THE OSMOTIC BEHAVIOR OF ROD PHOTORECEPTOR OUTER SEGMENT DISCS

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ABSTRACT

The permeability properties of frog rod photoreceptor outer segment discs were investigated in preparations of purified, dark-adapted, outer segment fragments by the techniques of direct volume measurement and electron microscopy. Outer segment discs were found to swell and contract reversibly in response to changes in the osmotic pressure of the bathing medium in accordance with the Boyle-van't Hoff law. By use of the criterion of reversible osmotic swelling, the disc membrane is impermeable to Na^+, K^+, Mg^{+2}, Ca^{+2}, Cl^-, and $(PO_4)^{-3}$ ions, whereas it is freely permeable to ammonium acetate. The disc membrane is impermeable to sucrose, although its osmotic behavior towards this substance is different from its behavior towards impermeable ions. Electron microscopy showed that the osmotic effects on the rod outer segment fragments represent changes in the *intradiscal* volume. Fixation with glutaraldehyde did not abolish the permeability properties of the disc membrane, and fixed membranes were still capable of osmotic volume changes. It is concluded from this study that the frog's rod photoreceptor outer segment discs are free-floating membranous organelles with an inside space separate and distinct from the photoreceptor intracellular space.

INTRODUCTION

Vertebrate rod and cone photoreceptor cells can be divided into three main parts: a synaptic terminal; a cell body containing the nucleus, mitochondria, ribosomes, and other components of the cellular metabolic machinery; and an outer segment containing the light-sensitive system. (For a comprehensive review of photoreceptor structure, see [7]). The cell body and the outer segment are joined by a narrow connecting cilium. In both rods and cones, the outer segments are filled with a closely packed array of membranes which appear in electron micrographs as a neatly arranged stack of discs. The rods and cones differ in the over-all shape of their outer segments. While the rod shape is a uniform elongated cylinder, the cone has a conically shaped outer segment. Moreover, while the rod discs appear to be free-floating double membranes not connected to the membrane enveloping the outer segment, the cone discs often appear to be a continuation (invagination) of the enveloping membrane. The rod and cone discs thus seem to be fundamentally different: the rod-disc double membrane seems to enclose an intraorganellar space, while the cone discs appear to be open to the extracellular space.

Scattered reports in the literature indicate that the rod discs are indeed enclosed organelles and that their membranes show selective permeability to various ions and nonelectrolytes (2, 6, 8).[1] That visual pigments are structural components of the rod outer segment membranes is generally accepted (1, 9, 11, 21). Thus, it is of interest to in-

[1] Cohen, A. I. 1970. Manuscript in preparation.

vestigate the properties of rod outer segment discs and to find out whether the membrane properties change upon illumination. In addition, since the outer segment discs bear some resemblance to other intracellular organelles such as mitochondria and chloroplasts, it would be instructive, in attempting to gain an insight into their structure and function, to compare the behavior of all of these membrane systems under varying conditions.

The present work attempted to answer the questions of whether the rod outer segment discs enclose a real space, and whether the permeability properties of these membranes may cause the discs to act as osmometers towards certain solutes. This report will show that the rod disc membrane is impermeable to certain solutes and that the discs behave as osmometers. Our results confirm the findings of Brierley et al. (4) published in a preliminary note.

MATERIAL AND METHODS

Osmotic Swelling Experiments

PREPARATION OF ROD OUTER SEGMENT PELLETS: Frog rod outer segment fragments used in the osmotic behavior experiments were isolated according to a procedure modified from that of Heller (10). The frogs used were mostly *Rana pipiens* and, in some experiments, *Rana catesbeiana*. Results obtained with the two frog species were similar. All steps were performed under dim red light at 4°C. Frogs were dark adapted overnight, and after decapitation their retinas were immediately removed and chilled on ice. The retinas were homogenized (approximately 10 retinas/ml) in a medium containing (final concn.): 1.05 M sucrose, 2 mM $MgCl_2$, 1 mM Tris-HCl buffer, pH 7.5, and 1 mM adenosine triphosphate (ATP) (sucrose-buffer). The retinas were homogenized with a Teflon pestle and glass tube, (0.4–0.5) mm clearance), by making three up-and-down strokes at a speed setting of 2.6 (Tri-R Instruments, Inc., Rockville Centre, N.Y.). The pestle and tube were then washed with the same medium, bringing the volume to approximately 5 retinas/ml, and the homogenate was next shaken on a Vortex mixer for 30 sec (Scientific Industries, Inc., Queens Village, N.Y.). The homogenate was centrifuged for 15 min at 27,000 *g*. The ROS[2] floated to the top, and, after they were loosened from the tube wall with the tip of a pipet, the whole supernatant was collected by decantation. The remaining pellet was resuspended in 1.05 M sucrose buffer and refloated as before. This step was repeated twice more, each time collecting the ROS float. The four resulting ROS floats were diluted 1:4 with a medium containing 2 mM $CaCl_2$, 3 mM $MgCl_2$, 1 mM Tris-HCl, pH 7.5, and 1 mM ATP and then combined. The diluted ROS float was centrifuged at 12,000 *g* for 10 min. The ROS pellet was resuspended in the 1.05 M sucrose buffer, and the floatation was repeated twice as above. The ROS float was again diluted with the sucrose-free medium and centrifuged at 12,000 *g* for 10 min. The resulting pellet was used for osmotic swelling experiments. The whole procedure took approximately 3 hr.

To ascertain purity, representative pellets were fixed for 2 hr in a 1% formaldehyde, 1% glutaraldehyde mixture in 0.085 M phosphate buffer (pH 7.2). Following aldehyde fixation, they were postfixed for 1 hr in 1% OsO_4 in the same buffer. The fixed pellets were dehydrated in graded ethanols, and embedded in Araldite 502 (Ciba Products Co., Summit, N.J.). Sections with light-gold interference colors were cut through the entire thickness of each pellet. The sections were stained with uranyl acetate and lead hydroxide, and viewed at 80 kv in a Siemens Elmiskop IA electron microscope.

MEASUREMENT OF PELLET VOLUME CHANGE: Two methods were used to measure osmotic swelling of ROS discs.

Initial experiments were performed in 3-ml glass Bauer-Schenck protein tubes with long narrow tips calibrated in 0.004 ml graduations. The ROS pellet was suspended in 3-ml salt solutions of appropriate molarity. The tubes were centrifuged in specially made Teflon adapters in a Sorvall RC2-B centrifuge equipped with an SS-34 rotor at forces up to 5000 *g* for 20 min.

In later experiments all volume determinations of the pellet were done by weighing. The pellet was suspended in a preweighed 3 ml polycarbonate centrifuge tube. The suspending medium, generally 2.5 ml, contained an appropriate amount of solute in addition to 1 mM ATP and 1 mM Tris-HCl buffer, pH 7.5. The suspended ROS were centrifuged at 27,000 *g* for 10 min at 4°C and the clear supernatant was decanted. The tube wall and the pellet's rim were carefully dried of all excess liquid with small pieces of lint-free tissue paper. The tube and the pellet were then weighed with a Magni-Grad model FH microbalance (Ainsworth, Denver, Colo., rated sensitivity of 1 μg), using class S (g) and M (mg) weights. The weighing was performed at 22°C. The reproducibility of weighing the same pellet subjected to repeated suspension and centrifugation in the same medium was better than 1%. Each experimental point was measured at least twice; each time the pellet was suspended in a given medium and then centrifuged. Weighing experiments were performed on dark-adapted ROS pellets in a lightweight alumi-

[2] Abbreviation used: ROS, rod outer segments.

num container. In some experiments the ROS pellets were illuminated before and during the osmotic swelling experiments. All solute concentrations were molar, measured at 22°C. Solutions of sodium acetate, sodium phosphate, ammonium acetate, and ammonium phosphate were adjusted to pH 7–7.2. ATP·Na_2 (Sigma Chemical Co., St. Louis, Mo.) was dissolved in H_2O, adjusted to pH 7.2 with KOH, and kept frozen until used. Protein was determined by the method of Lowry et al. (14), with bovine serum albumin as the standard. ROS pellet dry weight was determined by weighing pellets before and after heating at 80°C for 24 hr and then drying in an evacuated desiccator over P_2O_5.

