sulfate Precipitate

This was generally employed with unfixed retinas. The dissected retinas were exposed to 0.1 M sodium sulfate in 0.1 M cacodylate buffer, final pH 7.2, final osmolarity 426 milliosmols. After 10 min in this solution, the retinas were exposed to 0.1 M barium chloride in the same buffer, final pH 7.4, final osmolarity 441 milliosmols, for periods from 10 min to 24 hr. This was followed by fixation in osmium tetroxide. Other retinas were fixed in glutaraldehyde prior to sulfate treatment. Controls included absence of barium or of ions precipitable by barium.

Fixed retinas were rapidly dehydrated in neutral alcohols and embedded in epoxy resin (Durcupan, Fluka AG, Basel, Switzerland.). Stained (uranium-lead) or unstained sections were examined and photographed with a Siemens Elmiskop 1A electron microscope.

RESULTS

Electron micrographs of osmium tetroxide- or permanganate-fixed outer segments of amphibian photoreceptors have been repeatedly illustrated elsewhere (39–41, 55) and are not shown here. For the most part, these were micrographs of longitudinal sections. However, since longitudinal sections may easily miss a narrow neck by which a saccule of an outer segment might be connected to the cell membrane and the saccule lumen to the extracellular space, typical cross-sections of outer segments are demonstrated (Figs. 1 and 2). It will be noted that the infolding of the cell membrane to form a cone saccule is discernible, but no sign of a similar process is seen in the case of a rod saccule. However, it is difficult to follow the entire perimeter of the cell membrane without encountering small regions where membrane definition is vague.

It is, therefore, of obvious value to attempt the infiltration of the saccule lumina with various inert materials of differing dimensions and chemical properties. Attempts with ferritin, gold colloid, horseradish peroxidase, and lanthanum hydroxide suspensions (44) failed. However, since these tracers did not reach the receptor surfaces, it seemed likely that the experiments themselves had not succeeded because a natural gel and residue of pigment cell processes in the vicinity of the receptors had filtered out the tracer materials. Revel

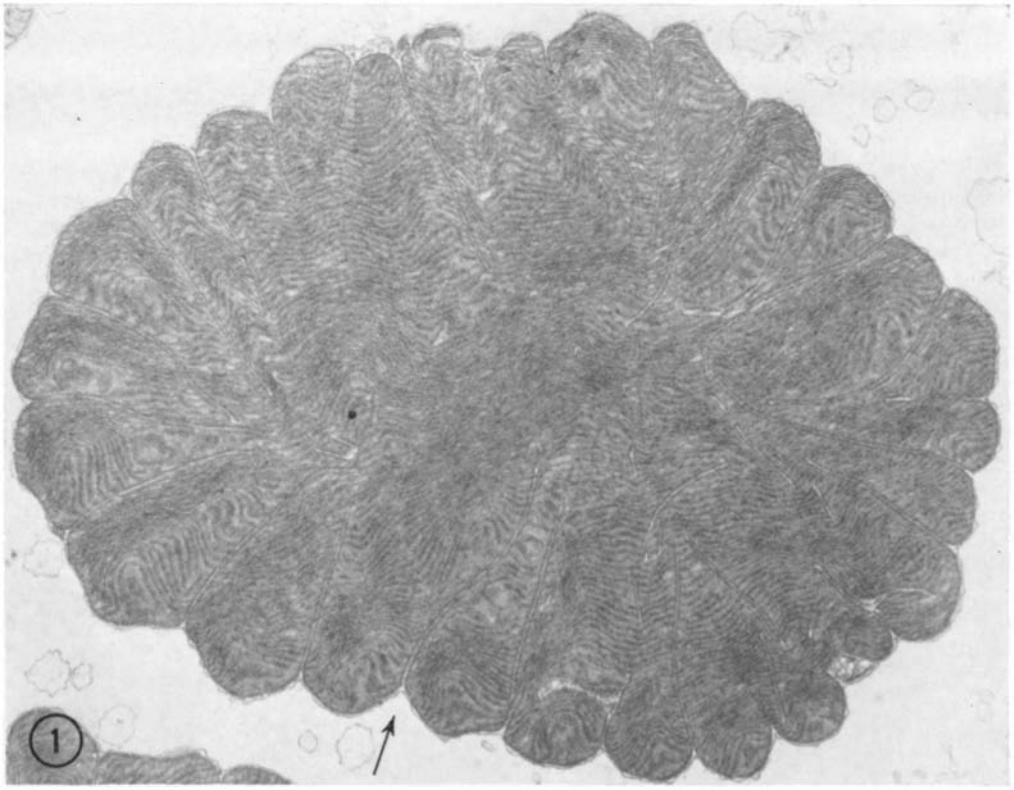

FIGURE 1 Electron micrograph of a cross-section of the outer segment of a frog rod. The thin cell membrane (arrow) can be seen as separate from saccule membrane at almost all points of the perimeter. The saccules virtually fill the area of the cross-section, and their nonplanarity produces a striping effect as the section plane cuts many saccules. OsO_4-fixed, lead and uranium stained. × 18,500

and Karnovsky (44) similarly note the superficial penetration of lanthanum hydroxide suspensions. It was therefore decided to try to form a lanthanum precipitate in the vicinity of the receptors by a modification of a method of Revel and Karnovksy (personal communication).

Lanthanum

Exposure of glutaraldehyde-phosphate–fixed retinas to mildly alkaline lanthanum and then to osmium tetroxide yielded the following findings. If the pigment epithelium was absent, then the receptors typically were found to be surrounded by a dense black material (Figs. 4 and 5). This was in continuity with similar material in most, if not all, of the saccules of cones (Figs. 4 and 8), but rod saccules were lacking in this material, with the occasional exception of a few most basal saccules. Similar material was observed piled heavily at the external limiting membrane of the retina, i.e. outside the terminal bars surrounding the glial cells of Müller and bases of the inner segments of the photoreceptors. In some limited regions the material variably penetrated the extracellular space of the retina proper and could be found at any level. The dense material was also seen in the cleft between the outer and inner segments of both principal varieties of photoreceptors. Since in this study no behavioral distinction was observed between red and green rods or among the various cone types, the observations are therefore reported for rods and cones. When the pigment epithelium adhered to the retina, the dense material could be found on the outer aspect of the pigment cells and between them (Fig. 3) up to the level of the terminal bars at their inner aspect, facing the photoreceptors, but not past this level except in regions of pigment cell damage. Even

the persistence of only the inner face of the pigment cells severely reduced lanthanum penetration. On the vitreal surface of the retina, the typical result was a feeble lanthanum penetration which yielded limited extracellular densification or deposition around the foot processes of the glial cells of Müller in all retinal regions. Considerable precipitate was seen in adherent vitreal gel well off the retina, and this probably accounts in part for limited penetration from this aspect.

While the above findings suggested that the dense material was confined to the extracellular compartment and to the lumina of the saccules of cones compared to rods, a critical examination of glutaraldehyde-osmium tetroxide–fixed material sometimes revealed the presence of what appeared to be a precipitate in the lumina of some rod saccules including the expanded intrasaccular space at the saccule edges. However, such precipitates were also observed in similar frequency within some regions of rod saccules of control specimens that had not been exposed to lanthanum. It seemed possible that this material was a residue of glutaraldehyde-osmium tetroxide interaction. It was particularly troublesome in uranium- and lead-stained sections. Its particular persistence in rod saccules (Fig. 6), as opposed to cone saccules (Fig. 7), is also consistent with the idea either that rod saccules are sealed off or that any opening into them is very small.

