ELECTRON MICROSCOPE OBSERVATIONS
ON FORM CHANGES IN PHOTORECEPTOR OUTER
SEGMENTS AND THEIR SACCULES IN
RESPONSE TO OSMOTIC STRESS

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ABSTRACT

Isolated retinas of rats or frogs were incubated in various salt or sucrose solutions over a wide range of osmolarities and then fixed in 1% osmium tetroxide solutions of matching osmolarities. Light and electron microscope observations were concentrated on the outer segments of the intact photoreceptors. These became globular and of increasing size with increasing hypoosmolarity and irregularly linear and condensed in hyperosmotic solutions. Isoosmotic incubations of rat retinas in solutions containing potassium as the only cation also produced swelling of the outer segments when chloride or acetate was present; but swelling was less when the cation was sodium and it was not seen with either cation when the anion was methylsulfate. The effects of various metabolic and membrane poisons are also reported. The behavior of the saccules within the outer segments was equivocal. While there was a tendency toward more saccules with wider lumina with hypoosmolarity, most of the saccules were not swollen. Surprisingly, intrasaccular space was consistently enlarged in rat retinas exposed to hyperosmotic sucrose but not to salt. The saccules or their derivatives within swollen outer segments tend to maintain their intersaccule spacing and approximation to the cell membrane. It was also noted that the ciliary connectives resist swelling and that retinal Müller cells swell readily.

INTRODUCTION

The following report deals with alterations in the form of rat and frog photoreceptors in response to osmotically variant solutions of different kinds, the changes being observed by light and electron microscopy. The study was planned with several objectives in mind. The first was to learn if form changes induced by osmolarity variations would, at least in part, survive processing for electron microscopy. The first objective being realized in preliminary studies, a second objective was to see whether one could employ osmotic stress to learn something of the permeability and structural organization of these cells. Particular interest was directed to the compartments of the outer segments, the active site for initiating the visual signal (Penn and Hagins, 1969). It has been claimed that outer segments exhibit little osmotic response (Bonting, 1969) and have very porous membrane so that a relatively high concentration of sodium surrounds the saccules (Bonting and Bangham, 1968). It is also not clear whether there is an isolated volume within the saccules in the outer segments of rods or whether this saccular volume, in life, is confluent with the extracellular volume.
However, there is currently no evidence for tubular connectives of the saccules and plasma membrane which open at the cell's surface (Cohen, 1968, 1970). It has become important to learn as much as possible about the permeance of the outer membrane and of the enclosed saccules or discs.

Most of the observations on saccules in this study were directed to the question of their osmotic sensitivity—in particular whether they would expand as the cytoplasm about them was presumably being diluted by entering water. Particular attention was paid to the saccules of cones of frogs, because, as the lumina of these saccules clearly connect to extracellular space (Cohen, 1968, 1970), they should not be osmotically responsive to the dilution of outer segment cytoplasm. In considering saccule responses, we further hoped that one might dissociate effects of ionic strength from osmotic effects.

Methods

Retinas were obtained from freshly enucleated eyes of dark-adapted albino rats or leopard frogs (Rana pipiens). They were isolated under red illumination in ice-cold physiological salines appropriate for the animal as described below. Every effort was made to minimize retinal trauma during the isolation.

Retinas of postweanling rats were employed in most of the experiments to be described, but frog retinas were employed in other experiments in order to permit a comparison of the behavior of rods and cones.

The isolation medium for rat retinas was either Earle's (1943) physiological saline (Baltimore Biological Division of Becton-Dickinson & Co., Cockeysville, Md.) or the artificial cerebrospinal fluid (CSF) of Ames and Hastings (1956). The latter is essentially similar to the solution employed for rat retinas by Weinstein et al. (1967) to demonstrate electrical activity in vitro except that, as the incubations were brief, all plasma was omitted. For frogs, the usual medium was a dilution of Earle's saline (DES) prepared by adding 24 ml of water to 76 ml of the mammary medium. This had an osmolarity of 225 milliosmols.

Preliminary studies included phase and Nomarski interference microscope examinations of slices of rat retinas. The slices were made on retinas after the standard incubations (see below) in normal (290 milliosmols) or dilute CSF (120 milliosmols), or slices of fresh, unincubated retinas were employed. The slices in a drop of appropriate medium were placed on slides under cover slips sealed with Vaseline and were rapidly examined.

As the literature contains brief, uncontrolled observations on form changes in photoreceptors, presumptively related to the osmolarity of fixatives, other preliminary studies were carried out by direct fixing freshly isolated retinas for 1 hr at 25°C under normal room illumination in fixatives which contained 1% osmium tetroxide but which varied in osmolarity. The osmium tetroxide was added to dilutions of artificial CSF (rats) or of DES (frogs), or in a few cases to dilute sucrose solutions, to provide final osmolarities ranging from 324 to 111 milliosmols in the case of rats and from 262 to 60 milliosmols in the case of frogs, with intervals of 30–40 milliosmols. Normal toxicity (erythrocytes) was presumed to be 285 milliosmols for rats and 225 milliosmols for frogs. Two of these experiments employed 1% glutaraldehyde instead of osmium tetroxide, with or without postfixation in osmium tetroxide.