MEASUREMENT OF PELLET INTERSTITIAL SPACE The ROS pellet dead space was determined according to Nobel (16). The ROS pellet (prepared as above) was incubated at 4°C with dextran-^{14}C (New England Nuclear Corp., Boston, Mass., mol wt 60,000–90,000) in either 135 mM or 60 mM KCl including 5 mM $CaCl_2$, 5 mM $MgCl_2$, 1 mM Tris-HCl, pH 7.5, and 1 mM ATP. The dextran concentrations (constant specific activity) were adjusted with cold dextran to 1, 2, and 3% (w/v, final). After centrifugation at 27,000 *g* for 10 min, a sample was taken for counting from the supernatant and the pellet. The samples were counted in the Packard Model 3375 Tri-Carb Scintillation Counter with Beckman Biosolv-3 added to the PPO/POPOP toluene scintillation mixture. To determine what fraction of the pellet was penetrated by the supernatant fluid, the results were expressed as counts per minute/milligram of pellet divided by counts per minute per milligram of supernatant fluid.

Ultrastructure Studies of Disc Volume

PREPARATION OF PELLET: Rod outer segments prepared for electron microscopy were isolated by a modification of a procedure developed by Lolley and Hess (13). *Rana pipiens* were dark adapted overnight at 23°C. The frogs were decapitated, and the retinas were removed from their eyes under dim red illumination. The retinas were suspended in 30 ml of medium containing various amounts of NaCl (see Results), 1 mM Tris-HCl buffer, pH 7.5, and 1 mM ATP at 4°C. The rod outer segments were detached by stirring with a magnetic stirrer for 5 min in a 125 ml trypsinization flask kept in an ice-water bath. The homogenate was filtered through a fine nylon screen (Nitex, 95 μ mesh, Tobler, Ernst and Traber, Inc., New York), and the filtrate was collected into a 50 ml polycarbonate centrifuge tube. The detached rod outer segments were fixed by adding glutaraldehyde to a final concentration of 0.15% (15 mM) and allowing the preparation to stand for 1 hr at 4°C. This concentration of glutaraldehyde was previously shown to be adequate for fixation of organelles in suspension (18). At the end of the fixation time with glutaraldehyde, the rod outer segments were centrifuged at 1085 *g* for 2 min and then at 3020 *g* for 3 min. The supernatant was decanted and the resuspended pellet was postfixed with 0.12% (5 mM) OsO_4 dissolved in the same medium as the glutaraldehyde (18).

ELECTRON MICROSCOPY: Following postfixation in OsO_4, the ROS were centrifuged at 27,000 *g* for 10 min. The pellets were dehydrated and embedded in Araldite 502. Sections with silver interference colors were cut through the thickness of each pellet. All other details were as described above.

RESULTS

Purity of ROS Preparation

The purity of ROS preparations used for direct measurement of disc volume was judged by making random sections through a number of pellets and preparing electron micrographs. As can be seen from Fig. 1, *a* and *b*, the purified pellets contained ROS fragments, individual discs, and fragments of discs. No intact ROS were observed in this preparation. The disc fragments were mostly reorganized into vesicles of various sizes. An occasional mitochondrion or cluster of mitochondria was found. The ROS pellet was judged by electron microscopy to be about 90% pure.

Reversible Osmotic Swelling of ROS in Salt Solutions

The initial measurements of ROS pellet volume as a function of the osmolarity of the bathing medium were performed in volumetrically calibrated glass centrifuge tubes. These experiments showed a linear relationship between pellet volume and the inverse osmolarity in solutions of NaCl and KCl. The direct determination of pellet volume in calibrated glass tubes was abandoned in favor of pellet weighing, because the accuracy of the latter method is much greater. In addition, the glass tubes could not stand forces greater than about 5000 *g*, and even then frequent breakage occurred.

The results obtained by weighing ROS pellets suspended in various concentrations of NaCl are shown in Fig. 2. A linear relationship exists between pellet weight (volume) and the inverse osmolarity of the suspending medium, as predicted from the Boyle-van't Hoff law. Thus, the ROS discs behave as osmometers and adhere to the relation $V = k/c + b$, where V is the pellet volume, c

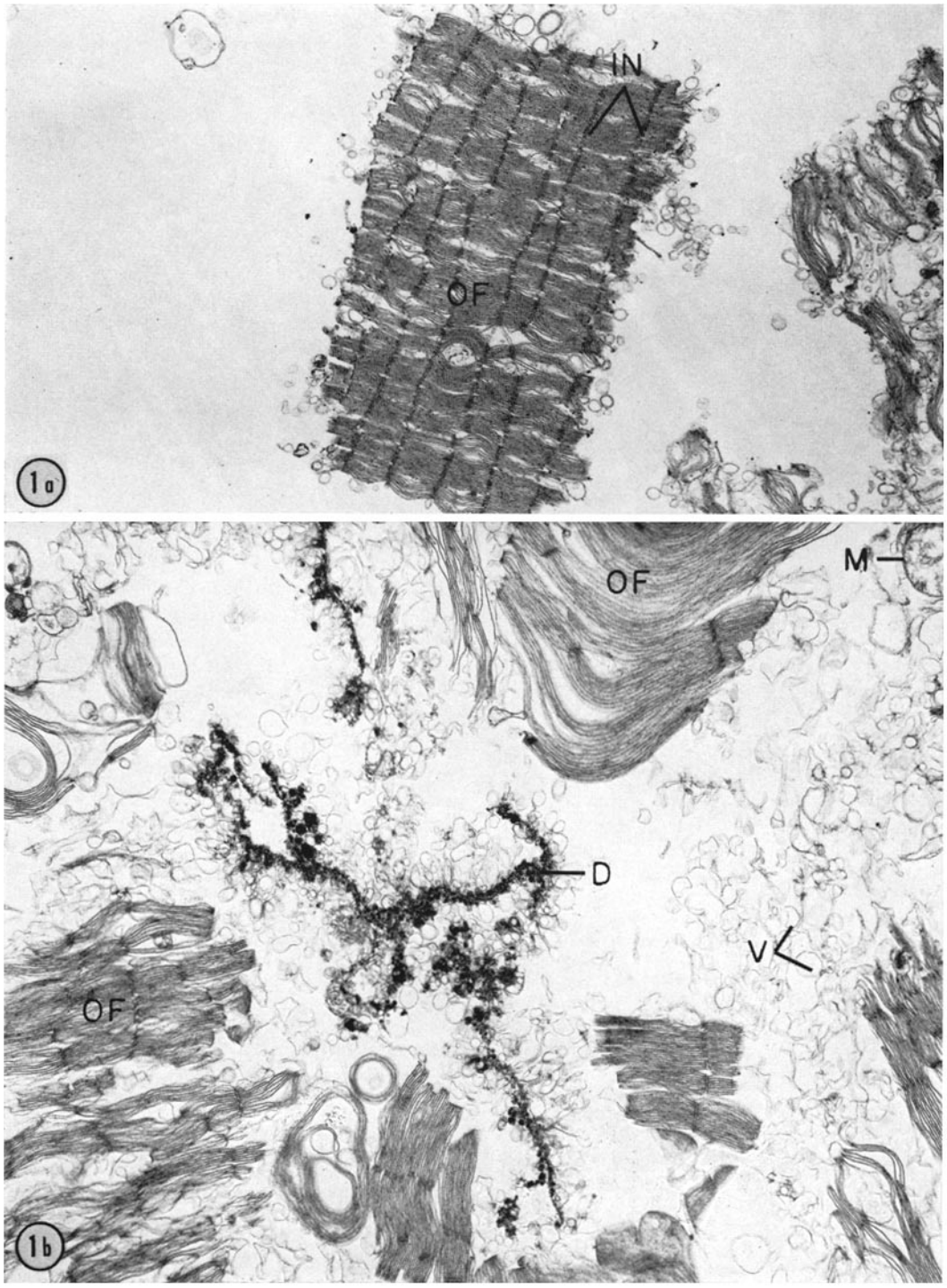

FIGURE 1 Electron micrographs of rod outer segment pellets used for osmotic swelling experiments (by weighing). (1 *a*) An outer segment fragment (*OF*) consisting of about 250 discs. The discs are observed here as lamellar components of the cylindrical ROS fragment. The striated pattern in the rod outer segment is produced when individual disc incisures (*IN*) fall into register along the long axis of this organelle. (1 *b*) A random assortment of components observed in these preparations. They include outer segment fragments (*OF*), mitochondria (*M*), assorted vesicles (perhaps produced by disc-membrane reorganization) (*V*), and debris (*D*) of an undetermined origin, Fig. 1 *a*, × 7800; Fig. 1 *b*, × 5900.