However, in view of the objectives of this study it was decided to attempt to eliminate all treatments which might obscure or confuse a minor deposit of material of lanthanum origin. Prolonged washing between glutaraldehyde and osmium tetroxide fixations did not provide this result, and accordingly thin sections of non–OsO_4-fixed tissue were examined with and without lanthanum exposure. For a time, sections of glutaraldehyde-fixed but non–OsO_4-fixed tissue were stained with

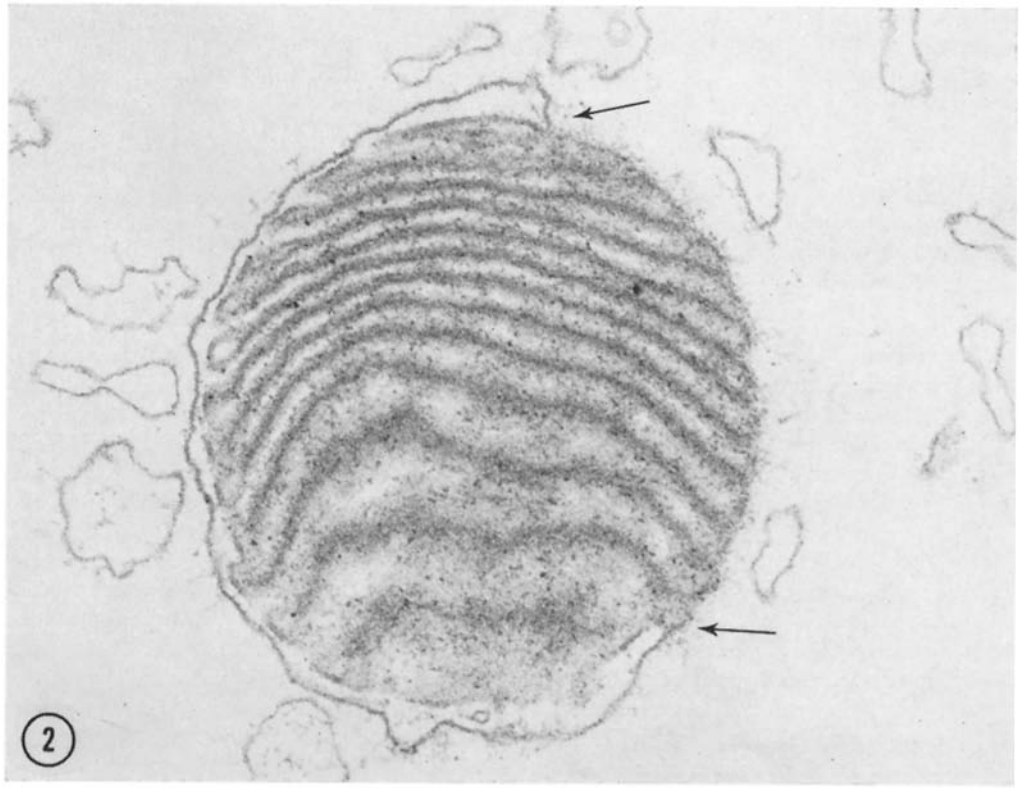

FIGURE 2 Electron micrograph of a cross-section of the outer segment of a frog cone. The saccule planes are not strictly perpendicular to the receptor axis. The cell membrane is confluent with the saccule membrane at two points (arrows). OsO_4 fixed, lead and uranium stained. × 80,750.

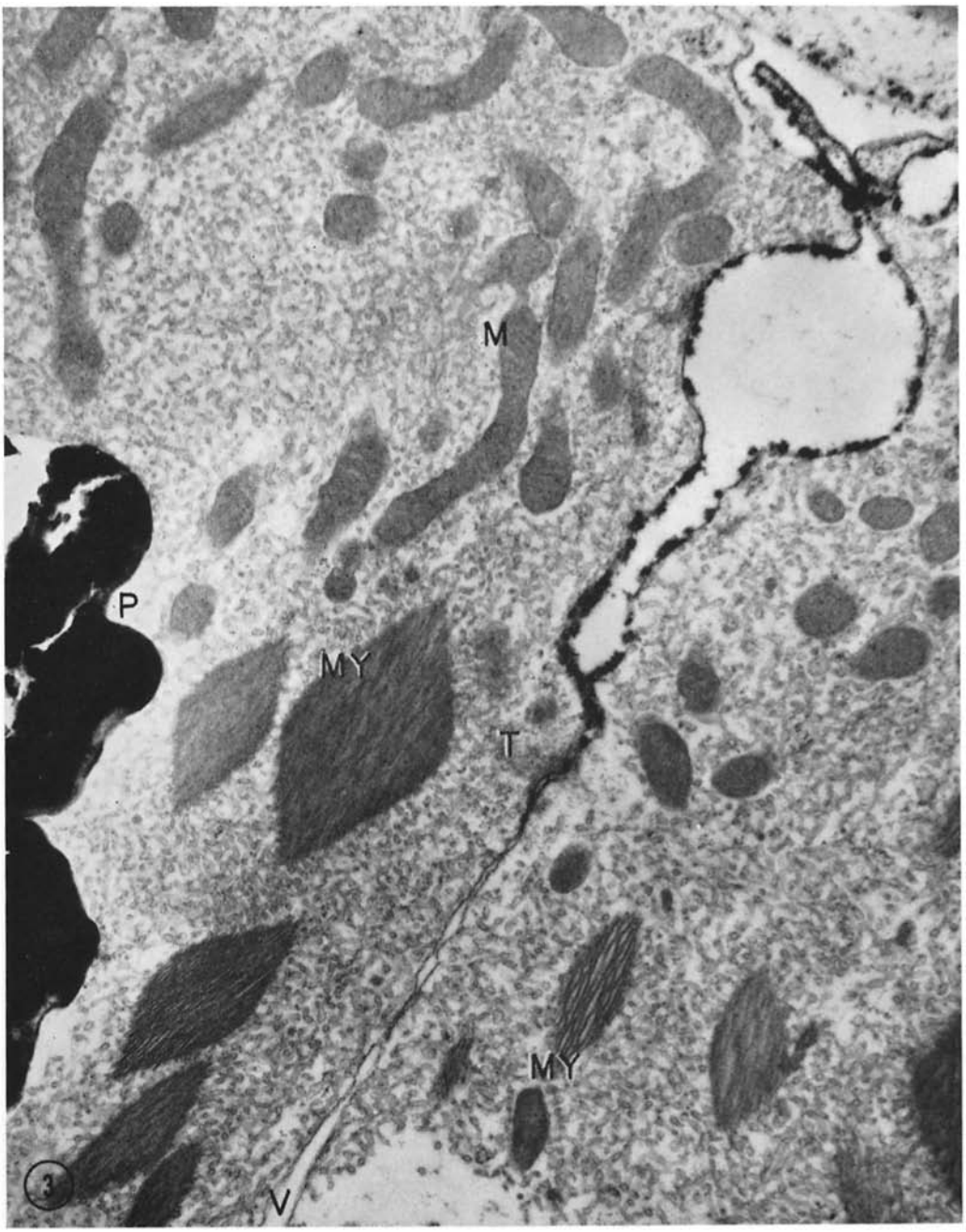

FIGURE 3 Electron micrograph of the junction of two cells of the pigment epithelium following lanthanum treatment. The black, extracellular precipitate has not passed the terminal bar area (*T*) joining the two cells. Also seen are myeloid bodies (*MY*), mitochondria (*M*), pigment granules (*P*), and the ventricular space (*V*) into which the receptors protrude. Glutaraldehyde-OsO_4-fixed, lead and uranium stained. × 18,750.