However, in the bulk of the experiments to be reported, retinas of dark-adapted animals were removed under deep red illumination and were first incubated in various test solutions and then fixed in the dark for 1 hr in matching solutions in which 1% osmium tetroxide was included. This addition increased the toxicity by about 33 milliosmols.

The major categories of solutions tested were:

(a) Dilutions of balanced salt solutions to hypotonic levels ranging from 5 to 290 milliosmols at 30–40 milliosmolar intervals. (b) Dilutions of sucrose solutions to hypotonic levels ranging from 22 to 318 milliosmols at 40–50 milliosmolar intervals. (c) Hyperosmotic solutions of sucrose or of physiological salines to which sucrose or sodium chloride had been added. Thus rat retinas were tested in sucrose solutions of 689, 675, and 318 milliosmols, in artificial CSF with sucrose added to 567, 440, and 326 milliosmols, and in artificial CSF with sodium chloride added to give 547 and 675 milliosmols. (d) Isoosmotic solutions containing one salt plus, for buffering purposes, the requisite small amount of a bicarbonate which had the same cation. (e) Various isotonic solutions to which were added such metabolic inhibitors or membrane poisons as CN−, dinitrophenol, F−, ouabain, gramicidin, and valinomycin.

In the above incubations two retinas were placed in flasks containing 5 ml of medium. The flasks had been previously equilibrated with a 5% CO2, 95% O2 atmosphere and were briefly regassed after adding the retinas and before sealing. Rat retinas were incubated in the dark for 10 min at 38°C, and frog retinas were similarly incubated for 20 min at 20°C. The flasks were shaken at low amplitude and speed. Dilutions of physiological saline or sucrose

1 As employed in this report, isoosmotic, hypotonic, and hyperosmotic solutions relate in freezing point depression to the tonic solutions for erythrocytes of rats or frogs without reference to their effects on photoreceptors.
CELLS, SUCH SEGMENTS SOMETIMES COULD BE DISCERNED."  

The amount of water or saline carried into the flasks by the retinas or adherent vitreous humor did not significantly affect the osmolarity before or during the incubation, but a trivial drop in osmolarity due to osmium binding was observed following the hour of fixation.

With reference to studies with a single cation species, these contained the chloride, acetate, or methylsulfate of sodium or potassium plus 23 mM of either sodium or potassium bicarbonate to hold the pH in the usual range under 5% CO₂. Solutions of about 290 milliosmols by freezing point depression were achieved with bicarbonate and the following concentrations (g/l) of salts: NaCl, 8.0; CH₃COONa, 12.5; NaCH₃SO₄·H₂O, 17.5; KCl, 10.0; CH₃COOK, 12.5; and K₂(CH₃SO₄)₂·H₂O, 20.0.

When metabolic or membrane poisons were employed, they were added to yield the following concentrations: 0.01 M CN⁻, 0.02 M F⁻, 40 μM dinitrophenol, 10 μM ouabain, 3 μM gramicidin (Sigma Chemical Co., St. Louis, Mo.); 7 μM valinomycin (Calbiochem, Los Angeles, Calif.). The influence of added 0.01 M glucose on hypotonic effects or in combination with certain poisons was also studied.

All fixed material was dehydrated in ethanol and embedded in epoxy resin (Durcupan, Fluka AG, Basel, Switzerland). Sections from the region of the largest diameter of each experimental retina and from two other regions of the same retina were stained and evaluated by light microscopy, and the blocks were then trimmed to obtain sections of representative areas for electron microscopy. The thin sections were studied and photographed with a Siemens Elmiskop IA.

RESULTS

Outer Segments

When unfixed rat retinas were exposed to normal or dilute CSF (120 milliosmols), it was possible to observe the effects on photoreceptors in slices of these retinas by employing phase or Nomarski interference microscopy. Many outer segments break off of the photoreceptors during the manipulations for microscopy but, whether they break off or remain attached, they proved to be rod-like after isotonic incubations in CSF but were spheres of various dimensions after the hypoosmotic incubation (Figs. 1, 2). While the thickness of the slices makes it difficult to discern individual outer segments still attached to their parent cells, such segments sometimes could be discerned at folds (Figs. 3, 4) and in all cases they conformed in general appearance to the separated segments.

The next experiments dealt with the ability of osmotically induced distortions to survive fixation. Comparisons were made between rat retinas isolated and immediately immersed in osmium tetroxide or glutaraldehyde fixatives, based upon a broad range of dilutions of balanced salt solutions, and other retinas isolated and incubated in a similar range of fixative-free solutions for 10 min and only subsequently transferred to identical solutions to which a fixation agent had been added (see Methods for details of isolations, incubations, and solutions).