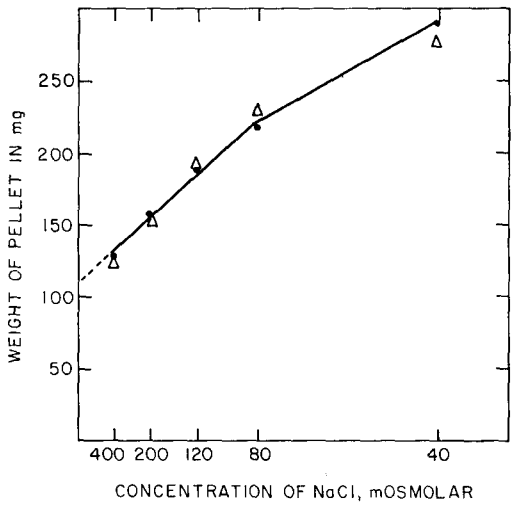

FIGURE 2 A plot of ROS pellet volume (weight) as a function of the osmolarity of the suspending medium. ROS discs were suspended in 1 mM Tris-HCl buffer, pH 7.5, 1 mM ATP, and various concentrations of NaCl. The ROS were centrifuged at 27,000 *g* for 10 min and the weight of the pellet was measured. The pellet went through a cycle of osmotic shrinkage (•) and swelling (△).

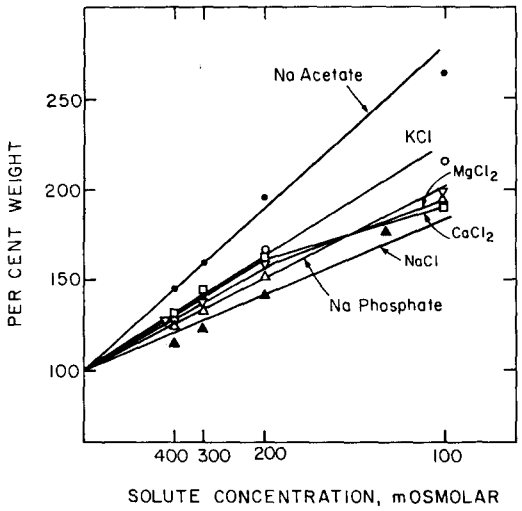

FIGURE 3 A plot of ROS per cent volume (weight) as a function of the osmolarity of the suspending medium. The extrapolated weight at infinite osmolarity (generally about 100 mg) is taken as 100%. Symbols are: NaCl, ▲; Na acetate, ●; Na phosphate, △; KCl, ○; $MgCl_2$, ▽; and $CaCl_2$, □. Experimental details are as in Fig. 2.

is the concentration of solute (in osmoles) and b and k are constants. The constant b represents the osmotically dead space and is equal to the intercept with the ordinate. In other words, b is equal to the volume of the totally "collapsed" ROS discs at infinite solute osmolarity and is made up of the volume of the disc membranes, the volume of all other solids inside the discs, and the interstitial volume. The constant k is related to the colligative thermodynamic parameters of the system (19).

Osmotic swelling in hypotonic solutions of NaCl was perfectly reversible. By progressively increasing the osmotic pressure of the bathing medium, it was possible to reverse the volume changes caused by hypotonicity of the medium (Fig. 2). The cycle of volume changes could be repeated more than once, and the same volumes were obtained whether initial osmotic pressure was high or low. Since these experiments lasted about 8 hr, it is clear that, at least for this length of time, the discs retained their semipermeable properties. In very dilute salt solutions the volume increase was smaller than that predicted from the linear relationship. This deviation from theory is apparently not due to bursting of the ROS discs with concomitant loss of osmotic behavior, because when the salt concentration was increased the ROS discs regained their former volume. If the deviation were due to bursting, the pellet volume would be expected to return to some volume smaller than the initial volume at the same osmolarity.

The results obtained with other salts are shown in Fig. 3. It appears that the osmotic swelling brought about by sodium phosphate, $MgCl_2$, and $CaCl_2$ is very similar to that caused by NaCl or KCl. The swelling in sodium acetate seems to be greater, and this might be related to the unique permeability properties of the acetate ion. On the other hand, when the ROS discs were suspended in ammonium acetate, a 2.5-fold swelling occurred. The swelling in ammonium acetate was independent of the salt concentration. Replacing the ammonium acetate with ammonium chloride led to a dramatic shrinkage of the ROS pellet to the same volume occupied by the pellet in NaCl solutions of the same osmolarity. Additional experiments revealed that solutions of ammonium phosphate had the same effect on the ROS pellet as did NaCl. Thus, it seems that while the cations, Na, K, Mg, and Ca, and the anions, Cl and phosphate, are nonpenetrating and result in osmotic behavior of ROS pellets, ammonium acetate is freely penetrating and causes very large swelling.

The permeability properties of the majority of these ions with respect to ROS and mitochondrial membranes are thus very similar (5). Phosphate is the major exception in being freely permeable through mitochondrial membranes (5), but not through ROS disc membranes (4).

The linear reversible relationship between the inverse osmotic pressure of the medium and the ROS disc volume was valid whether the experiments were conducted in the light or in the dark.

Reversible Osmotic Swelling in Solutions of Nonelectrolytes

Initial experiments with media containing 1 mM Tris-HCl buffer, pH 7.5, 1 mM ATP, and variable amounts of sucrose (50–300 mosmol) encountered unexpected difficulty in that only a very loose and fragile ROS pellet was formed after the usual centrifugation. Longer periods of centrifugation did not change the fragile nature of the pellet. The ROS pellet regained its firm consistency and could be brought down easily when centrifuged at 27,000 *g* for 10 min when low salt concentrations were added to the sucrose. In 300 mosmol sucrose, both $CaCl_2$ and $MgCl_2$ were equally effective, and the minimal effective concentration was 2.5 mM (ionic strength = 0.0075), whereas NaCl was effective only above 30 mM (ionic strength = 0.03). On an ionic strength basis, Ca^{+2} and Mg^{+2} are about fourfold more effective than Na^{+} in maintaining a firm pellet in nonelectrolyte media.

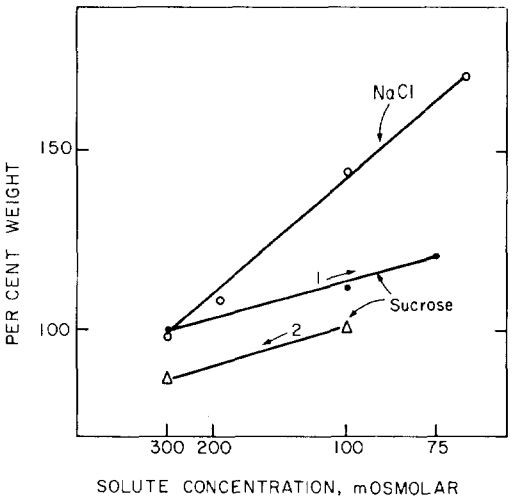

FIGURE 4 Comparison of osmotic swelling of ROS discs in NaCl and sucrose solutions. All sucrose solutions contained 10 mM $CaCl_2$ in addition to 1 mM Tris-HCl buffer, pH 7.5, and 1 mM ATP. Results are plotted as per cent change of ROS pellet weight. Arrows 1 and 2 refer to direction of initial swelling and subsequent contraction in sucrose media. The weight of the pellets was approximately 100 mg, at 300 mosmolar.

Osmotic swelling in sucrose solutions (containing 10 mM $CaCl_2$) was much less than in salt solutions (Fig. 4). The swelling was reversible, although the pellet volume after a cycle of expansion-contraction was smaller than the initial volume by approximately 10%.

Ultrastructure Studies on Disc Volume

Rod outer segments which were isolated in 150 and 100 mM NaCl retained considerable organization at the ultrastructural level. In 150 and 100 mM NaCl solutions, long, highly organized fragments of rod outer segments were frequently observed (Fig. 5, *a* and *b*). These fragments contained several hundred discs in register. In 50 mM NaCl, there was a tendency toward disaggregation of disc arrays and curling of the outer segments (Fig. 5 *c*). In 10 mM NaCl, there was considerable disruption and well-organized ROS fragments were rarely observed (Fig. 5 *d*).