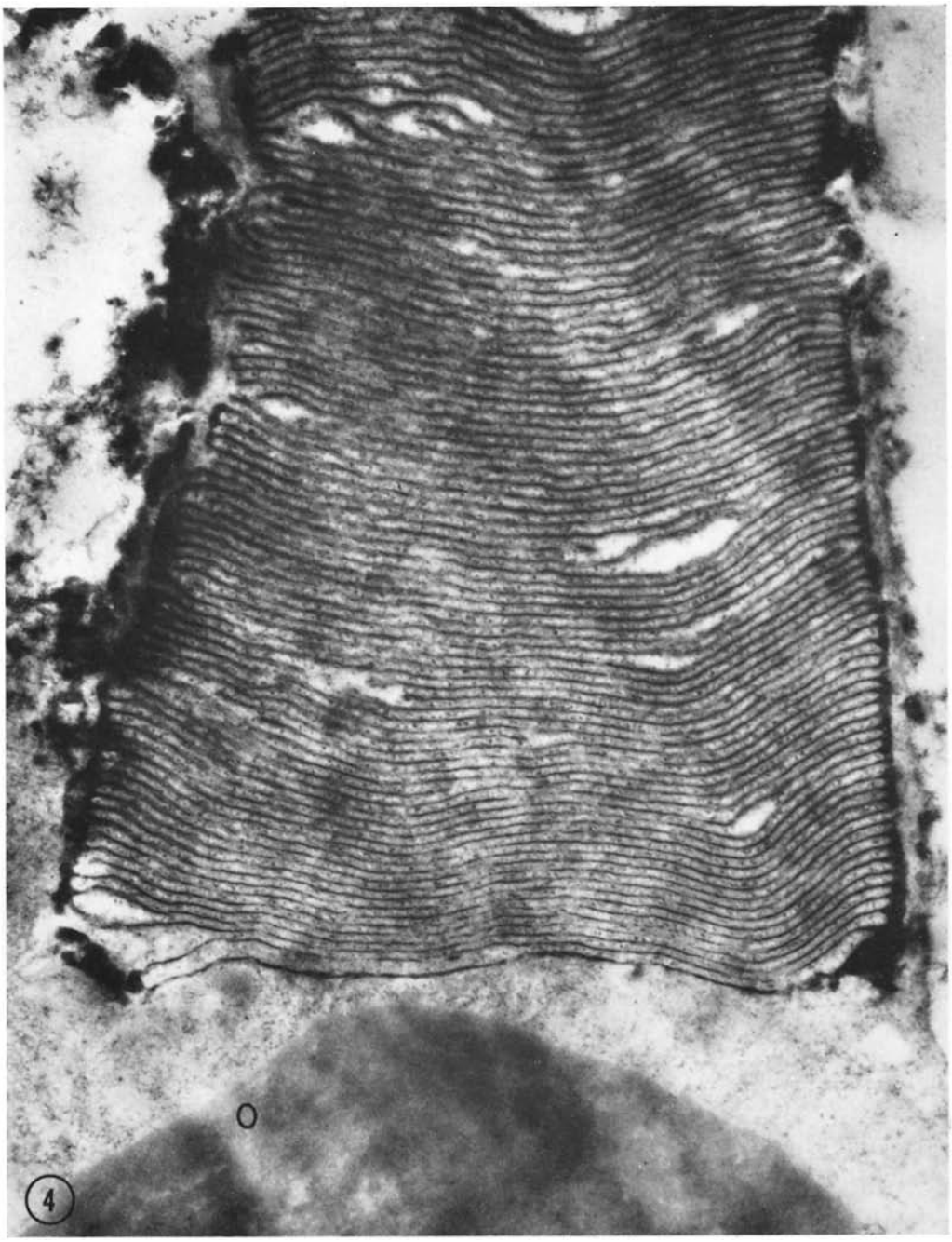

FIGURE 4 Electron micrograph of an outer segment of a cone from a lanthanum-exposed retina. Note the dense material coating the outside of the outer segment and penetrating it in layered fashion from one or both sides. An oil drop is also evident (*O*). Glutaraldehyde-OsO_4-fixed, unstained. × 62,000.

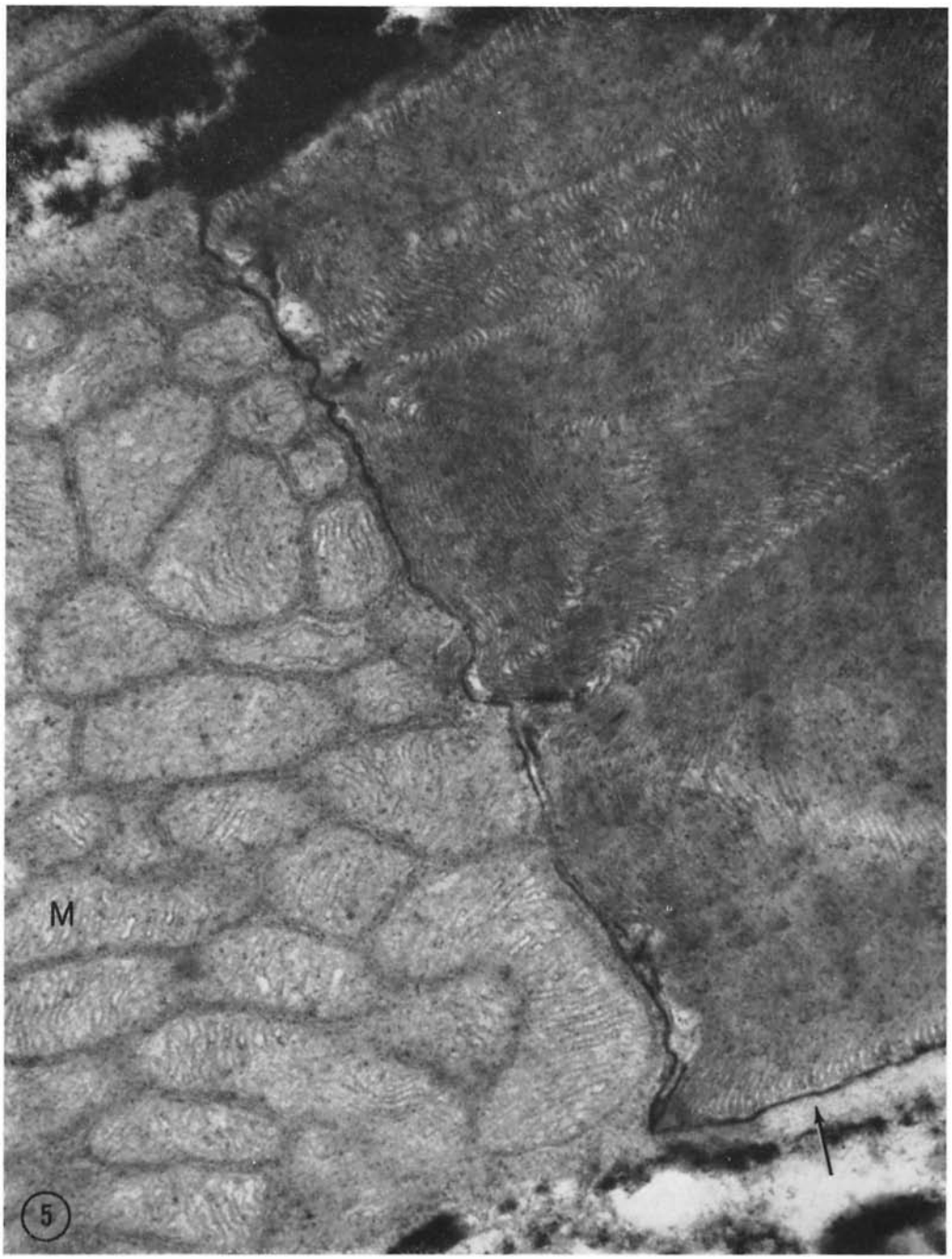

FIGURE 5 Electron micrograph of an outer segment of a rod, at its junction with the inner segment, from a lanthanum-exposed retina. The dark line separating the two segments represents lanthanum in the extracellular cleft in this region. Note the lanthanum-free saccule loops (arrow) and the numerous mitochondria of the inner segment (*M*). Glutaraldehyde-OsO_4-fixed, lead and uranium stained. × 49,000.

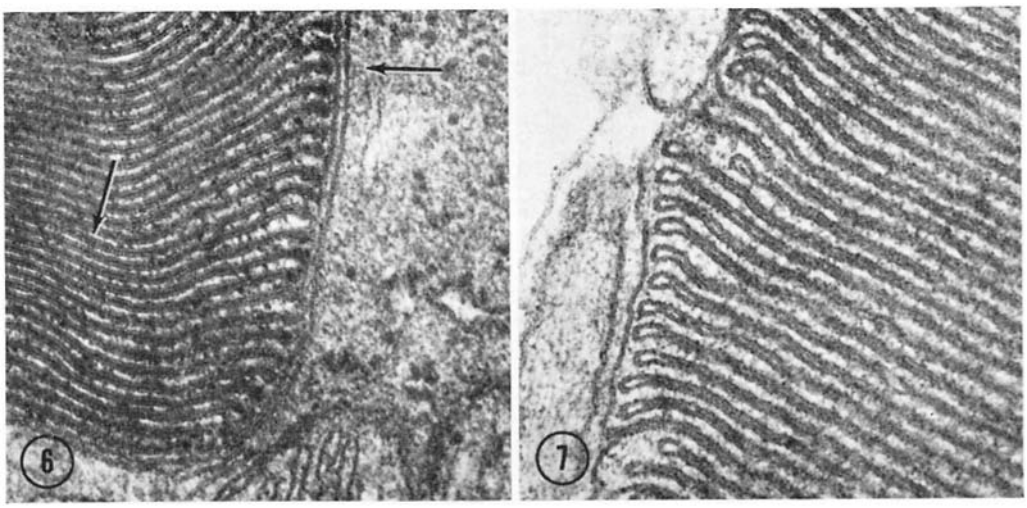

FIGURE 6 Electron micrograph of the edge of a rod outer segment from a control retina to illustrate densification of nonlanthanum origin sometimes seen in rod saccules and loops (arrows). Glutaraldehyde-OsO_4-fixed, lead and uranium stained. × 87,000.

