

MEMBRANE CHARACTERISTICS AND OSMOTIC BEHAVIOR OF ISOLATED ROD OUTER SEGMENTS

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ABSTRACT

Freshly isolated frog rod outer segments are sensitive osmometers which retain their photosensitivity; their osmotic behavior reveals essentially the same light-sensitive Na^+ influx observed electrophysiologically in the intact receptor cell. Using appropriate osmotic conditions we have examined freeze-etch replicas of freshly isolated outer segments to identify the membrane which regulates the flow of water and ions. Under isosmotic conditions we find that the disc to disc repeat distance is almost exactly twice the thickness of a disc. This ratio appears to be the same in a variety of vertebrate rod outer segments and can be reliably measured in freeze-etch images. Under all our osmotic conditions the discs appear nearly collapsed. However, when the length of the outer segment is reduced by hyperosmotic shocks the discs move closer together. This markedly reduces the ratio of repeat distance to disc thickness since disc thickness remains essentially constant. Thus, the length reduction of isolated outer segments after hyperosmotic shocks primarily results from reduction of the extradisc volume. Since the discs are free floating and since they undergo negligibly small changes in volume, the plasma membrane alone must be primarily responsible for regulating the water flux and the light-sensitive Na^+ influx in freshly isolated outer segments. On this basis we calculate, from the osmotic behavior, that the plasma membrane of frog rod outer segment has a Na^+ permeability constant of about 2.8×10^{-6} cm/s and an osmotic permeability coefficient of greater than 2×10^{-3} cm/s.

INTRODUCTION

Recently, Korenbrot and Cone (1972) have shown that outer segments isolated by gently shaking a retina are sensitive osmometers: after hyperosmotic shock with NaCl the outer segments shrink and then recover in volume, and the rate at which they recover is markedly reduced by light. This behavior demonstrates a light-sensitive Na influx, and the photosensitivity of this Na influx appears to be the same as in the intact receptor cell. In addition, isolated outer segments are impermeable

to K since no volume recovery occurs after hyperosmotic shocks with KCl. These results are consistent with the electrophysiological characteristics of the rod photoreceptor (Tomita, 1970; Hagins et al., 1970). Thus, in freshly isolated outer segments the mechanism of visual excitation appears intact.

Rod outer segments consist of a stack of free-floating discs surrounded by a plasma membrane (Cohen, 1963). Hence, two osmotically active

volumes could exist in the outer segment: disc volume and extradisc volume. The semipermeable properties of disc membrane could regulate disc volume, whereas extradisc volume could be regulated by the semipermeable properties of plasma membrane. Osmotic behavior attributable to either or both membranes has been described (Brierley et al., 1968; Clark and Branton, 1968; Heller et al., 1971; Cohen, 1971) and the osmotic behavior of freshly isolated outer segments described by Korenbrot and Cone might therefore be regulated by either or both of these membranes. We have used freeze-etch techniques to determine which membrane regulates osmotic behavior under the conditions used by Korenbrot and Cone. Our results indicate that plasma membrane is primarily responsible for changes in length of freshly isolated outer segments under the various osmotic conditions tested.

Freeze etch was selected as the best technique available for this study since it should reliably reveal changes in the disc and extradisc volumes. The speed with which experiments are completed is important since under our conditions (Korenbrot and Cone, 1972) outer segments isolated for longer than about 15 min begin to undergo structural, and presumably functional, changes (Robertson, 1966). With freeze-etch technique, outer segments can be gently isolated, osmotically shocked, and frozen all within 12–16 min after separation from the retina. Freeze etch does, however, have some limitations: a high density of isolated cell organelles is required, possible effects of the cryoprotective agent must be considered, and uncertainties can arise in analyzing platinum shadows in the replicas. Comparison of images of freeze-etched outer segments with X-ray diffraction analysis of the same structure can be used to overcome some of these limitations.