At higher magnification, the osmotic behavior of individual discs could be readily observed. In 150 mM NaCl solutions (Fig. 6), the ultrastructure of ROS discs was similar to that observed in many published electron micrographs of these organelles (7, 17). Discs showed two apposed parallel membranes, resulting in the well-known pentalaminar arrangement of alternating dark and light lines. The only points at which intradiscal spaces were observed with regularity were at disc edges where the membrane folded upon itself, resulting in a hairpin configuration (see also Fig. 7 *a*). Occasional interruptions in the pentalaminar patterns could be observed. The interruptions were produced by separation of the two apposed disc membranes over short distances with the subsequent formation of small intradiscal spaces. For purposes of orientation, to determine what an *intradiscal versus* an *interdiscal* space was, it was convenient to use the edges of individual discs as a guide. At the disc edge or at disc incisures, the disc membrane was folded back upon itself, forming a hairpin loop. This loop contained an observable space, regardless of the osmolarity of the medium, and served as a useful guide for tracing intradiscal spaces. The hairpin loop at the disc edge tends to

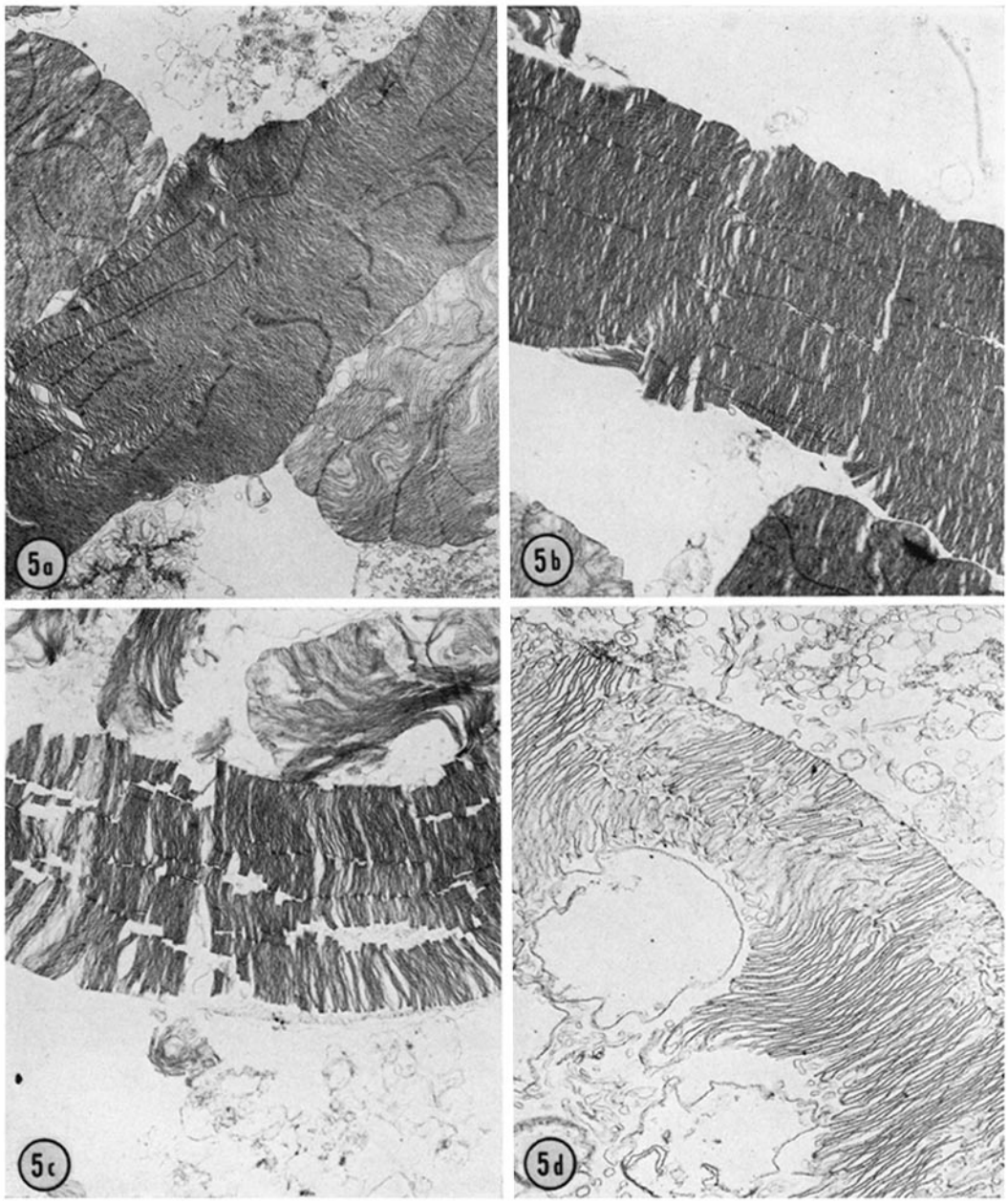

FIGURE 5 Electron micrographs of rod outer segments prepared according to the method of Lolley and Hess (13) in media containing various concentrations of solute. Similar outer segments were used for ultrastructure studies of disc volume: (5 *a*) 150 mM NaCl; (5 *b*) 100 mM NaCl; (5 *c*) 50 mM NaCl; and (5 *d*) 10 mM NaCl. As the solute concentration decreased, there was a trend toward disaggregation of outer segment disc arrays and the formation of vesicles. × 6000.

retain its shape and size regardless of the osmolarity of the medium (Fig. 7).

In 100 mM NaCl solutions, the size and frequency of intradiscal spaces increased significantly (Fig. 8). At this molarity, an intradiscal space could be followed for a considerable distance along the plane of individual discs. In 50 mM NaCl solutions, the trend was towards a gradual