FIGURE 7 Electron micrograph of the edge of a cone outer segment, away from the zone of infolding. Glutaraldehyde-OsO_4-fixed, lead and uranium stained. × 87,000.

uranium and lead salts. However, this was also abandoned since, without reference to lanthanum treatment, thick sections sometimes exhibited a reversal of contrast which is analogous to that sometimes seen in freeze-dried materials (33, 52), with the lipid-free membranes appearing lighter than the cytoplasmic background. The non-OsO_4-fixed and unstained sections showed unequivocally in lanthanum-exposed material a densification of the lumina of cone saccules (Figs. 8 and 9) and only a few basal saccules of occasional rods. However, occasional rod outer segments which had been exposed to lanthanum showed a narrow band of densification across a random region of the outer segment (Fig. 10). Upon detailed examination this was revealed, in the majority of cases, to be an extrasaccular but intracellular densification (Figs. 12 and 13); presumably this density occurs when something enters through a tear in the external membrane and spreads more readily across, rather than up and down, the segment because tears in this direction are more likely. In only two or three instances, intrasaccular (Fig. 12) as well as extrasaccular density was observed in this narrow zone. Yet these few instances suggest that the lumen of the rod saccules in all outer segment regions is potentially patent. It might be argued that density is not seen in rod saccules because these have narrower lumina than cone saccules (compare Figs. 6 and 7) and are too tight for the penetration of a fine precipitate. However, one must note that no dense material was ever found in the enlarged space at the margins of rod saccules in intact, outer segments.

Finally, attempts were made to see the effects of lanthanum on unfixed photoreceptors. Unfixed retinas were soaked in phosphate buffer, then in cacodylate-buffered lanthanum nitrate or control buffer, and finally fixed in cacodylate-buffered glutaraldehyde. The tissues were then examined without further fixation or staining. In the majority of cases, cone outer segments, *but not rod outer segments*, were destroyed following lanthanum exposure. In three instances, recognizable cone outer segments were observed, and these all showed densification of, or precipitate within, the saccule lumina (Fig. 15); but rod saccules were again free of any densification (Fig. 14). 5-min lanthanum exposures yielded more surviving cone outer segments, but then the cone saccules exhibited only trace amounts of material. The exposure of retinas to high concentrations of phosphate or lanthanum ions is admittedly unphysiological. However, pilot experiments showed that little precipitate was formed when the concentration of either of these ions was greatly reduced. When an undamaged

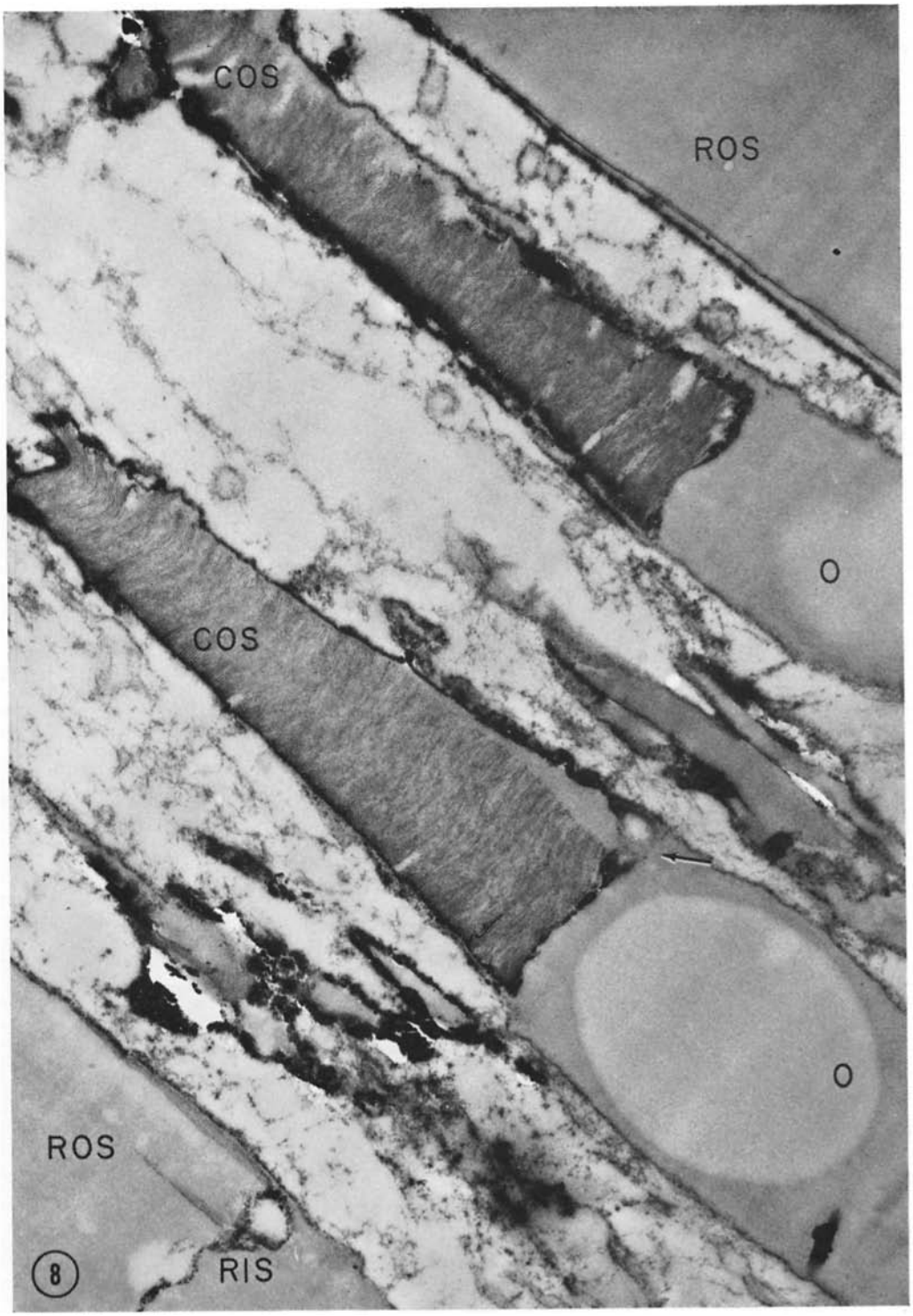

FIGURE 8 Electron micrograph of a lanthanum-exposed retina, showing densified outer segments of cones (*COS*) but not of rods (*ROS*). A rod inner segment (*RIS*) and two cone inner segments with oil drop spaces (*O*) are also evident. The arrow points to the ciliary stalk connecting the segments. Glutaraldehyde fixed, unstained. × 13,500.

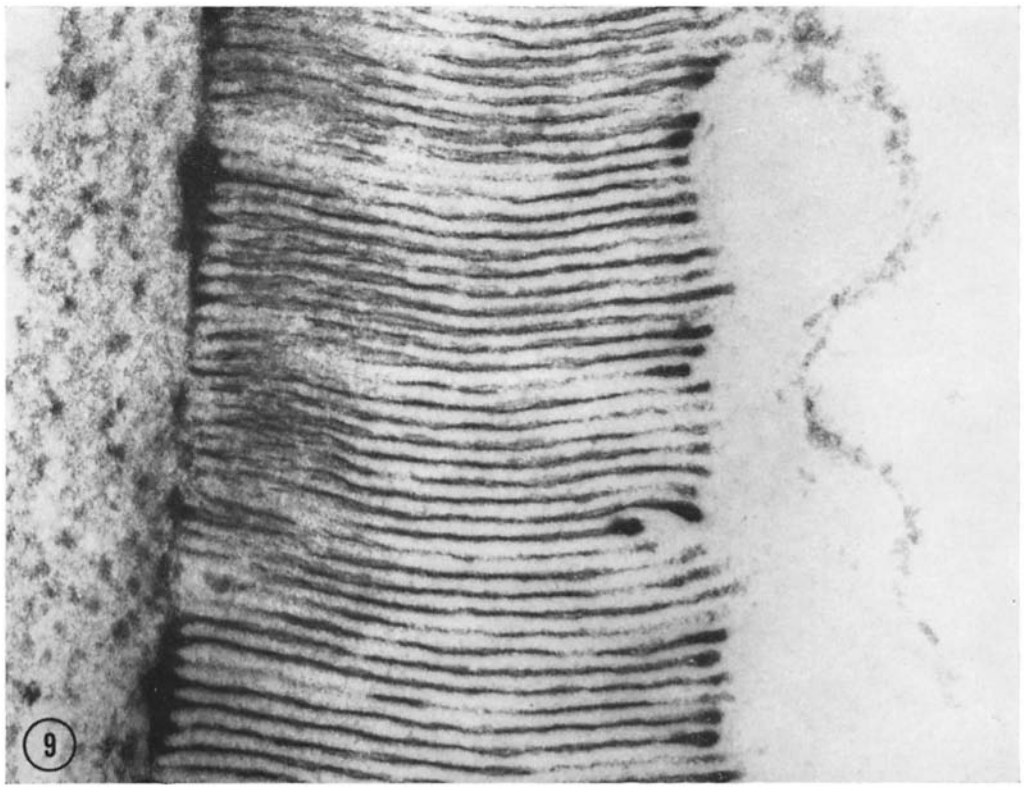

FIGURE 9 This electron micrograph shows, in contrast with Fig. 4, a similar cone outer segment from a lanthanum-exposed retina, but, without having been fixed in OsO_4. Only the lanthanum filling is demonstrated. Glutaraldehyde fixed, unstained. × 98,500.