The results of these experiments were that, following those hypoosmotic incubations adequate for inducing form changes in outer segments, at least some portion of these presumed osmotic swellings (Figs. 5-10) survived the fixation process and the degree of presumed osmotic swelling seen in the electron micrographs roughly paralleled the degree of hypoosmolarity of the matched incubating and fixing solutions. However, after direct fixation in osmium tetroxide, presumed swellings were only exhibited after exposure to rather dilute solutions (below 110 milliosmols without fixative) containing 1% osmium tetroxide. As a 1% concentration of osmium tetroxide adds only 33 milliosmols, the final fixative osmolarities were still in the general osmotic range of the incubation solutions on which they were based. The above results suggest that the permeance of the outer segments is rapidly increased by osmium tetroxide fixation, and that at least some portion of any pre-fixation deformation does persist.

On the other hand, adding 1% glutaraldehyde to incubation solutions ranging down to 30 milliosmols raised their osmolarity by about 260 milliosmols (i.e. to isosmolarity or above), but, despite this, retinas directly fixed in hypoosmotic incubation solutions with an addition of 1% glutaraldehyde showed only moderately less swelling than retinas which had first been incubated and then fixed. The degree of presumed swellings in the latter case again roughly paralleled the degree of hypoosmolarity of the incubating media. The volume of fixative employed was such that a drop of only 10 milliosmols was seen after an hour of fixation. This result suggests that glutaraldehyde rapidly enters cells, possibly by virtue of its lipid solubility, and largely equilibrates with respect to its external concentration, but that such speed of equilibration is not paralleled by equally rapid...
Figures 1-4

Fig. 1. Outer segments broken from rat retina in isotonic CSF medium. Erythrocytes added. Fig. 2. Spherical outer segments broken from rat retina after 10 min incubation at 38°C in dilute CSF (106 milliosmols). Fig. 3. Intact outer segments at edge of rat retina in isotonic medium. Fig. 4. Spherical outer segments (arrow) at edge of rat retina briefly incubated in dilute CSF (133 milliosmols).

Figs. 1, 3, 4. Nomarski interference microscopy of slices, × 460. Fig. 2. Phase microscopy of slice, × 400. Bars equal 20 μm.
FIGURES 5-7  Outer segments of rat retinas incubated in CSF dilutions at 290, 153, and 63 milliosmols, respectively, and fixed in matching solutions. × 600. Bar equals 15 μ.

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changes in cell permeability. Thus, in terms of modifying cell permeance, osmium tetroxide was adjudged to be the more rapid agent.

In the general experiments employing hypotonic incubations, rat retinas were exposed to diluted CSF at osmolarities ranging from 32 to 290 milliosmols at intervals of about 40 milliosmols, and frog retinas were exposed to diluted Earle's saline at osmolarities ranging from 5 to 239 milliosmols with intervals of about 20-30 milliosmols. In addition, frog retinas were exposed to sucrose solutions ranging from 22 to 217 milliosmols and rat retinas to sucrose solutions of 31 to 318 milliosmols. The nature of the experiments made it difficult to obtain an accurate idea of the time course of swelling. Retinas exposed to hypotonic incubating solutions for 2 min before fixation were not distinguishable from those incubated for up to 10 min before fixation; in view of the surface position of the photoreceptors, it seems reasonable to expect that the times and temperatures of incubation employed allow a maximal response.

The most striking observation was that both rod and cone outer segments exposed to hypotonic solutions became essentially spherical in form (Figs. 11, 12). They also showed other signs of fluid imbibement. In rods, to judge from moderately hypotonic incubations, swelling of outer segments apparently begins as a focal bulging occurring somewhere in the length of the outer segments (Fig. 13), often at the tip. Occasionally, two foci of expansion were noted. The fact that the presumed swelling did not initially occur in a uniform manner everywhere in the length of the outer segment, suggests that structural forces within this limb, perhaps the stacked discs and interdisc material, resist its deformation by water uptake until some weak point gives way. In support of this view, at certain moderate hypotonicities the relatively unstructured inner segments were swollen without signs of swelling of the outer segments. If the dilutions of the balanced media were carried out in a manner preserving the calcium ion concentration as well as the bicarbonate concentration, there was evidence that it took a higher dilution to achieve the same morphological effect as when calcium was absent from the diluting fluid. The minor osmotic contribution of the calcium salt could not account for this effect, but precise quantification of this result was not attempted, nor were efforts made to learn whether the calcium ion was exerting its effect prior to fixation.

There was variation in effects within each retina, some areas showing receptors not conforming to the general appearance in being either more or less swollen. However, it was rarely difficult to classify the average appearance of the outer segments. As to possible sources of the variation, receptors with artifically perforated outer membranes might be expected to show little swelling of osmotic origin, and, where the retina was folded during incubation, the receptors covered by a fold could have had less opportunity for free fluid exchange. In this regard it was noted that, when a frog retina which was only partly denuded of pigment epithelium was incubated in a hypotonic solution, the receptors protected by the epithelium were less swollen. This source of variation in frog retinas was compounded by the fact that some additional pigment epithelium comes off during incubations. In addition, obviously persistent vitreous humor on the inner retinal face was apparently associated with somewhat reduced swelling in the ganglion cell region. The possible sources of anomalous local regions with greater swelling are less obvious. Because it is clearly possible to obtain transient swelling, one could not assume that unswollen receptors were never swollen during the incubation. However, experiments in which retinas were given successive 10-min exposures to hypotonic followed by isotonic media showed that reversals of those swellings, which originally were severe enough to have deformed the outer segments, left permanent changes in saccule orientation and saccule deformations although outer segment swelling was reversed.