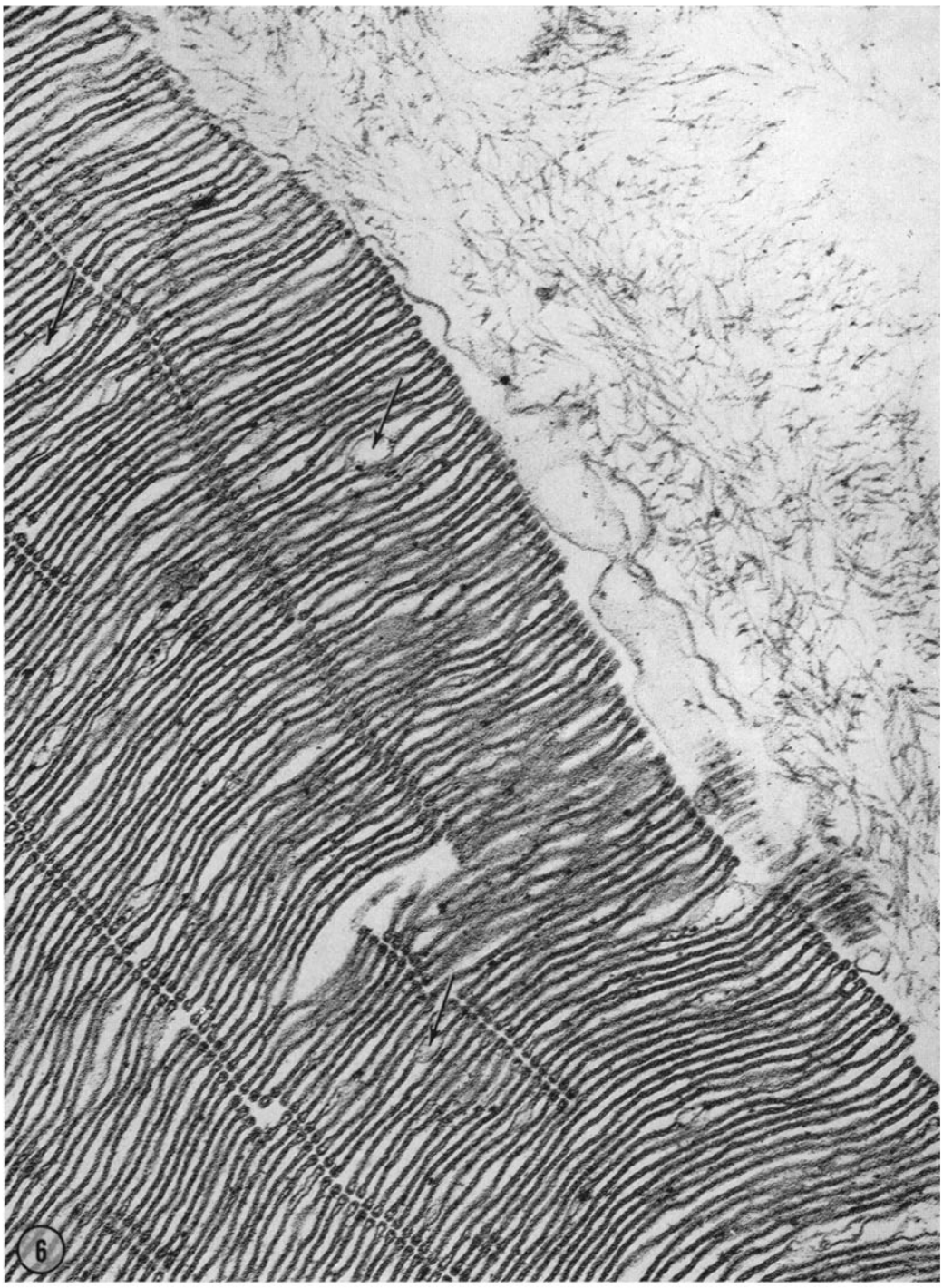

FIGURE 6 Portion of a rod outer segment isolated in 150 mM NaCl. At this solute concentration, individual disc ultrastructure reveals two apposed parallel membranes consisting of five layers of alternating dark and white lines. At certain isolated places along the disc profile, the apposed membranes separate to form an intradiscal space (*arrows*). × 65,000.

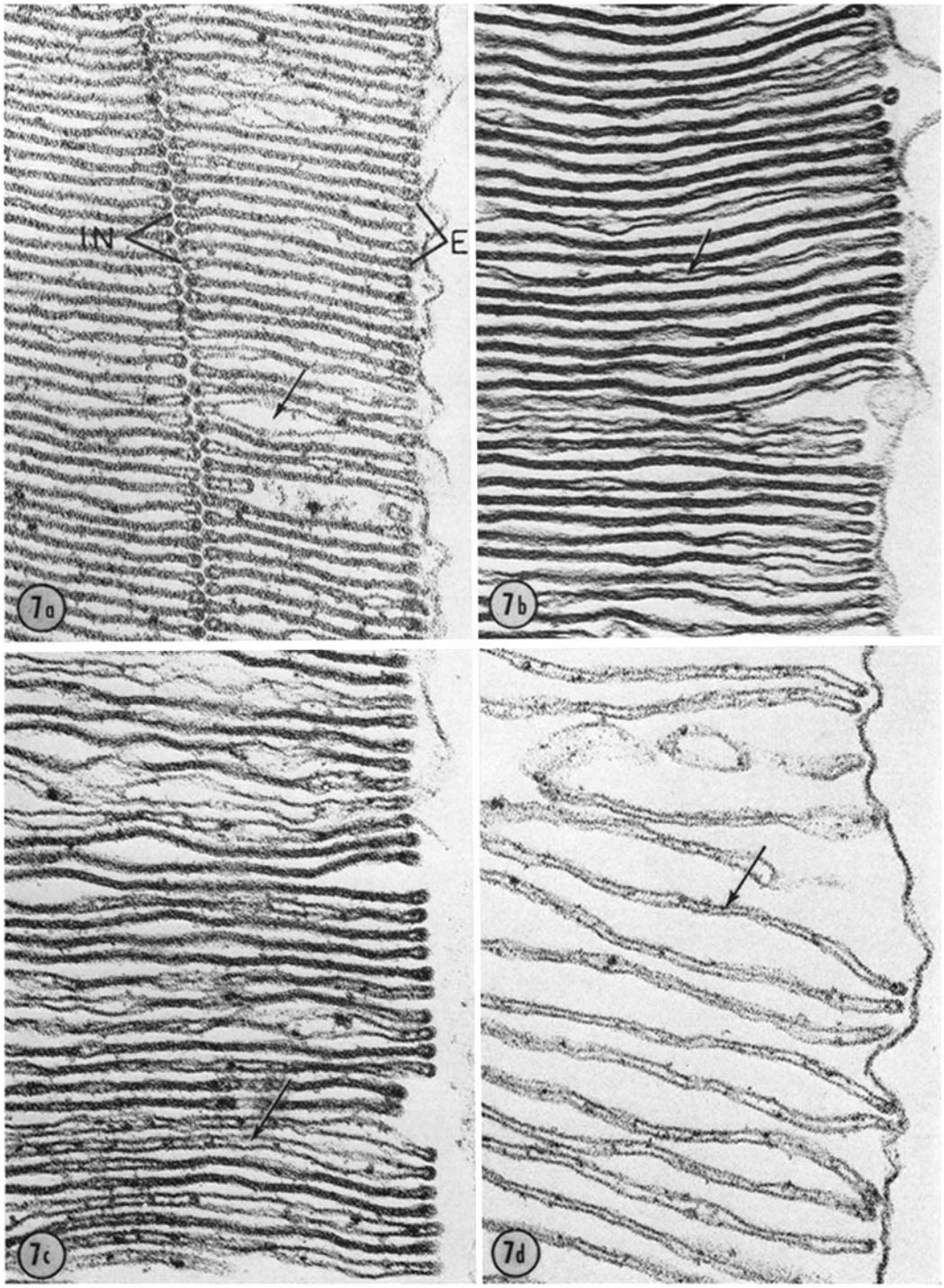

FIGURE 7 Edges of rod outer segments isolated in varying concentrations of solute: (7 *a*) 150 mM NaCl; (7 *b*) 100 mM NaCl; (7 *c*) 50 mM NaCl; and (7 *d*) 10 mM NaCl. At disc edges (*E*), or at disc incisures (*IN*), the disc membrane folds back upon itself to form a hairpin loop. This loop was useful as a guide to the intradiscal space (*arrows*), which grew larger with decreasing concentrations of solute. The interdiscal space (i.e., the distance between individual discs) also increased with decreasing solute concentrations. × 100,000.