pigment epithelium was present on the retina, no densification of any sort was seen with respect to the photoreceptors; and cone outer segments were now largely preserved. It is probable that this protection against lanthanum ion by the pigment epithelium again relates to the terminal bars at the inner (vitread) surface of these cells (20, 36).

Note must also be taken of occasional results with lanthanum which are more difficult to explain. In a few instances no precipitate or densification was seen except for a band of extracellular material at a certain retinal level. In other instances external, dense material was restricted to outer (sclerad) levels of the surface of rod outer segments, as if a gel kept the dense material or the ions from which it had originated from penetrating deeper. In one experiment a narrow zone of dense material was observed solely just above the external limiting membrane. With prolonged exposures (days) to lanthanum, crystalloids larger in size and entirely different in character than the amorphous densities described here were observed both intra- and extracellularly throughout the retina.

The failure to find uniform or consistent lanthanum deposition within the retina is puzzling, and occasionally finding it in a zone within the retina may mean either that the stain or deposit is readily eluted in later processing or that it forms where lanthanum and phosphate ion gradients interact. Trivalent lanthanum is said to exhibit certain resemblances to calcium (31), and the concentrations applied might strengthen postulated intercellular "cement" substances and modify intercellular movement of ions or fine precipitates. A fine, tortuous channel into a saccule might similarly be blocked. Local changes in chemical background could also affect the size of a fine colloid or perhaps its charge. Another possible source of difficulty is the oxidation of some

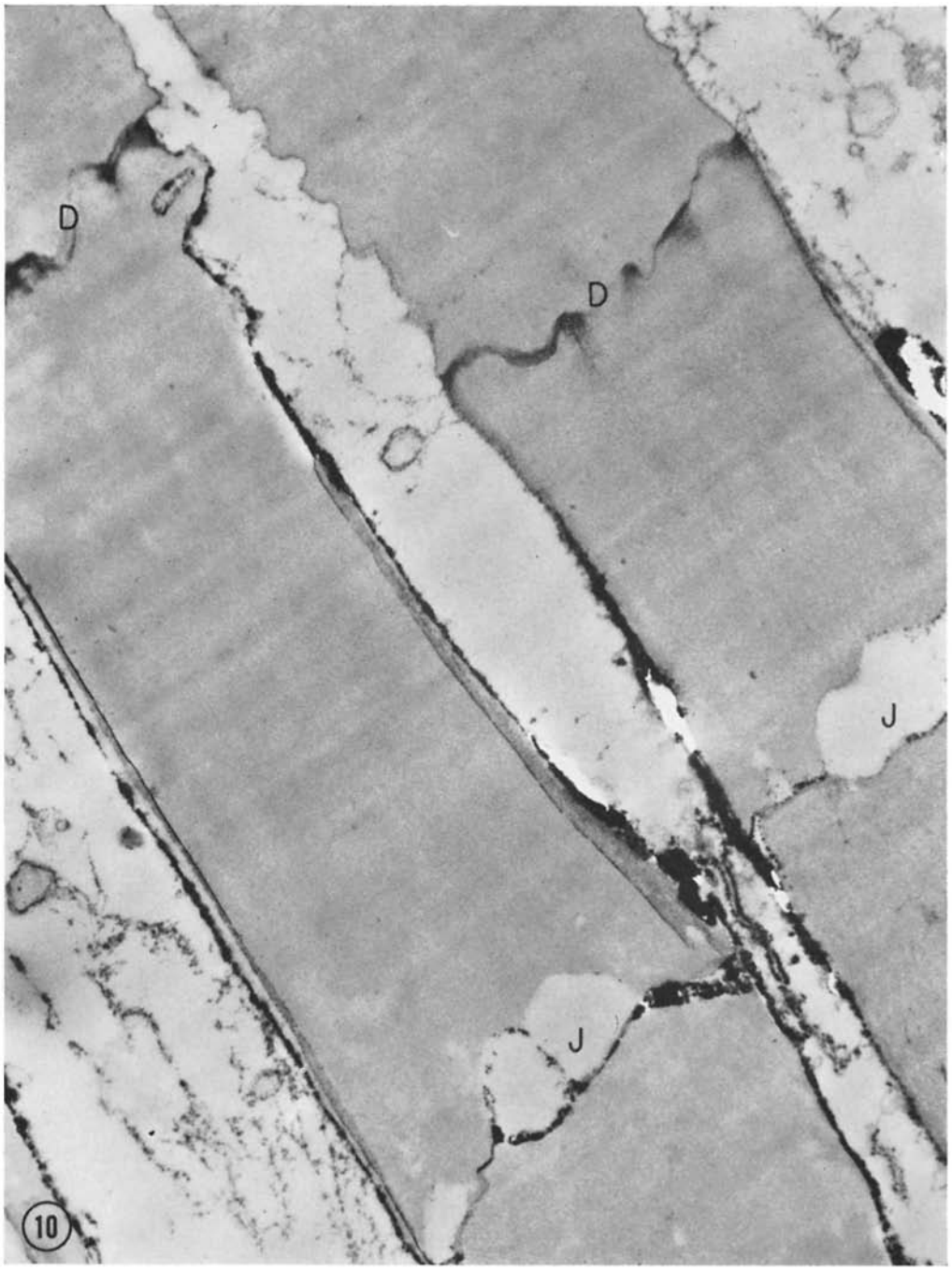

FIGURE 10 This electron micrograph shows two rods from a lanthanum-treated but non-OsO_4-fixed retina showing the absence of transverse density except at the junctions of outer and inner segments (*J*) and in two presumptive defect zones (*D*), not seen in most rod outer segments of this and similar preparations. Glutaraldehyde fixed, unstained. × 13,500.