MATERIALS AND METHODS

Biological Material

Frogs (*Rana catesbeiana*) 5–7 inches in body length were used in all experiments. The animals were kept in constant darkness and were killed by decapitation immediately preceding the experiment. The eyes were enucleated and the retinas dissected free of pigment epithelium. The entire procedure was carried out under dim red light.

Solutions

All solutions were prepared in distilled demineralized water and were buffered to pH 7.4. Reagent

grade salts were used and the solutions were prepared as previously published (Korenbrot and Cone, 1972). In general, hyperosmotic solutions were made by adding the appropriate salt to the standard isosmotic solution. All solutions which contained glycerol were prepared by adding the appropriate salts to a 20% vol/vol glycerol in water solution. Osmotic pressures were determined from freezing-point depression data (Weast, 1967). Concentrations of nonisosmotic solutions are expressed in units of isosmotic pressure (one unit, 1 Is, is 232 mosmol).

Osmotic Shock and Preparation of Material for Freeze Etch

One difficulty encountered in the freeze-etch technique when working with isolated cell organelles is low specimen density in the fracture plane. The following procedure was found successful in producing replicas with adequate specimen density. Under dim red light two retinas were dissected in the standard isosmotic solution and each was cut into 4–6 smaller sectors. Using forceps, each sector was slowly and gently shaken about twice a second for about 45 s into a 300 μ l conical glass receptacle containing the solution whose osmotic effect on the outer segments was being investigated. To help reduce fragmentation of the outer segments, care was taken to avoid contact between the retina and the sides of the receptacle. The receptacle was then centrifuged in a Sorvall GLC-1 centrifuge (Ivan

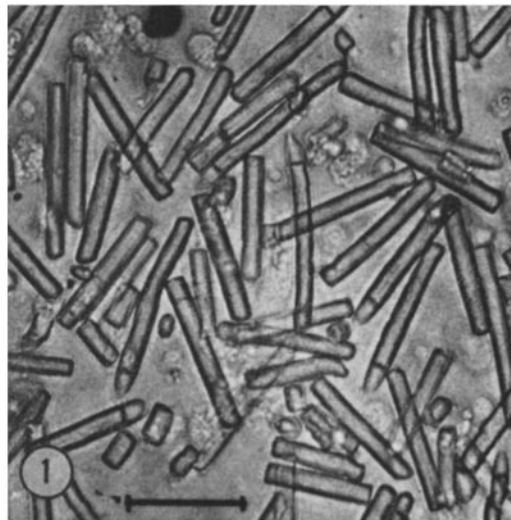


FIGURE 1 Typical field of isolated frog rod outer segments in standard isosmotic solution collected immediately preceding freezing (see text). It can be seen that most of the outer segments remain intact through the gentle preparative procedures used. The calibration bar is 50 μ m.

Sorvall, Inc., Norwalk, Conn.) with a rotor head adapted to hold the conical receptacle. Centrifugation lasted 2–3 min at 730 *g* in the absence of glycerol or at 1000 *g* in its presence.

After centrifugation the small pellet formed was transferred onto a modified gold-nickel specimen stage with the help of a 50 λ microcapillary. The specimen stage was modified by depositing a small drop of agar and trimming it to a flat surface. The agar (2% wt/vol) was dissolved in the same solution with which the outer segments had been shocked in order to avoid further osmotic changes. In addition, Fast Green histological stain was added to the agar to increase its visibility. The transferred drop of outer segments was allowed to sit on the stage for about a minute, in which time the outer segment settled to the agar interface. The stage was then quickly frozen in Freon 22 (-160°C) and stored in liquid nitrogen. The stage was frozen 12–16 min after the first sector of the retina had been shaken. This is more than sufficient time for the outer segment to attain equilibrium after an osmotic shock. Most of the outer segments remained unbroken through this gentle preparative procedure, as is shown in Fig. 1.