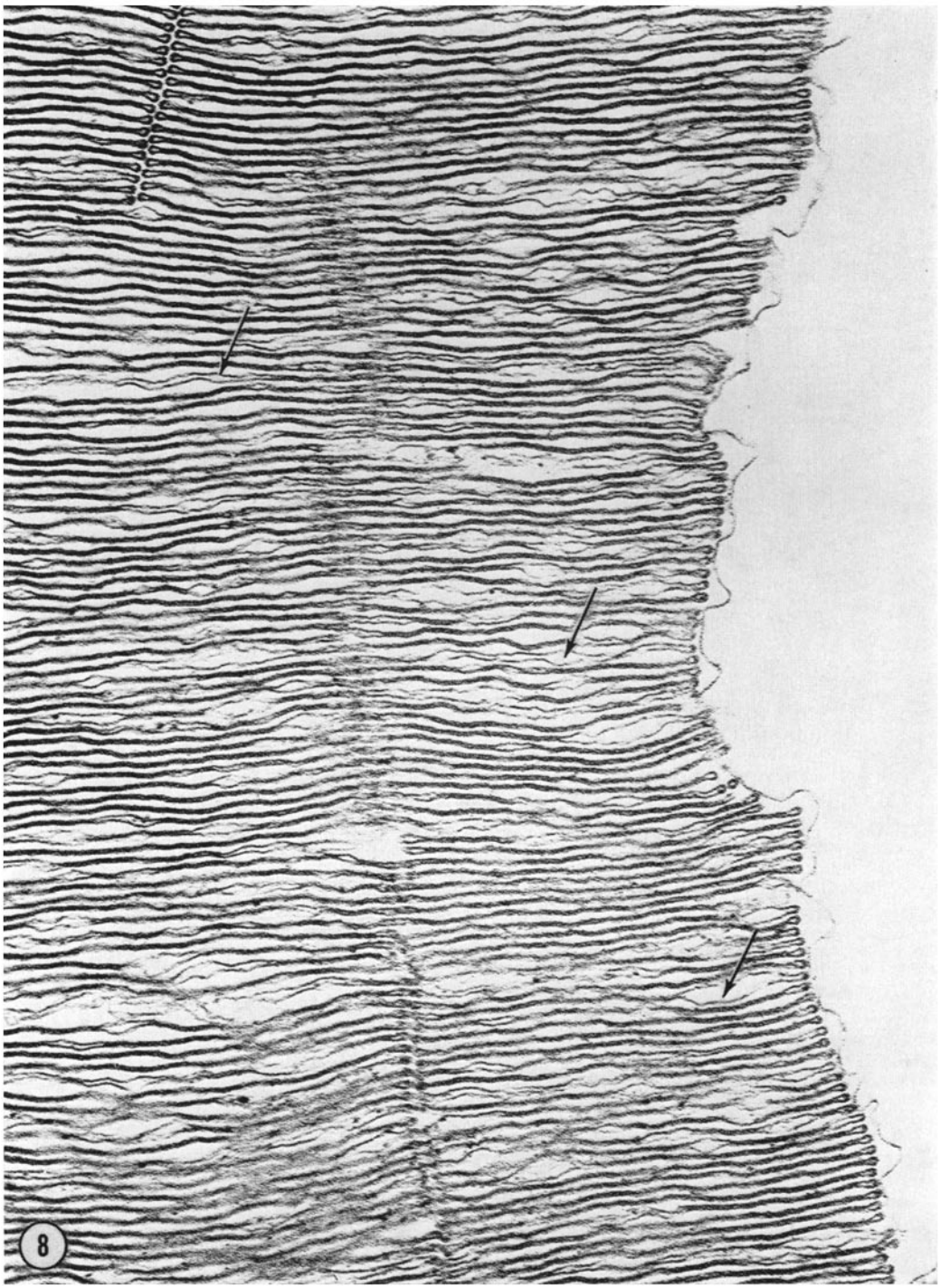

FIGURE 8 Part of a rod outer segment isolated in 100 mM NaCl. The size and frequency of intradiscal spaces (*arrows*) increased significantly over those of intradiscal spaces observed in 150 mM NaCl. However, discs were not completely open at this solute concentration, and apposed disc membranes were frequently observed. × 65,000.

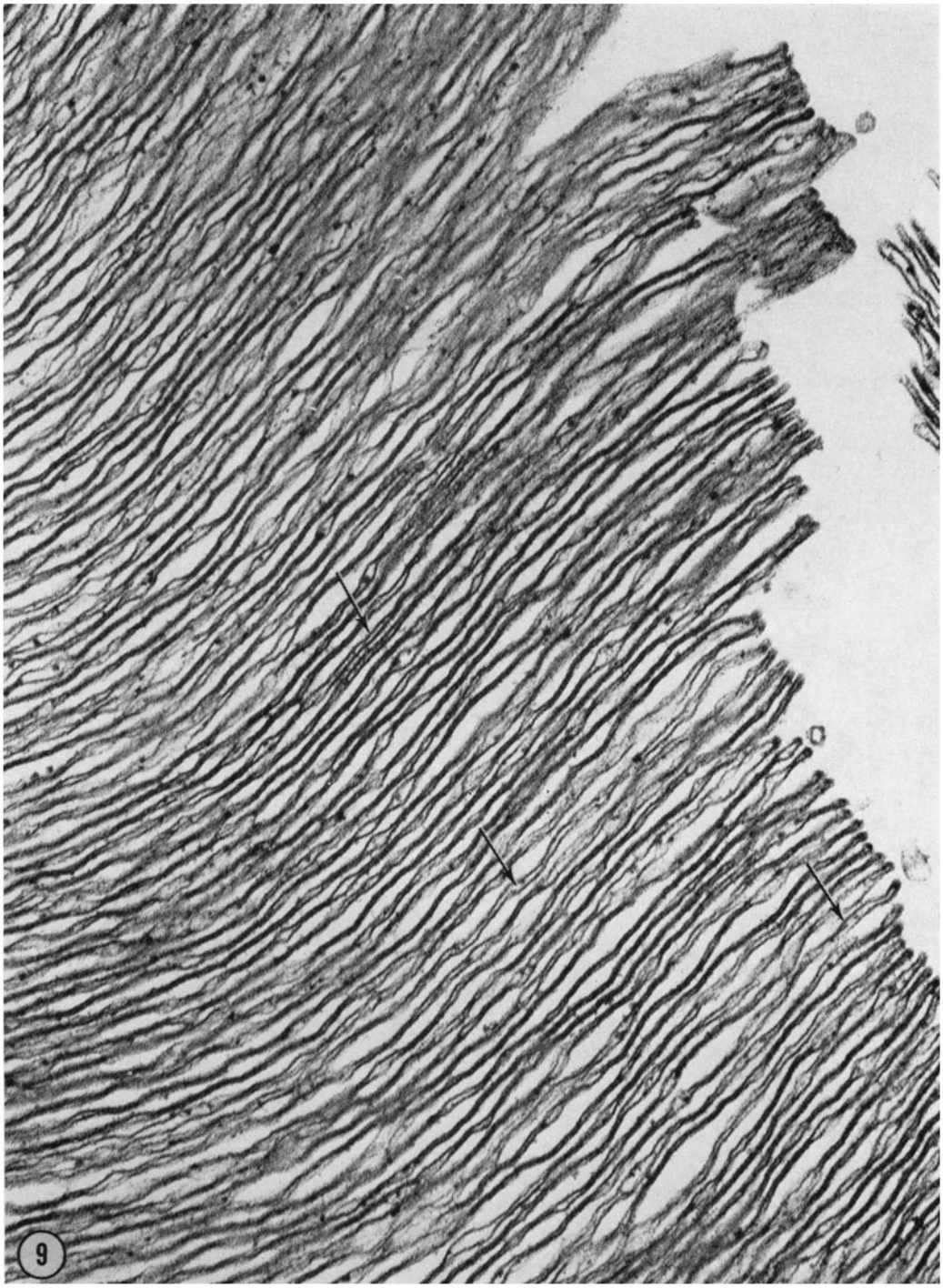

FIGURE 9 Portion of a rod outer segment isolated in 50 mM NaCl. The intradiscal spaces (*arrows*) have become more extensive and numerous than in isolation media with higher solute concentration. × 65,000.