Outer segments of rats or frogs were consistently swollen when incubated in hypotonic solutions whether these were based upon dilutions of sucrose or on dilutions of balanced, physiological salines. On the other hand, no swelling was ever observed in isoosmotic controls of balanced salt solutions or sucrose. In histological sections, outer segments from isotonically incubated rat retinas exhibited volumes of about 22 µm³ and those of frogs 2250 µm³. The volumes calculated from the diameters in histological sections of the swollen, spherical segments of retinas exposed to markedly hypotonic media indicated volumes of 60-70 µm³ for rats and 7400 µm³ for frogs. However, such measurements were not routinely made and globular deformation was presumed to indicate osmotic swelling. This presumption is made throughout this report. The additions of cyanide, ouabain, dinitrophenol, or
Figure 11 A swollen rod outer segment of a rat retina after incubation fixation at 110 milliosmols in dilute CSF. Note saccules (S) and ciliary connective (C). × 81,000. Bar equals 0.5 µ.
dinitrophenol plus gramicidin to balanced salt solutions likewise produced no obvious swelling of rat outer segments.

Moderate swelling of outer segments of rats was observed in isoosmotic potassium acetate–potassium bicarbonate solutions (Fig. 14). The addition of potassium cyanide, ouabain, or dinitrophenol had little additional effect on the outer segment swelling, but there was a marked swelling of receptor terminals in all cases. Adding valinomycin to...
dinitrophenol had no discernible effect on outer segments but may have produced a slight enhancement of terminal swelling.

Retinas exposed to isoosmotic sodium acetate–sodium bicarbonate solutions also showed swelling of outer segments, but less than that seen with potassium salts. This was not notably affected by ouabain or dinitrophenol, but was clearly enhanced by combined dinitrophenol and gramicidin. Ouabain and dinitrophenol again enhanced the swelling of receptor terminals. The addition of either sodium cyanide or sodium fluoride to the sodium acetate–bicarbonate solution somewhat reduced outer segment swelling, but the amounts required suggested that the action was by an increase in osmotic pressure rather than by metabolic alteration.

In the case of isoosmotic solutions including chlorides and bicarbonates, moderate to severe swelling of the outer segments and the remainder of the photoreceptors was seen with the potassium salts (Fig. 15), but only possible traces of swelling with the sodium salts (Fig. 16). With solutions including methyl sulfate and bicarbonate, all cells in the retinas exposed to either the sodium or potassium salts appeared normal by light microscopy. However, electron microscope examination of the retinas exposed to the potassium salts showed glial swelling in most regions and traces of neuronal and photoreceptor swelling in a few small zones.
With hyperosmotic solutions of either sucrose (in bicarbonate buffer), CSF plus sucrose, or CSF plus sodium chloride, the obvious finding in rat retinas was an increase in the density of the outer segments and large variations in diameter along their lengths. Thus it was not possible to judge on a gross basis whether they had lost volume. In comparing retinas incubated in 675 milliosmolar sucrose (in bicarbonate buffer) with retinas incubated in CSF with NaCl added to a matching hyperosmolarity a marked shrinkage of photoreceptor nuclei was seen with the saline but not with the sucrose.

Results with single cations and additives are summarized for outer segment responses in Table I.

In a considerable minority (ca. 20\%) of swollen outer segments of rats, but only rarely (< 1\%) in frogs, organelles characteristic of the inner segment were found in the expanded outer segment. Where the connecting cilium was observed, it did not appear to be expanded; thus some other route of entry for these organelles must have existed unless the ciliary channel is capable of temporary expansions. Rodent outer segments which were not clearly separated from inner segments have been reported as an abnormality (Tokuyasu and Yamada, 1960), and such segments were observed in this study, but Richardson (1969) regards them as possibly normal in mammals. The observations in this report clearly suggest a vesicle separate from the remainder of the cell formed by the swelling of outer segments, but it is possible that secondary, nonciliary linkages of inner and outer segments are severed by the forces involved in the deformations.

Saccules

With osmium tetroxide fixation, the typical appearance of a saccule is that of an apparently empty space enclosed by two dark lines. In the following description the “empty” space will be designated as the saccule lumen, but it is recognized that nonosmiophilic membrane materials may be present in this volume and that there is an arbitrary aspect in deciding where the surface of a cell membrane begins. Outer segments were sectioned at right angles to their length, to avoid artifactual saccule compression. Electron micro-

**Figures 14-16**: Outer segments of rat retinas which had been incubated in isoosmotic solutions: Fig. 14, potassium acetate-potassium bicarbonate; Fig. 15, potassium chloride-potassium bicarbonate; Fig. 16, sodium chloride-sodium bicarbonate and then fixed in matching solutions which contained 1\% osmium tetroxide. X 600. Bar equals 15 \(\mu\).