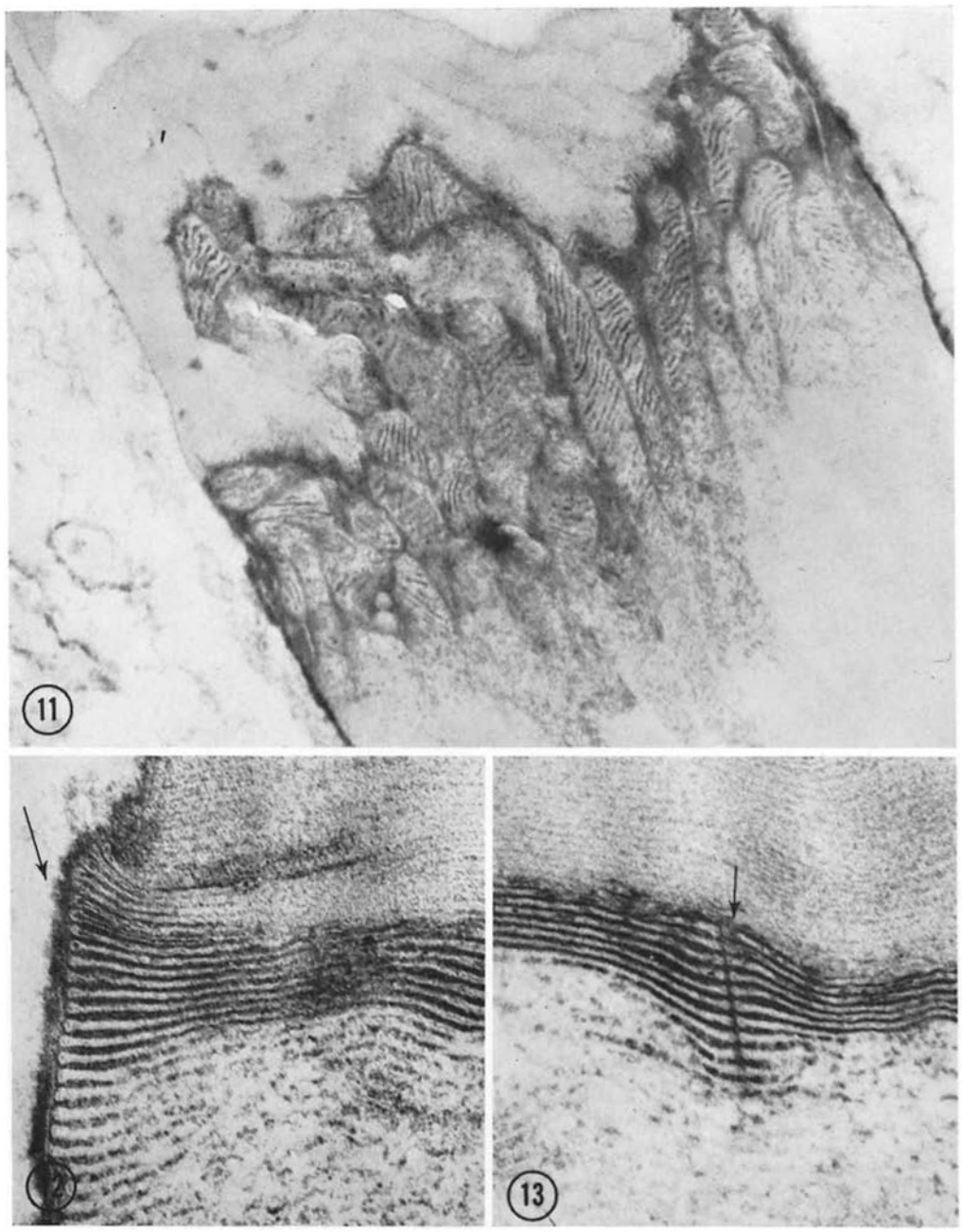

FIGURE 11 Electron micrograph showing a presumptive and rare defect in a rod inner segment of a lanthanum-exposed retina. Note the density in the cytoplasm and apparent filling of cristae of the mitochondria. Glutaraldehyde fixed, unstained. × 31,750.

FIGURES 12 and 13 These electron micrographs show areas of presumptive defect zones from two rod outer segments of lanthanum-exposed retinas. Note that most of the densification is between saccules, but dense material is present in some saccules (arrows). Glutaraldehyde fixed, unstained. × 90,000.

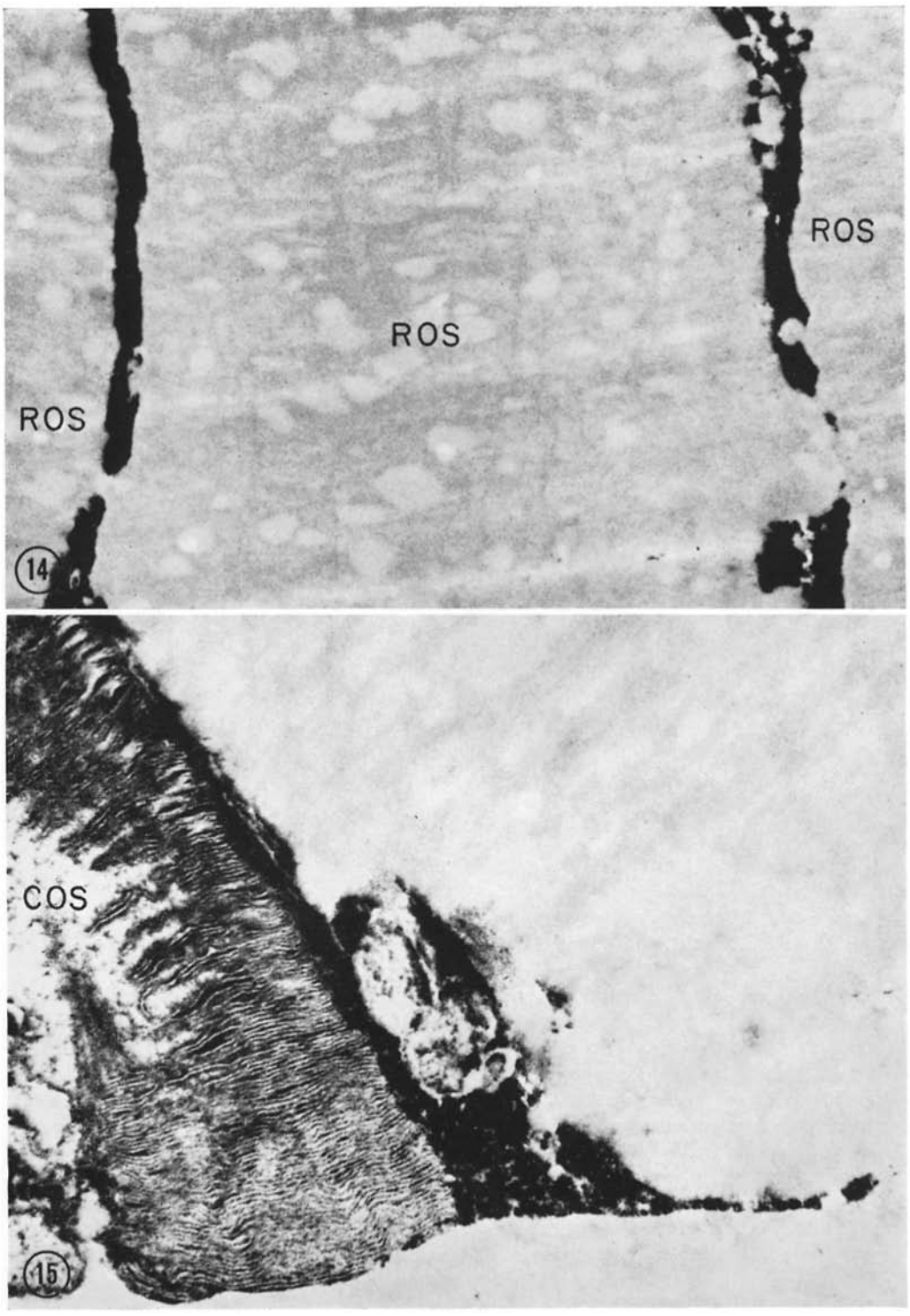

FIGURE 14 Electron micrograph showing regions of three rod outer segments (*ROS*) from a retina exposed to lanthanum prior to fixation. Glutaraldehyde postfixed, unstained. × 17,400.

FIGURE 15 Electron micrograph showing a rare surviving cone outer segment (*COS*) from a retina exposed to lanthanum before fixation. Glutaraldehyde fixed, unstained. × 24,400.

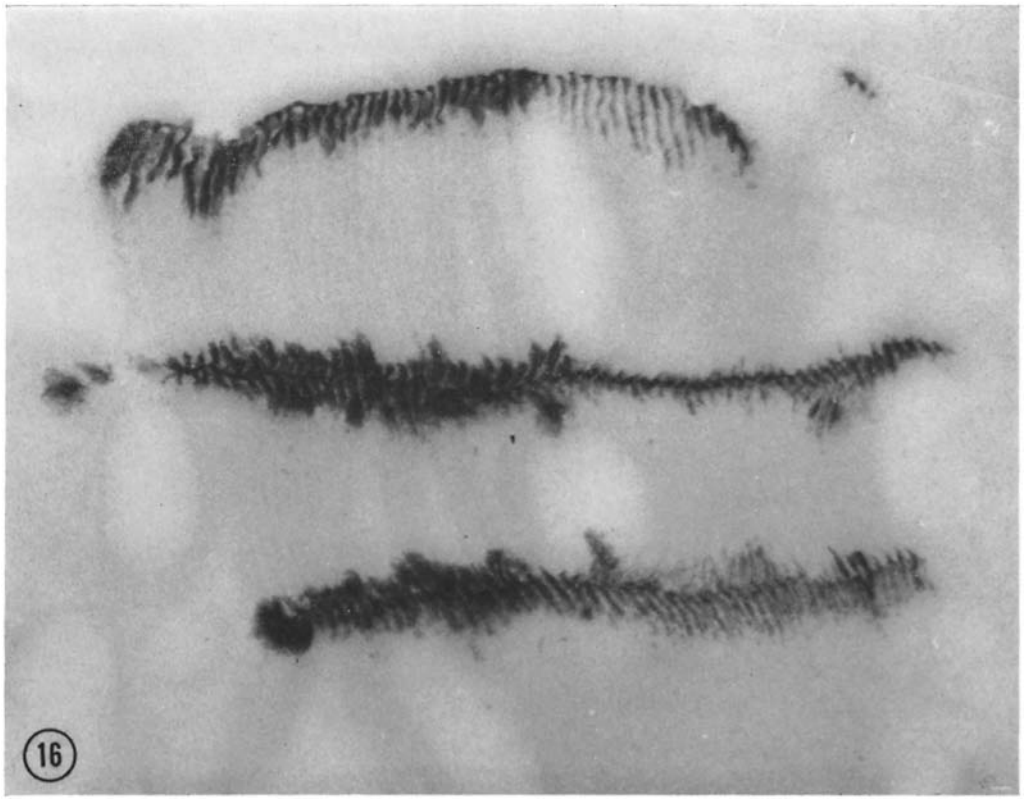

FIGURE 16 Electron micrograph showing the interior peripheral filling of occasional rod outer segments exposed to barium sulfate while unfixed. Glutaraldehyde postfixed, unstained. × 65,600.