Light Exposure

While still attached to the retina, the outer segments were exposed to three flashes of light delivered by M-3 flashbulbs through a filter combination which passed light between 560 and 750 nm (Wratten 23-A, Schott KG-3, and IR absorbing filters from Edmund Scientific Co., Barrington, N. J.). The flashes bleached more than 50% of the visual pigment.

Freeze Etch and Electron Microscopy

Freeze etching (Moor, 1966) was carried out in a Balzers 360-M device (Balzers AG, Balzers, Furstentum Leichtenstein). The specimen was cleaved at -100°C . After etching for 1 min at $2-4 \times 10^{-6}$ torr, a replica of a surface near the agar interface was produced by evaporation of the mixture of platinum and carbon followed by evaporation of pure carbon. The replica was cleaned of organic matter with 70% sulfuric acid dichromate.

The replicas were observed with a Siemens Elmiskop 101 electron microscope at 80 kV. The microscope was calibrated with a grating having 2160 lines/mm.

Analysis of Freeze-Etch Images

Freeze-etch replicas of isolated outer segments in each of the following solutions were prepared: 1 Is standard solution, 3 Is KCl solution, and 3 Is NaCl solution both in the dark and after bleaching more than 50% of the visual pigment.

In the freeze-etch method tissues are often fractured along planes which lie tangent to the surface of the membranes (Clark and Branton, 1968; Leeson, 1970). However, in this report we investigated images of cross-fractured outer segment membranes (Fig. 2). Freeze-etch image of cross-fractured membranes have recently been shown to reliably represent the dimensions of the original membranes (Lickfeld et al., 1972). Electron microscope images of cross-fractured outer segments were enlarged and printed, and the printed images were measured with an Edscorp comparator (Edmund Scientific Co.) calibrated in 0.1 mm. Repeat distances from disc to disc, and disc thickness, were measured on the prints according to the following protocol: (a) rod outer segments were positively identified by the presence of multiple incisures which are characteristically absent in cone outer segments (Moody and Robertson, 1960); (b) only carefully selected images where the plane of fracture appeared to be nearly perpendicular to the disc membranes were measured; (c) discs and extradisc spaces were identified by searching for clearly defined loops at the rims of the discs; (d) a straight line, perpendicular to the plane of the discs, was drawn on the enlarged print and all measurements were taken along this line in part to insure that each repeat distance and disc thickness were measured only once. The repeat distance was measured between successive corresponding edges of the discs, and the disc thickness was measured between the outer edges of each disc.

By making a large number of measurements, statistical fluctuations were reduced to less than 2%. However, more significant errors can arise from systematic uncertainties such as variations in the angle of the plane of fracture and the difficulty in determining the position of the edge of the membranes due to the granularity which results from irregularity in water sublimation and platinum deposits. Since, for fracture planes essentially perpendicular to the disc membrane, the ratio of major repeat distance to disc thickness obtained from each image should be nearly independent of the angle of the plane of fracture, errors due to small variations of this angle can be minimized by considering only the "repeat to disc" ratio in comparing images, instead of the absolute dimensions as measured on the print. The position of each membrane edge was determined by eye and was thus subject to systematic bias on the part of the observer. Such bias has little effect on measurements of the repeat distance, but is probably the limiting source of error in measurements of disc thickness. The observer attempted to determine both the minimum and the maximum possible thickness of each disc in a given image. From this procedure we estimate the systematic bias can be no larger than 10%.