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scope views of rodent saccules after conventional fixations are available in papers of Sjöstrand (1953, 1959), Cohen (1960), and Dowling and Gibbons (1961).

When saccules were examined from retinas exposed to moderately hypotonic to moderately hypertonic solutions, a clear and relatively uniform intrasaccular space was always seen in saccules within rods of rats. On the other hand, this space was observed in most saccules of red rods of frogs in hypotonic solutions only. In confirmation of Nilsson (1965), only the basal zone of saccules of frog rods exhibited a clear intrasaccule lamina at the osmolarity of amphibian Ringer's. In rods of both rats and frogs the intrasaccule lamina did not show a clear expansion quantitatively related to the degree of hypoosmolarity of the incubation media. In maximally deformed, spherical outer segments of rats, no consistently expanded laminae were evident within the saccules, although isolated examples of expanded saccule derivatives were not hard to find. In frogs, while the lumen within the saccule was often larger in spherical outer segments, and while regions could be found in which saccules were expanded to the point where the distance between adjacent saccule walls was markedly narrower than the intrasaccule laminae, other regions showed saccules with minimal expansions (Figs. 17-19). The results suggested that mechanical deformations, acting on saccules in an environment of diminished ionic strength, could as easily account for the results as direct osmotic action. In confirmation of De Robertis and Lasansky (1961), saccule edge regions deform or expand less readily.

In the case of cones, the saccule light interval did not expand and perhaps narrowed with increasing dilutions of the incubating media. The saccule mass typically decreased, presumably by degeneration of the discs into tubular membranes. It was considered, inasmuch as the membrane of cone saccules is a continuum of the cell membrane, that the saccule membrane might "flow" back into the expanding cell membrane or that the "pleats" be pulled apart. If the saccule membrane "flowed" into the cell membrane during swelling, then one might expect the wide saccule openings on the cell membrane to move apart. This was not observed, nor was there a pulling apart of "pleats."

To judge from progressively more dilute exposures, a postulated sequence of events accompanying outer segment swelling is as follows: The saccules become oriented along the inner surface of the forming spheres and then stretch into shelf-like arrangements. These tend to retain their intersaccule spacing, radial orientation, and adhesion to the cell membrane. In extreme dilutions, they further deteriorate into small discs or ribbons. Occasional swollen discs are seen, as well as tubular formations. Cone saccules in frogs showed considerable tubular degeneration.

At extreme dilutions, fragments of swollen outer segments were found. These might be trapped fragments of hypotonically ruptured outer segments or fragile outer segments ruptured during processing. In any event, almost all disc residues in these fragments were double walled and often adherent to fragments of outer cell membrane (Fig. 20). No differences in the behavior of saccules were seen in

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### Table I

**Behavior of Outer Segments in Artificial CSF or Single Cation Solutions (Isoosmotic)**

|                | — | CN | DNP | DNP + Gr | DNP + V | O   | F | F + G | G   |
|----------------|---|----|-----|----------|---------|-----|---|-------|-----|
| **CSF Na**     |   | N  | N   | N        | N       |     |   |       |     |
| **Cl⁻, HCO₃⁻** |   | T  | N   | N-T      | M-S     | M-T | N | N-T   | N-T |
| **CH₃COO⁻, HCO₃⁻** |   | T-M| N   | N-T      | M-S     | M-T | N | N-T   | N-T |
| **CH₃SO₄⁻, HCO₃⁻** |   | N  |     |          |         |     |   |       |     |
| **K**          |   |   |     |          |         |     |   |       |     |
| **Cl⁻, HCO₃⁻** |   | S-M|     |          |         |     |   |       |     |
| **CH₃COO⁻, HCO₃⁻** |   | M  | T   | T-M      | M-S     | M   | S-M|       |     |
| **CH₃SO₄⁻, HCO₃⁻** |   | N  |     |          |         |     |   |       |     |

Normal (N), or trace (T), moderate (M), or severe (S) swelling. CN, 0.01 M cyanide; DNP, 40 µM dinitrophenol; Gr, 3 µM gramicidin; V, 7 µM valinomycin; F, 0.02 M fluoride; O, 10 mM ouabain; G, 0.01 M glucose.
FIGURE 17 A portion of a swollen outer segment of a rod of a frog retina, incubated-fixed in sucrose-bicarbonate at 54 milliosmols. × 15,600. Bar equals 0.1 µ.
instances of outer segment deformations produced
by dilutions of saline or sucrose, with isoosmotic
swelling, or in the presence of the various metabolic
or membrane poisons noted earlier.