glutaraldehyde to glutaric acid. A residuum of the latter may chelate applied lanthanum. Such oxidation could occur in stock solutions or during exposure of the fixative to tissue. Finally, if one adds alkali to precipitate lanthanum hydroxide from a lanthanum nitrate solution while attempting to follow the pH in the alkaline range with a glass electrode, the electrode behaves erratically; this suggests that the electrode is poisoned by surface films. This in turn suggests that under certain conditions lanthanum deposits may act as barriers in tissue to further lanthanum penetration.

On the basis of the continuity of extracellular space in electron micrographs, the lanthanum infiltration of fixed material exactly confirmed the predicted patency of saccules of fixed retinas of frogs. No differences in penetration were observed when light- and dark-adapted retinal preparations were compared.

The occasional survival and infiltration of unfixed cone outer segments demonstrate that prior glutaraldehyde fixation is not required for the infiltration. The survival of many rod outer segments, however, with no sign of infiltration by lanthanum, could also relate to a postulated difference in saccule patency.

An artifact of possible significance for ascertaining the fineness of orifices, which can at times be permeated, occurs when a rare photoreceptor inner segment is seen to contain dense cytoplasmic material; presumably this is a consequence of a damaged cell membrane. In these instances this dense material occupied the cytoplasmic space of the ellipsoid region (Fig. 11), and the density was apparent in the cristae of the numerous mitochondria in this region. All mitochondria in the area of cytoplasmic blackening exhibited this phenomenon; this suggests that this labeled space may be a compartment continuous with the intracellular space. Both Andersson-Cedergren (1) and Sjöstrand (50) have noted instances of junctions of outer and inner mitochondrial membranes.

FIGURE 17 Electron micrograph illustrating a typical cross-section of a rod outer segment entered by barium sulfate while unfixed. Glutaraldehyde postfixed, unstained. × 19,300.

Such junctions could constitute pores. Some of Robertson's conjectures (45) concerning mitochondrial structure also could explain pores. Doggenweiler and Frenk (28) also mention frequent staining of mitochondria in frog retinas stained with lanthanum; and Revel and Karnovsky (personal communication), using lanthanum hydroxide, observed an identical phenomenon in mitochondria of liver cells. In the current investigation, material or stain was never seen within mitochondria unless there was some blackening of the cytoplasm about them.

Barium Deposition

Experiments similar to those employing lanthanum were attempted with unfixed retinas by soaking the retinas in sulfate-containing solutions and then exposing them to buffered solutions containing barium ion. While a dense deposit was

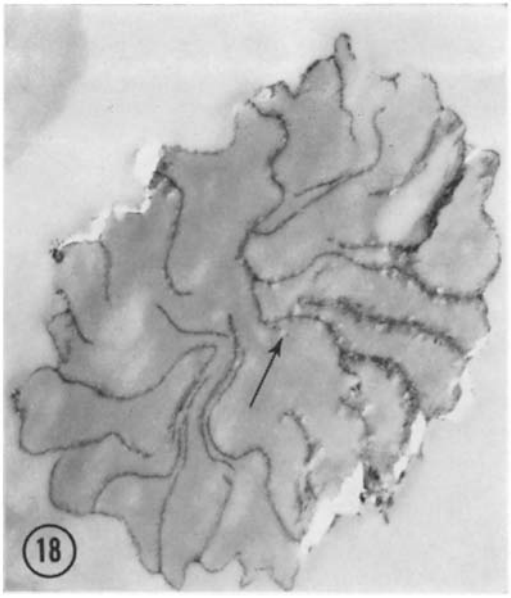

FIGURE 18 Electron micrograph illustrating an exceptional instance where an outer segment locally entered by barium sulfate shows complete outlining of the saccules. Note that some sectors (arrow) are completely encircled; this suggests that they are connected to something not in the plane of section. Glutaraldehyde postfixed, unstained. × 13,900.

consistently found at the external limiting membrane, it was not typically found in the receptors and only in limited extracellular patches in the retina. However, an occasional rod outer segment showed a restricted but random zone where dense material, presumably barium sulfate, had entered the outer segment. This material remained strictly extrasaccular and could surround the entire saccule perimeter and penetrate between the lobulations of the saccules to a variable degree (Figs. 17 and 18). However, while the precipitate or its precursors always failed to penetrate the saccule lumina in this zone, it also had, in contrast with lanthanum, only rare success in penetrating between the outer walls of two adjacent saccules; this indicates some kind of structure in this intersaccular region (Fig. 16).

DISCUSSION

In this study dramatic differences were obtained with respect to the outer segments of frog rods *versus* frog cones. But certain clear cautions must be kept in mind. Although the densification of sac-

cules of fixed cones is a positive result, the saccules could have been modified while being fixed. In the case of unfixed cones, the exposure to high phosphate and then to high lanthanum concentrations, while necessary to achieve dense surrounding precipitates, is admittedly most unphysiological. This was further evidenced by the low survival of unfixed outer segments of cones after the lanthanum step. If one argues that previously impervious cone saccules were opened by this treatment, one must advance some special hypothesis to explain the failure of rod saccules to open. Certain fixations, such as permanganate (39, 51), yield views of saccules in which the two membranes have apparently "fused" to give a pentalaminate appearance. The ready lanthanum infiltration of cone saccules and the occasional lanthanum infiltration of rod saccules in basal and damage zones, even in permanganate-fixed material (28), suggest that this fusion is more likely a close approximation. This view is also supported by the ready reappearance of the saccule lumen under certain experimental conditions such as extreme hypotonicity.

The negative findings for rod saccules demand even greater caution. As noted in the Introduction, while these experiments might tend to disprove isolation of rod saccules by showing that these saccules can be infiltrated, they could never prove isolation. The failure to achieve more than patchy infiltration of the retina from either face, could serve to illustrate a mechanism which might block the infiltration of rod saccules. Studies still in progress seem to implicate extracellular gels as one mechanism of removal by filtration of formed precipitates. But, in any event, one again is faced with explaining the absence of labeling in rods next to labeled cones. In addition, whenever any tracer enters cones but fails to enter rods, one may argue, with full justice, that a finer label may have entered rods. Or, in spite of the contrary evidence, considering the reconstruction from serial electron micrographs of rod outer segments of tadpoles (40), one could argue that prior to fixation, if not after fixation, rod saccules possess connections to the surface, but that these are so small that they are readily occluded or too tortuous for the significant diffusion of a given colloid or certain ions.

Lanthanum has recently been employed with two intentions in cytological investigations. Doggenweiler and Frenk (28) employed lanthanum in the ion form, as a stain. This stain was applied in the presence of permanganate ion which acted as both fixative and stain. Revel and Karnovsky (44), on the other hand, used lanthanum in the hydroxide form as a fine colloidal precipitate with the apparent ability to infiltrate extracellular space within glutaraldehyde- or osmium tetroxide–fixed tissue. The current investigation employed conditions in which a precipitate was caused to form either within or very near the impregnated structures. The results obtained are, therefore, comparable to those of Revel and Karnovsky, in that it is not necessary to postulate any affinity of lanthanum *ion* for a tissue component. Doggenweiler and Frenk, who also studied frog retinas, directed their use of lanthanum toward staining the tissue; they kept this ion in solution by maintaining a pH of 5.8. I have made three attempts to obtain staining at pH 5.8 (acetate buffer) with lanthanum nitrate applied before, during, or after washout of glutaraldehyde at this same pH, but I have observed no trace of densification or precipitate in receptors, retina, or extracellular space. Nor have I observed a precipitate when neutral or acid lanthanum nitrate was followed by strictly neutral potassium permanganate and washing solutions.