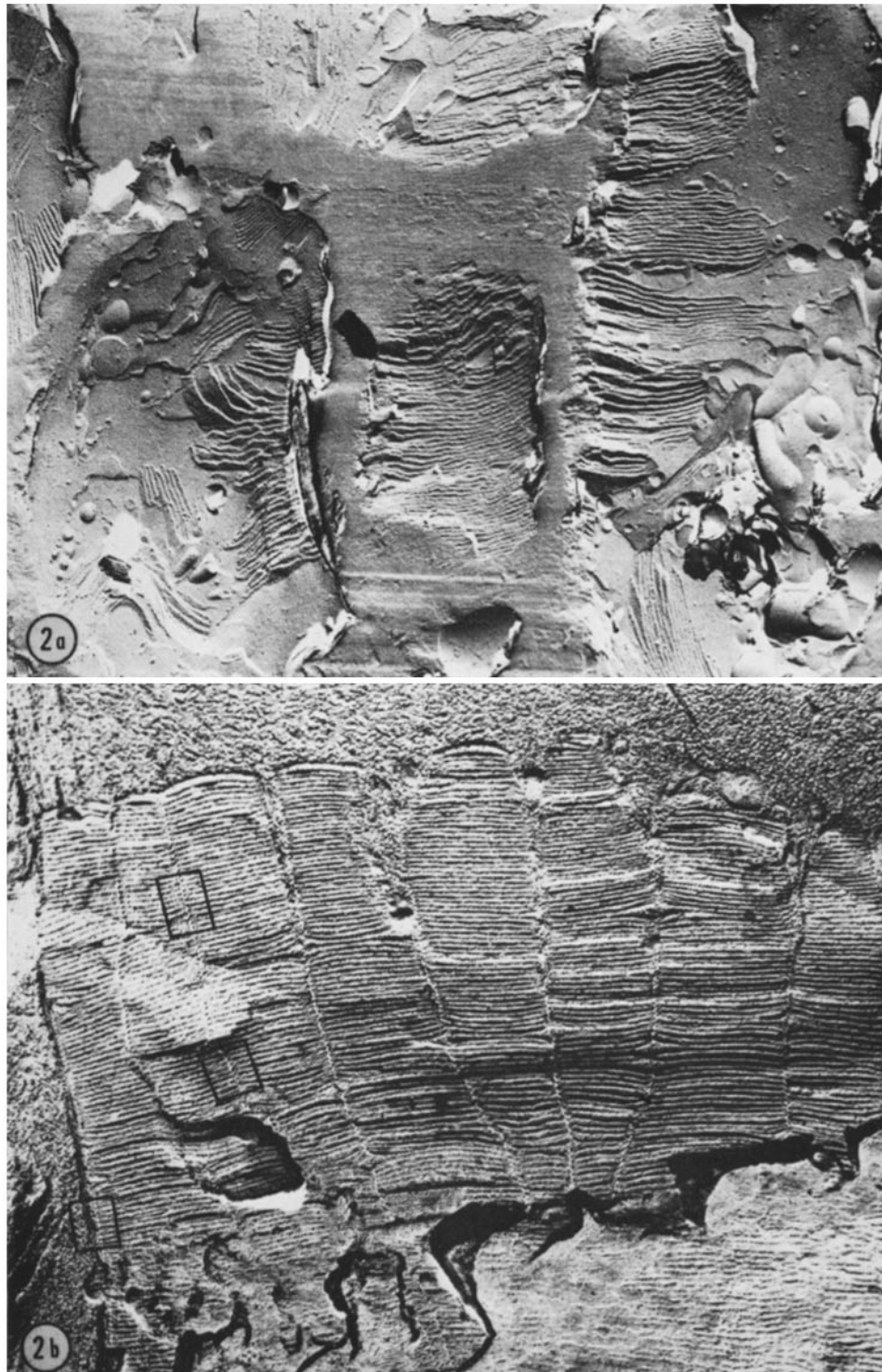


FIGURE 2 Images of freeze-etched outer segments in standard isosmotic solution in the presence of glycerol (20% vol/vol). (a) Commonly found field of outer segments. Fracture surfaces occur along various different angles $\times 13,500$. (b) Cross-fractured rod outer segments were identified by the presence of characteristic multiple incisures, and the disc and extradisc spaces in the outer segments were identified by searching for loops at the rims of the discs. However, the angle of the plane of fracture varied across the replica and only in a few areas (rectangles) did it appear nearly perpendicular to the plane of the disc, as revealed by the highly symmetrical image of the discs. $\times 26,750$.