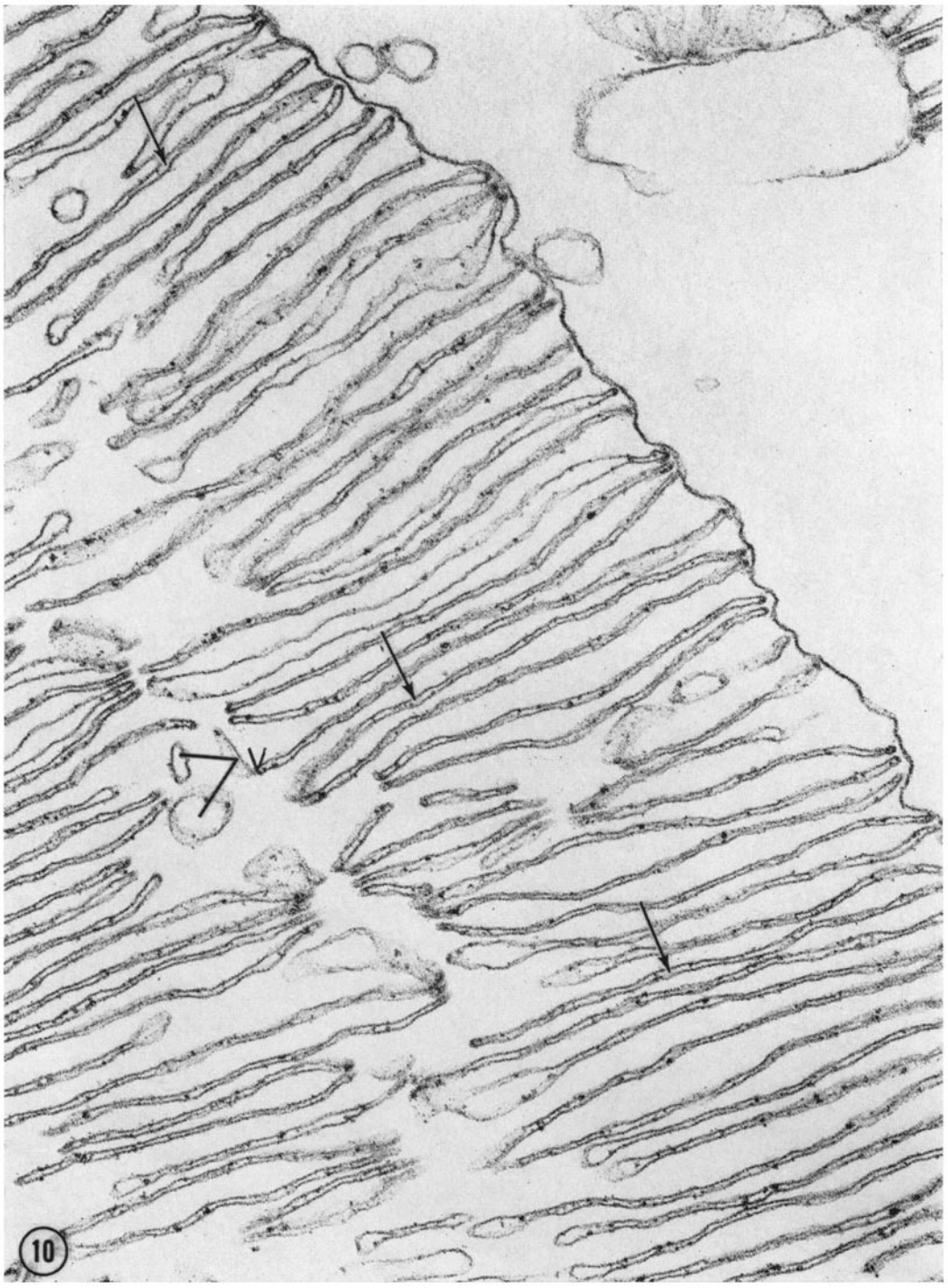

FIGURE 10 Part of a rod outer segment isolated in 10 mM NaCl. The separation of previously apposed disc membranes is complete. A continuous intradiscal space (*arrows*) is evident in very disc profile. In addition, there is a trend toward reorganization of disc membrane into vesicles (*V*). × 65,000.

increase in the size and extent of the intradiscal spaces (Fig. 9). However, examples which showed the space being present throughout the entire profile of any given disc were difficult to find. At this molarity, as well as at every other salt concentration employed, large areas of intact, enveloping cell membrane could frequently be observed. The presence or absence of these cell membrane fragments, however, had no effect on the degree of disc swelling for a given salt concentration.

Rod outer segments exposed to 10 mM NaCl concentrations showed a marked change from those exposed to the higher molarities (Fig. 10). In every case, separation of previously apposed disc membranes was complete, and a continuous intradiscal space was evident throughout the length of every disc profile. For the first time, there was a tendency toward reorganization of disc membranes into vesicles. Numerous vesicles of varying size were observed interspersed among intact and partially modified ROS discs. In addition, although there was only a moderate increase in the interdiscal space with decreasing osmolarity up to this point, the size of the interdiscal space increased considerably in 10 mM NaCl.

Throughout each experiment rigorous control of the salt concentration was necessary, even after glutaraldehyde fixation. If the osmolarity of the OsO_4 postfixation medium did not match that of the glutaraldehyde fixing solution, the final size of the intradiscal space was determined to a considerable extent by the OsO_4 medium. Fig. 11 shows an outer segment isolated in 150 mM NaCl and fixed with glutaraldehyde in NaCl of the same molarity. The segment was subsequently postfixed in OsO_4 dissolved in 10 mM NaCl. Although the outer segment is considerably more organized than those isolated in 10 mM NaCl, the presence of a considerable intradiscal space is obvious. Likewise, disc swelling induced by low salt concentrations, followed by fixation with glutaraldehyde, could be reversed by placing the swollen, fixed discs in OsO_4 dissolved in 150 mM NaCl.

Interstitial Space of ROS Pellet

As can be seen from Fig. 12, the interstitial space of the ROS pellet is 39% ± 1% at 305 mosmol, and 37% ± 1% at 153 mosmol. Nobel (16) showed that dextran adsorbs onto the membrane in chloroplast pellets, since the observed pellet/supernatant ratio decreases as the dextran concentration increases. The same phenomenon is observed with ROS pellets, and the extrapolated interstitial space was obtained from the intercept with the ordinate. As was mentioned earlier, the intercept represents the interstitial space at infinite dextran concentration.

Dry Weight and Protein Content of ROS Discs

Protein content (expressed as milligram equivalents of bovine serum albumin) of the wet pellet was 4–5% of the total pellet weight. When the wet pellet weight was corrected for a 38.5% interstitial space, the protein content was 6–8%. The dry weight of the pellet was 6–10% of the total wet weight, or 10–16% of the weight when the interstitial space was subtracted. The weight of the salts represents some 10% of the total dry weight (with 150 mM NaCl in the bathing medium).

DISCUSSION

The purpose of the experiments reported in this paper was to show (*a*) that ROS disc membranes enclose a space which is separate from the photoreceptor intracellular space, and (*b*) that the ROS disc membranes have specific permeability properties with respect to various ions and nonelectrolytes.

One difficult problem with ROS preparations is the degree of contamination by other subcellular organelles, especially mitochondria. This problem was not really solved in our study, although it appears from electron micrographs that mitochondrial contamination is on the order of 10% or less. Enzyme markers, it was felt, could not answer this question in a more quantitative way, since the extent to which any given enzyme is present or absent in "pure" preparations of ROS is unknown (13).

Osmotically Induced Changes in ROS Disc Volume

The results of the weighing experiments, in conjunction with the electron microscopy study, show that the discs are indeed enclosed spaces in rod photoreceptor outer segments. Since the enveloping cell membrane of the outer segment was torn and incomplete in our preparations, the volume changes measured by weighing can only reflect changes in disc volume. Further support

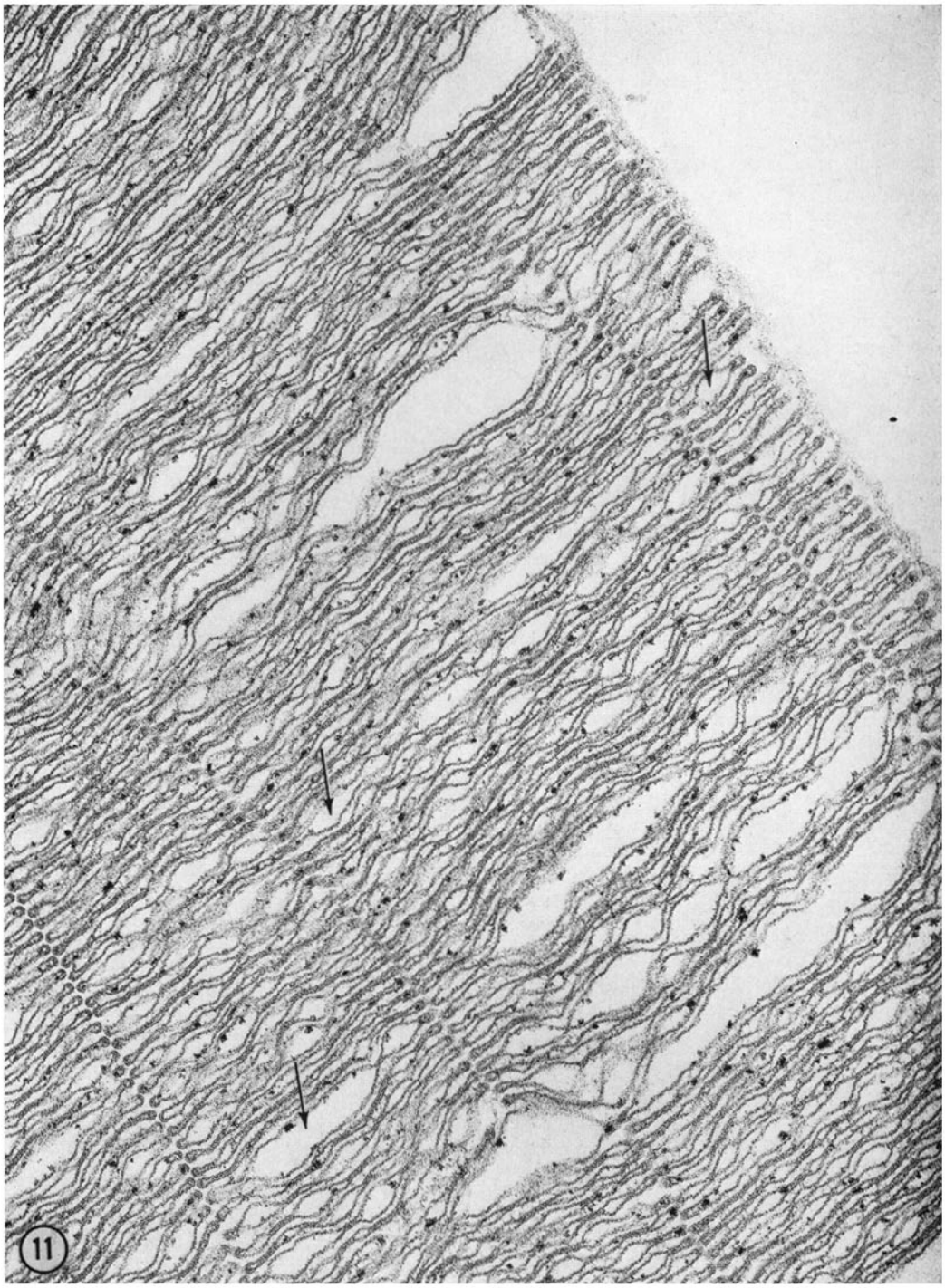

FIGURE 11 Portion of a rod outer segment isolated in 150 mM NaCl and fixed with 0.15% glutaraldehyde in the same medium. Following fixation, the outer segment preparation was suspended in 10 mM NaCl and postfixed in 0.12% OsO_4 in the same dilute medium. In comparison with Fig. 6, the size of the intradiscal space (*arrows*) has increased visibly in spite of prior fixation with glutaraldehyde. Thus, some of the disc membrane permeability properties are retained after glutaraldehyde fixation. × 65,000.