The preceding reports and the present one agree about the *extracellular* appearance of density or dense material, with the following possible exception in the report of Doggenweiler and Frenk (28). These authors state that an interlamellar matrix of retinal rod outer segments was heavily stained by lanthanum. They do not mention cone saccules. One might be tempted to draw the inference from their report (28) and from a preliminary note (31) that all rod saccules showed such deposition and that their Fig. 4 represented the typical result, since they do not mention any failure of saccule staining. In fact, Doggenweiler (personal communication) states that only 10% of rod outer segments in his preparations showed evidence of "staining." The fact that Fig. 4 of Doggenweiler and Frenk also shows very extensive *cytoplasmic* blackening, external to the saccules, strongly suggests that nearby cell membrane is not intact. Damage to photoreceptor membrane is common in permanganate-fixed material (21). Since only a few saccules are shown to illustrate the dense matrices, it is hard to decide whether saccule membrane is similarly damaged since basal saccules of outer segments of rods are known to

occasionally connect to extracellular space (39), and their Fig. 4 is identified as a basal region of an outer segment.

The apparent difficulty in getting barium sulfate to penetrate between saccules in damaged areas of unfixed receptors has been noted in this report. Whatever held back the barium salt or one of its precursor ions did not hold back the lanthanum precipitate or its precursor ions under similar circumstances. This is probably a question of dimensions of infiltrating colloids. Barium and lanthanum are similar in atomic number, atomic weight, and chemical properties.

Lesseps (37) has shown that pretreatment of isolated cells with the enzyme phospholipase C removes a material from membrane surfaces with an affinity for lanthanum. Since he applied the lanthanum at an alkaline pH (7.8), a precipitate could have been present although obscured by the permanganate in his fixative. Therefore, it is not clear from his results whether it is lanthanum ion or a lanthanum precipitate which no longer is held by something on or in the membrane. I have tried phospholipase treatment and obtained no effect; but, since some difficulty has been experienced in attempting to infiltrate the region around receptors with other proteins, possibly owing to a gel in the area, this negative result is equivocal. In a previous study (15) loosely organized material was seen coating the outer limbs of monkey receptors; although this material could be seen to superficially enter cone saccules, no deeper penetration could be established. Similar amorphous coats on the external membrane may continue into the saccules of frog cones, and coating material might exist within saccules of frog rods even if these saccules are not continuous with extracellular space. Indeed, if saccule membrane possesses certain properties of cell membrane, most of the saccules of the rods could contain a minute but isolated compartment which is ionically extracellular. It would be important to know the relative volumes of the intra- and extrasaccular compartments within rod outer segments, but fixation effects make it difficult to estimate these volumes. That the saccules of cones are densified only because they connect with the extracellular space, not because they possess a material unique to cone saccules is indicated by the densification of basal rod saccules and densification of rare rod saccules in presumptive damage zones as is demonstrated in both this work and that of Doggenweiler and Frenk (28).

Results consistent with the above findings are those of Scarpelli and Craig (48) who exposed unfixed frog retinas to media designed to test for nucleoside phosphatase activity. Subsequently, dense deposits, presumed to be lead phosphate, heavily stained saccule membranes of cone outer segments, but those authors noted that rod deposits were discrete and peripheral extrasaccular deposits. The current study suggests that these deposits were at points of damage of rod outer segments and that the lead ion and/or nucleoside entered cone saccules more directly. The illlustrations of Scarpelli and Craig are not convincing on the precise locations of the deposits, i.e., whether they are intra- or extrasaccular. It is also possible that their localizations demonstrate the extent of diffusion of lead phosphate, rather than sites of its formation.

Also consistent with the isolation of rod saccules is the statement of DeRobertis and Lasansky that these saccules exhibit osmotic swelling (27). However, no data or methodology has been published to support this important claim.

As noted earlier, as a consequence of having an isolated rod saccule containing visual pigment, a visual signal involving a spreading disturbance of outer membrane potential would have to be linked to saccule events by a diffusion step. No light-evoked potential change across the membrane of the outer segment of vertebrate photoreceptors has been demonstrated. Although such attempts may have failed because of great technical difficulties, alternatively one could consider that the outer segment functions as a quasi-solid state apparatus. As has been pointed out (55), there are great difficulties in viewing the outer segment as a semiconductor, and this simply postpones the problem of transduction to the site of ciliary insertion of the outer segment into the inner.

Although, in fact, our experiments were planned and executed in the belief that the early potentials from the frog retina were largely of rod origin, a recent report (32) states that both the rate of recovery of the ERP in the frog after pigment bleaching and the action spectrum for the frog ERP make it highly likely that most of the response derives from the cones. Since it has been made even more probable by the current investigation that, in fact, frog cone saccules are connected to the extracellular space, and since the data best fit the view that most frog rod saccules are isolated from the cell membrane, this result is in full accord

with the hypothesis of Lettvin (38) because dipoles forming in isolated rod saccules would not be detectable and the sole response would derive from cones and possibly from a few basal rod saccules.

These findings are also consistent with recent demonstrations in invertebrate photoreceptors in which similar early potentials are seen and in which the dipoles indeed arise within cell membrane (34, 53).

On the other hand, with extracellular or intracellular electrodes a potential analogous to the ERP may be detected after illumination of the pigment epithelium of the frog eye (9, 13, 14), and this potential seems to relate to absorption of light by melanin in melanin-bearing organelles. The migration of the melanin-containing organelles within processes of the pigment cells during the course of light and dark adaptation would tend to suggest their physical independence from the cell membrane. But, as Brown and Crawford (13, 14) note, it is not clear whether the complex melanin molecules possess a favored orientation with respect to the cell or one effect of illumination is to bring about a uniform orientation of dipoles. One would assume a relationship of such an orientation to the light path. But, as in the case of the photoreceptors (8), Crawford et al. have recently reported (23) that at a given pH the front surfaces of the melanin-containing cells exhibit the same early potential polarity, whatever the direction of light. Hagins and McGaughy (34) have recently shown how "thermal" effects may produce potentials by influencing nearby membranes, and these effects may be involved here. Arden et al. (2, 3) have also discerned, with extracellular electrodes, the presence of comparable potentials from the pigment epithelium of the eye of pigmented but not albino guinea pigs, and also from frog skin, rabbit and guinea pig iris (6), and green leaves (3). The same investigators (2) have shown that, while glutaraldehyde does greatly modify the early receptor potential of the eye of the albino rat, a small, very fast, vitreous-positive response remained. The considerations for detecting this residual response are not different from those bearing on detecting the full ERP. For saccules giving rise to early potentials, Lettvin (38) states that "we must suppose that the interior of each disc is still connected to the extracellular medium. Under such conditions, and given the high resistivity of cell membrane, external electrodes see in parallel all dipoles set up by bleaching. The dipoles are ordered in parallel electrically by the membrane as a boundary." However, Lettvin also notes that for brief, transient responses membrane capacitance is probably more significant than resistance. The early positive signal surviving glutaraldehyde treatment could be carried by capacitive current, and Cone (personal communication) states that all early signals may be carried by such currents. Brindley and Gardner-Medwin (8) found that in the frog's eye formaldehyde had more effect in reducing the earlier positive ERP manifestations and little effect on a later negative phase. It would be useful to know the effects of glutaraldehyde and formaldehyde on membrane capacitance and resistance in order to interpret these findings. If all outer segment membrane became very leaky, then, in effect, all arguments requiring membrane resistance and based on the dipoles existing at the border of a continuous intracellular-extracellular junction become more difficult to support. If fixations sever connections of saccules to the cell membrane and possibly bar lanthanum infiltration by this mechanism, then the detection of surviving early potentials cannot readily be attributed to mechanisms requiring such connections. While it is hard to establish the presence or absence of connections in unfixed material, the absence of such connections in fixed material rests on firmer grounds.

If the report of Goldstein (32) that frog rods are not major contributors to the frog ERP is confirmed, then this confirmation would greatly strengthen the view that rod saccules are indeed isolated, and it would have serious implications for receptor physiology. It also becomes important to look further into the possible occurrence and extent of rod saccule connections in those retinas where the ERP appears definitely related to rhodopsin, presumably in rods.

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