RESULTS AND DISCUSSION

Osmotic Behavior and Effects of Glycerol

The isolated outer segments were subjected to osmotic shocks under essentially the same conditions which we have previously described (Korenbrodt and Cone, 1972). However, most osmotic shocks were carried out in the presence of the cryoprotective agent glycerol (20% vol/vol). To make valid comparisons, therefore, it was necessary to determine whether glycerol altered the osmotic behavior. Isolated outer segments are permeable to glycerol, and as can be seen in Table I, when glycerol is added to the standard isosmotic solution the equilibrium length is unchanged from that in the standard solution. Table I also presents the results of hyperosmotic shocks in the presence and absence of glycerol. In the dark, the length of outer segments is reduced in hyperosmotic KCl solutions. On the other hand, outer segments shocked with hyperosmotic NaCl shrink at first and, if fully dark adapted, they recover in length returning essentially to their starting value. In contrast, outer segments which are flash bleached before the hyperosmotic NaCl shock behave indistinguishably from KCl shocked outer segments i.e., they shrink to the same extent and no length recovery ensues. Glycerol did not affect any of the equilibrium lengths shown in Table II. In addition, we observed no significant differences in the structure of freeze-etch outer segments pre-

pared in the presence or absence of glycerol. Thus glycerol, which was added as a cryoprotective agent to facilitate the production of good quality replicas, does not affect the osmotic behavior considered here.

Structure of the Rod Outer Segments

Schmidt (1935) proposed, on the basis of their birefringence properties, that rod outer segments consist of a regularly spaced lamellar array. Sjostrand (1953) reported the first thin-section microscope observations of intact rod outer segments, and the model proposed at that time was later modified (DeRobertis and Lasansky, 1961; Sjostrand, 1961) to what has become the accepted model for the structure of rod outer segments as observed in thin sections: the outer segment consists of stacked discs surrounded by a plasma membrane. Each disc is a flattened saccule with its membranes closely apposed to each other and lying in a plane perpendicular to the long axis of the outer segments. The discs appear to be free floating, except for a few near the ciliary connection (Moody and Robertson, 1960; DeRobertis and Lasansky, 1961), since no continuities between disc and plasma membrane have been observed. Moreover, Cohen (1968; 1970) has reported that discs in intact rods cannot be infiltrated by lanthanum, whereas cone discs, which are continuous with the plasma membrane, are infiltrated. Similarly Laties and Liebman (1970; P. A.

TABLE I
Repeat Distance to Disc Thickness Ratio in a Number of Vertebrate Rod Outer Segments

Animal	Repeat distance		Reference
	Disc thickness	Technique	
Frog			
<i>R. catesbeiana</i>	2.04	X-ray diffraction	Gras and Worthington, 1969
<i>R. temporaria</i>	2.00	X-ray diffraction	Blaurock and Wilkins, 1969
<i>R. pipiens</i>	2.00	X-ray diffraction	Corless, 1972
<i>R. catesbeiana</i>	1.99	Freeze etch	This report
<i>R. pipiens</i>	1.84	Thin section O ₃ O ₄ , 1%	Nilsson, 1965
Cattle	2.06	X-ray diffraction	Worthington, 1971
Rat	2.08*	Freeze etch	Leeson, 1970
Guinea pig	1.95	Freeze etch	Clark and Branton, 1968
Catfish	1.99*	Thin section glutaraldehyde-O ₃ O ₄	Dunn and Adomian, 1971
Banjo ray	1.96*	Thin section O ₃ O ₄	Dunn and Adomian, 1971

* Measured from the published micrographs.