Turning to hyperosmolarity, when the osmolar-
ity approached 700 the rat outer segments became
dense, and neither the extrasaccular space nor
intrasaccular "lumen" was clearly evident in the
majority of regions of most outer segments. How-
ever, this was in large part due to the fact that the
usual difficulty in finding saccules, whose planes
were perpendicular to the plane of section, was
being compounded by a distortion of the outer seg-
ments. With patient exploration, regions with
resolvable saccules could be found (Figs. 21, 22),
and a consistent difference was revealed between
retinas incubated and fixed in hypertonosmotic
sucrose (in bicarbonate buffer) and those incu-
bated and fixed in CSF plus NaCl at the same
hyperosmolarity (675 milliosmols). The saccules in
sucrose-treated retinas of rats were often expanded
to a point where their adjacent walls greatly mini-
mized the extra-saccular space (Fig. 21). On the
other hand, in the hyperosmotic saline (Fig. 22)
the saccule lumen was obviously reduced but not
eliminated. While in some regions saccules were
compacted against one another, in other regions
they were not, and extrasaccular space persisted.
Perhaps invisible adhesions of saccule edges and
the cell membrane retard the ability of saccules to
shift vertically within the outer segment. It was
obvious from the loss of both intra- and extra-

**Figures 18 and 19** Two regions of swollen outer segments of frog retinas incubated and fixed in dilute
salines at 5 and 17 milliosmols, respectively. X 40,000. Bar equals 0.5 µ.
saccular volume that the outer segment volume must be considerably diminished. A portion of an isotonically incubated and fixed rod is presented for comparison (Fig. 23). This region was selected to show that saccules with expanded lumina can even be found with isotonic incubation although the saccules with the minimal lumina shown are by far the most typical. Note also that hyperosmolarity tends to suppress the tubular degeneration of saccules commonly seen with osmium tetroxide fixation. In any event, there is clearly some mechanism, natural or artifactual, which can result in expanded saccules even in hyperosmotic media. The low ionic strength of the sucrose media or a low concentration of some specific ion such as calcium may be the significant factor.

Table II gives some dimensional characteristics of the significant outer segment volumes and areas in isotonically fixed rods of rats and frogs. One should note that, in comparison to dimensions measured on unfixed rods by phase and interference microscopy, there is a reduction in diameter of some 25% in outer segments in histological sections, but it is not clear that all dimensions and volumes are equally affected by fixation, dehydration, and other processing. The shrinkage is consistent with that reported in *Nature* by Brown et al. (1963). Finally, it is again noted that the light intrasaccular dimension is being treated as a volume but could include, in whole or part, materials usefully regarded as part of cell membrane, including bound water and other osmotically inactive netties.

**Retina**

While the preceding studies were not primarily directed at other than the photoreceptors, the various osmotic or toxic situations did produce changes in other retinal cells. The striking observation was that the glial cells of Müller were consistently found to be more sensitive to swelling than retinal neurons and that apparent swelling of the apex of the Müller cell at the external limiting membrane was sometimes seen when the basal region of these cells, facing the vitreous humor, seemed essentially normal.

**DISCUSSION**

The preceding results indicate that photoreceptors exhibit hypotonic swelling which includes swelling of their outer segments. They similarly swell in isosmotic salt solutions when certain permeant anions are present and the cation is potassium, and they swell to a noticeably lesser degree when the cation is sodium. The nonuniformity of initial swelling of outer segments and the indications of prior swelling in inner segments suggest that, at least in the low hypoosmotic range, outer segments would not be perfect osmometers.

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**Figure 20** Debris of swollen rod outer segments of a rat retina with incubation fixation at 80 milliosmols in dilute saline. Note fragments of the cell membrane (arrows). X 28,800. Bar equals 0.5 µ. 

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The results also indicate that statements in the literature to the effect that outer segments are osmotically insensitive (Bonting, 1969) are probably in error. Those statements reflect observations on retinas directly placed in hypoosmotic fixatives, and it seems likely that the fixatives were either insufficiently hypoosmotic or that the fixatives modified the membrane permeance before an osmotic effect could occur. It is, however, possible, although unlikely, that the membrane of the outer segment differs from the membrane in other regions of the same cells, and that the deformations seen in outer segments in the current study were wholly or partially indirect results of water entering other regions of this cell. In this case, isolated outer segments might behave in a different manner. The results reported here also cast doubt on the view that the membrane of the outer segment is normally highly permeable to the extracellular milieu including particles the size of ferritin (Bontaing and Bangham, 1968), although this could apply to certain preparations of isolated outer segments. A study with particulate tracers (Cohen, 1970) also suggests conventional permeability.

The observations on isosmotic swelling suggest that the plasma membrane of receptors is similar to that of most nerve cells in being more permeable to potassium than sodium. The results also suggest relative impermeance to methylsulfate and bicarbonate, and permeance to chloride and acetate. Isosmotic swelling of cerebral cortex, related to potassium and chloride concentrations, has been studied by Bourke (1968).