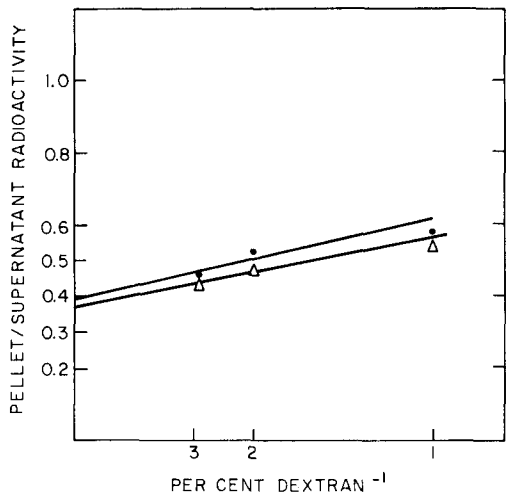

FIGURE 12 Interstitial space of ROS pellets determined with dextran-^{14}C. For experimental details, see Methods. Experimental points are at 305 mosmolar (•) or at 153 mosmolar (△). The pellet was prepared as described for the osmotic swelling experiments.

for this conclusion comes from the correspondence between osmotic pressure of the suspending media and swelling of the ROS discs as seen by electron microscopy. The disc membrane is semipermeable, and the disc volume corresponds to the osmotic pressure of the bathing medium. The ROS discs are thus free-floating sacs with an inside space separate and distinct from the photoreceptor intracellular space. These results confirm the previous finding by Brierley et al. (4) and the various hints in the literature indicating that ROS discs behave as semipermeable sacs. (For review, see Cohen[1], also references 2, 6, and 8).

An alternative explanation for swelling of the ROS pellet at low osmolarities would involve the hydration of gels which might be present in the interdiscal space. Three lines of evidence militate against this explanation: (*a*) The swelling represents a specific response to certain substances. Thus, changes in NaCl and KCl concentration cause a reversible, moderate swelling, while ammonium acetate causes a tremendous swelling independent of its concentration. This specificity is very similar to that known for other membrane systems, such as the mitochondrion (5), and is not to be expected for a gel. (*b*) With substances such as KCl and NaCl, the ROS pellet swelling is a perfectly reversible function of the linear function of the inverse salt concentration (osmotic pressure). Again, this behavior is to be expected with a semipermeable membrane, but not with a gel. (*c*) Mild sonication of an ROS pellet leads to a loss of over 80% of its weight at a given salt concentration (Heller and Ostwald, unpublished). This, again, can be explained as the destruction of the organized membrane system, resulting in a "leak" in the disc membrane. Again, it is difficult to see how mild sonication of a gel would reduce its hydration.

The sequence of events which lead to disc swelling as observed by electron microscopy is somewhat different than that anticipated. Rather than the entire disc opening up at once, portions of the disc appear to open up separately and at different times. In this light, the following hypothesis is advanced. The mechanism could perhaps be compared with the inflation of an air mattress. During the initial stages of inflation, certain areas of an air mattress fabric remain apposed while other areas bulge. Eventually, the entire mattress is inflated and its walls no longer touch. In the ROS disc, the internal membrane surfaces remain close to one another until they are separated by hydrostatic forces. Ultimately, the membranes are separated along their entire length.

A second hypothesis is presented with respect to vesicle formation at low salt concentration. It is conceivable that a process of budding might bring about the apparent reorganization of disc structure into vesicles at low salt concentration. In this scheme, hydrostatic forces building up within individual discs would eventually exceed forces holding the membranes intact. As a result, a small fluid-filled vesicle would bud off from the disc, thereby reducing the pressure within the disc. The authors are aware that disc membranes are frequently reorganized into vesicles when chemical fixation is delayed for a considerable period of time. Militating against this possibility, however, is the fact that elapsed time prior to fixation was identical for all experiments. The absence of significant membrane reorganization at the higher molarities would argue against fixation delay as a causative factor for vesicle formation at low molarities. The budding hypothesis, therefore, would appear to be the most acceptable one.

Is There an Intradisc Space In Vivo?

Electron micrographs of ROS isolated in the presence of 150 mM NaCl (isosmotic) show a small open space at the disc edge. Throughout the remainder of the disc, the two membranes are apposed, seemingly without any intervening space. Solids were found to represent some 10–16% of the disc wet weight. Thus, approximately 84–90% of the disc weight is due to water. We know that the membrane itself is composed mostly of hydrophobic proteins and lipids and contains very little water. Thus, a considerable fraction of the disc weight in isosmotic media is due to water. It seems reasonable to assume that most if not all of this water is present inside the disc. *We suggest, then, that in vivo the disc possesses not only a potential but a real internal space.*

Two arguments can be advanced for the lack of an intradiscal space in electron micrographs of ROS isolated in isosmotic media. Whatever space was originally there might have been obliterated during the rather harsh methods employed in the histological procedure. In addition, the limits of resolution imposed by sectioned biological material (10–20 A) could prevent detection of an intradiscal space.

Arguments for the large increase in the interdiscal space at low osmolarity (Fig. 7) are speculative. We have observed that the low osmolarities produce a general loss of organization, an appearance of vesicular forms, and the transformation of large ROS fragments into much smaller assemblies of discs. Since the ROS enveloping membrane is no longer present to hold the discs in register, it is most likely that, in addition to the changes enumerated above, the low osmolarity leads to a separation of the stacked discs. Thus, the increase in the interdiscal space involves a mechanism which is coincidental and unrelated to the osmotic swelling of the discs.

Permeability Properties of Disc Membranes

The disc membrane is impermeable to Na^+, K^+, Mg^{+2}, Ca^{+2}, Cl^-, and phosphate and is permeable to ammonium acetate. An identical series can be written for mitochondrial membranes, with the exception of phosphate, which permeates mitochondria but not ROS discs (4, 5). When the same type of experiment was tried with nonelectrolytes such as sucrose, a minimum amount of electrolyte such as NaCl or $CaCl_2$ was necessary. In the absence of salts, the ROS formed a very soft fragile pellet that could not be adequately weighed. This salt effect on the physical characteristics of the ROS pellet is partly an ionic-strength effect, since NaCl could substitute for $CaCl_2$ or $MgCl_2$, yet the divalent cation seemed to be at least four times more effective than NaCl on the basis of ionic strength. In line with the well-known effect of Ca^{+2} on membranes and on the osmotic behavior of microsomes and chloroplasts (12, 20), it is possible that Ca^{+2} and Mg^{+2} have a specific stabilizing effect on the ROS disc.

The semipermeability properties of ROS discs are preserved, remarkably enough, even after glutaraldehyde fixation for electron microscopy. During staining with OsO_4 the osmolarity of the osmium tetroxide solution has to correspond to that of the glutaraldehyde fixation medium, otherwise the final volume of the ROS disc will be largely determined by the OsO_4 staining medium. Other investigators also have observed the preservation of the semipermeability properties of tissue after glutaraldehyde fixation (3, 15).

Experiments reported and concepts developed in this paper make it possible to study the effects of illumination on the permeability properties of rod outer-segment discs, in the hope of elucidating the mechanisms of visual excitation. We are presently investigating the effect of illumination on the permeability properties of isolated ROS discs.

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