The observation of isosmotic swelling in the current report contrasts with results obtained on "fragments" of bovine outer segments by Brierly et al. (1968). These workers concluded that their fragments were relatively impermeable to sodium and potassium and that, even in the presence of the permeant acetate ion, potassium showed little support of osmotic swelling unless both dinitrophenol and valinomycin or gramicidin were present. The fragments were said to have a limited permeability for chloride. The reasons for the discrepancies are probably based on the absence of an intact plasma membrane of the outer segments in their preparations as implied by their term "fragments," and this suggests that the phenomena they observe were largely based upon the behavior of the sac-

**Figures 21-23** Portions of outer segments of rat retinas. Fig. 21, incubation fixation in sucrose-bicarbonate (675 milliosmols). Fig. 22, incubation fixation at isotonicity (290 milliosmols). X 46,700. Bar equals 0.25 µ.
TABLE II

Some Parameters of Elements* of Outer Segments (OSE) Fixed at Isotonicity

| Thickness | Surface Area | Volumes |
|-----------|--------------|---------|
|           | OSE          | CM      | OSE | ES | S | IS† |
| µ         | µ            | µ²      | µ¹  | µ¹ | µ² | µ³ |
| Rat rod   | 0.036        | 0.016   | 0.92×2 | 0.15 | 0.04 | 0.025 | 0.015 | 0.006 |
| Frog rod (red) | 0.025  | 0.010   | 40×2 | 0.56 | 1.0  | 0.6  | 0.4   | 0.16 |

* An element (OSE) is defined as a volume of cross-section containing one saccule and half the distance to each adjoining saccule. S, saccule, CM, cell membrane, ES, extrasaccular, IS, intrasaccular.
† See text for limitations on meaning.

However, the latter exhibited no discernible morphological response in our hands when receptors were exposed to isoosmotic potassium acetate in the presence of dinitrophenol and valinomycin. It may be that, with intact outer segments, gramicidin and valinomycin do not reach the saccules.

There is evidence for transparent material between the saccules (Cohen, 1968), or between the edges of adjoining saccules (Falk and Fatt, 1969). The binding of lanthanum colloid in cones (Cohen, 1968) also suggests intrasaccular gel. Thus, as some gels can exhibit quasiosmotic swelling (Ogston, 1966), one might consider that the swelling of fragments was based on gel swelling. However, it seems highly unlikely that gel swelling would be induced by dinitrophenol and valinomycin. Electron micrographs of the swollen preparations of Brierly et al. (1968) showed many vesicular elements, apparently derived from the saccules, and these authors conjecture that these could constitute a compartment capable of swelling. However, they made no attempt at establishing a quantitative relationship; vesicles were not found within swollen outer segments in the current investigation.

The prior reports in the literature on the behavior of saccules in hypoosmotic situations are based upon uncontrolled observations on retinas fixed under hypoosmotic conditions. Thus, De Robertis and Lasansky (1961) illustrated a portion of a rod outer segment from a hypoosmotically fixed retina of a toad. This segment exhibited expanded saccules, and the authors suggested that the saccules are "very osmotically sensitive." Lasansky (personal communication) informs me that this example came from a preparation fixed in conventional veronal-acetate-buffered osmium tetroxide from which sucrose was omitted, and that it was an extreme example selected to take advantage of the saccule expansions to show the saccular nature of the discs in the outer segment.

Robertson (1966) reported that an unspecified hypoosmotic environment caused discs of the outer segment of a frog to undergo vesicular degeneration, and he did not mention swelling. This was also an uncontrolled observation. While the discs in fixed preparations of outer segments are sometimes seen to have undergone vesicular degeneration, this result is often a function of the method of fixation (Eakin, 1965). The current report does not implicate osmotic stress as a direct cause of the phenomenon.

Zigman and Bagley (1970) have noted form changes in isolated outer segments of rods of dogfish with prolonged incubations (2-24 hr) in either light or dark. Some of the changes noted with prolonged incubations resemble globular deformations, and these were said to occur more rapidly with illumination; these authors, who often refer to the isolated outer segments as rod cells, state that light causes a shrinkage of outer segments but a swelling of inner segments.

Is there direct evidence of a lumen in the saccule? If there is a lumen, what are its dimensions? When Moody and Robertson (1960) studied discs of frog rods and cones after fixation in potassium permanganate, they concluded that the center of a double-membrane disc consists of the adhering outer surfaces of the two membranes (outer because the outer face of the cell membrane corresponds to the opposing surfaces within saccules if this membrane invaginates to form a disc or saccule). With permanganate fixation, which readily yields an appearance of trilaminate structure in an individual cell membrane, two trilaminate membranes appeared as if "fused" at their opposing surfaces to form a pentalaminate disc in either rods.
or cones. The same result was obtained with rods and cones of pigeons (Cohen, 1963). However, Cohen (1968) showed that this “fused” zone could be infiltrated with lanthanum salts in glutaraldehyde-fixed saccules of frog cones and sometimes even in rod saccules in zones of presumed damage. This shows that either the membranes are simply closely approximated after fixation, or that the “fusions” must be somehow interrupted so that a potential channel exists between any fusion points of the opposing membranes. Brightman and Reese (1969) have recently shown that some pentalaminar junctions in permanganate-fixed material are septalaminar junctions after other fixations and that they contain a 20–30 Å gap which can be infiltrated with lanthanum salts. When lanthanum-infiltrated “gap” junctions are sectioned tangentially, they exhibit a mosaic pattern which might suggest focal adhesions of the membranes, focally excluding the lanthanum colloid. Robertson (1964) had, in fact, presented a view of a tangentially sectioned disc of a frog rod, fixed in potassium permanganate, in which a mosaic patterning is suggested. However, even this may not mean that the postulated punctate adhesions exist in life, for Cohen (1970) succeeded in infiltrating some saccules of unfixed frog cones (in isotonic phosphate buffer) with particles of ferritin of 100 Å diameter. Unless the saccule clefts had been artificially enlarged prior to fixation by this non-physiological medium, this result does suggest that any “fusion” of the opposing membranes must be rather tenuous. Recent X-ray diffraction studies of unfixed frog rods by Blaurock and Wilkins (1969) also yield data suggesting that a lumen or at least a zone of greater hydration is present in the unfixed saccule.

Because the physical continuity of the area of saccule membrane and that of the cell membrane would modify the cell’s electrical capacitance, such measurements would provide a further test of whether saccules and cell membrane are discontinuous in rods. The data in Table II may be combined with an estimate that without the saccules the outer segment surface is between 40–60% of the total cell surface, and that adding the saccule area results in a maximum increase of 70× in the case of red rods of the frog but only 7× in the case of rods of the rat.

Table II also shows that, if the space in rod saccules is regarded as a lumen, then its volume is of the order of 25% of the extrasaccular volume in either species. The fact that the saccule surface is much more extensive than the cell surface in each elemental volume of outer segment suggests that water or ions entering through the cell surface could potentially equilibrate very rapidly with the contents of the saccules. The kinetics are likely complex because the rate of equilibration of the intrasaccular and extrasaccular compartments depends on the rate of equilibration of the extrasaccular and extracellular compartments. The problem might possess an additional complication. Cell membranes may not possess a symmetrical permeance for certain ions (Tasaki and Singer, 1966). Thus, an ion entering an outer segment and an ion entering a saccule from within the outer segment may not be in symmetrical situations since the outer surface of a saccule may correspond to the inner surface of the cell membrane.

It is also worth considering that regions such as outer segments with high surface-to-volume ratios could exhibit locally important changes in concentrations of ions associated with membrane double layers in response to changes in potential across these membranes.

While the findings reported here are strongly consistent with osmotic sensitivity of the photoreceptors as reflected in the behavior of the outer segments, the data are basically inconclusive regarding the behavior of the saccules within these segments. As instances of expanded saccules were found after both hyperosmotic and hypoosmotic incubations, and as the degree of expansion of saccules was highly variable within individual outer segments, and as many saccules, particularly in rats, showed no expansion, some uncontrolled variables seem to be operating.

Saccules could fail to swell because they may lack a significant osmotic volume. Or, perhaps incipient swelling perforates the saccules or otherwise renders them almost totally permeable. Slight swelling followed by subsequent shrinkage through processing for electron microscopy, could cause them to appear unwollen. The current study could not have detected transient changes. Since outer segments are swollen, the possibility that the saccules did not swell because they were opened by damage during retinal isolation seems improbable. The deformation of the saccules which accompanied outer segment changes does indicate that forces were acting upon the saccule membrane during these experiments, but no breaks in the membranes of deformed saccules were observed.
In theory, saccules could also fail to swell because there exists, in life, narrow connections between the interiors of the saccules and the exteriors of the cells, thus allowing for rapid equilibration with the extracellular volume. This is becoming increasingly unlikely in the light of recent evidence to the contrary (Cohen, 1965, 1968, 1970, Laties and Liebman, 1970).

Finally, while the current study is only a crude and indirect approach to studying the permeability of photoreceptor compartments, it is interesting to note that the indications of a greater permeability to potassium than to sodium, and of a greater permeability to chloride and acetate than to methylsulfate and bicarbonate, are what would be expected with most neurons. Other interesting observations, such as the conditions for the swelling of receptor terminals and for glial swelling, must be regarded as preliminary and worthy of a more detailed investigation. Since the glial cell of Müller is the principal glial cell of the vertebrate retina, the swelling of the glial cells of Müller prior to any swelling of retinal neurons requires an explanation and evaluation in the light of a postulated role for potassium as a buffer for neuronally released potassium (Kuffler, Nicholls, and Orkand, 1966; Trachtenberg and Pollen, 1970). The occasional swelling of one end of these tall cells before the other end confirms observations made on the ependymoglia of the Bowfin by Friede et al. (1969). It is conceivable that, in certain regions of cells of complex form, distinctions in local surface-volume ratios and the physical resistance of surrounding cells which are not swelling could influence the local retention of water and produce such results.

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