TABLE II
Equilibrium Length after Osmotic Shock in the
Presence or Absence of Glycerol (20% vol/vol)

	Glycerol (20% vol/vol) added	
	%*	%*
1 Is	100.	99.9 ± 1
3 Is KCl	74.6 ± 1	74.2 ± 1
3 Is NaCl (dark)	99. ± 1	99. ± 1
3 Is NaCl (bleach)	74.6 ± 1	74.0 ± 1

* Results are expressed as percentage units relative to the dimensions of the outer segment in the standard isosmotic solution in the absence of glycerol.

Liebman, personal communication) have found that neither intact nor isolated rod outer segments can be stained with Procion Yellow, whereas cone outer segments are readily stained. All vertebrate species investigated so far appear to have the same structure (Cohen, 1963). Recently, Nilsson (1965; 1964; see this paper for references to other studies) has made a thorough study of the rod outer segments in the frog retina. The ordered lamellar array of the outer segments has now also been studied by X-ray diffraction techniques (Blaurock and Wilkins, 1969; Gras and Worthington, 1969; Corless, 1972; Webb, 1972) as well as freeze etch (Clark and Branton, 1968; Leeson, 1970), both of which confirm the structure observed in thin sections.

At present, X-ray diffraction analysis appears to be the most reliable method to estimate the absolute dimensions of the membrane structures within the outer segments, particularly since it has now been shown that the intensity distribution of

the diffraction pattern of rod outer segments in the intact, living eye is essentially the same as that in the isolated retina (Webb, 1972). Although some controversy still exists in the detailed interpretation of the diffraction data, in all cases studied so far the major repeat period of the outer segment (the distance between the centers of two successive discs) is observed to be twice the thickness of the disc. Indeed Table I indicates that in a variety of retinas this repeat to disc ratio is nearly 2:1 regardless of the technique used in the investigation. Our observations also indicate that the repeat to disc ratio is 2:1 in isolated outer segments incubated in the standard isosmotic solution, both in the presence and absence of glycerol.

In all solutions used in this study, the discs appear essentially collapsed and an intradisc space is rarely discernable. The same observation has been previously reported in freeze-etch studies of the guinea pig retina (Clark and Branton, 1968). Also, most thin section studies describe the discs as "flattened" or "collapsed" and Dunn and Adomian (1971) report that when intradisc spaces to appear, they probably are the result of poor tissue preservation and post-mortem degeneration. However, a small disc space below the resolution of present electron microscope techniques must exist, since discs are osmotically responsive to both hypoosmotic (DeRobertis and Lasansky, 1961; Heller et al, 1971; Clark and Branton, 1968) and hyperosmotic shocks (Corless, 1972; Dowling, 1967; Blaurock and Wilkins, 1972), and X-ray data indicate that discs can shrink by about 15 Å when subjected to hyperosmotic shocks with sucrose solutions. Our results are also consistent with the presence of a small osmotically active disc volume.

FIGURE 3 Images of isolated rod outer segments shocked with different hyperosmotic solutions in the presence of glycerol (20% vol/vol). These are selected images in which the plane of fracture is nearly perpendicular to the plane of the discs, as revealed by the highly symmetrical image of the discs. (a) Standard isosmotic solution. All discs appear collapsed, although in a few of them the membranes occasionally separate. The repeat distance between successive discs is twice the disc thickness; (b) Hyperosmotic KCl (3 Is) shock. The discs remain collapsed, but they move closer together and the length of the outer segment is reduced; (c) Hyperosmotic NaCl (3 Is) shock of dark-adapted outer segments. The length of the outer segment recovers to nearly its starting value, and the ratio of repeat distance to disc thickness is nearly the same as in the isosmotic solution; (d) Hyperosmotic NaCl (3 Is) shock after flash bleaching of the outer segments. The length of the outer segment is reduced and no recovery ensues. The image is similar to that in the hyperosmotic KCl shock i.e., the discs remain collapsed, but the extra-disc space has been reduced. $\times 120,000$.

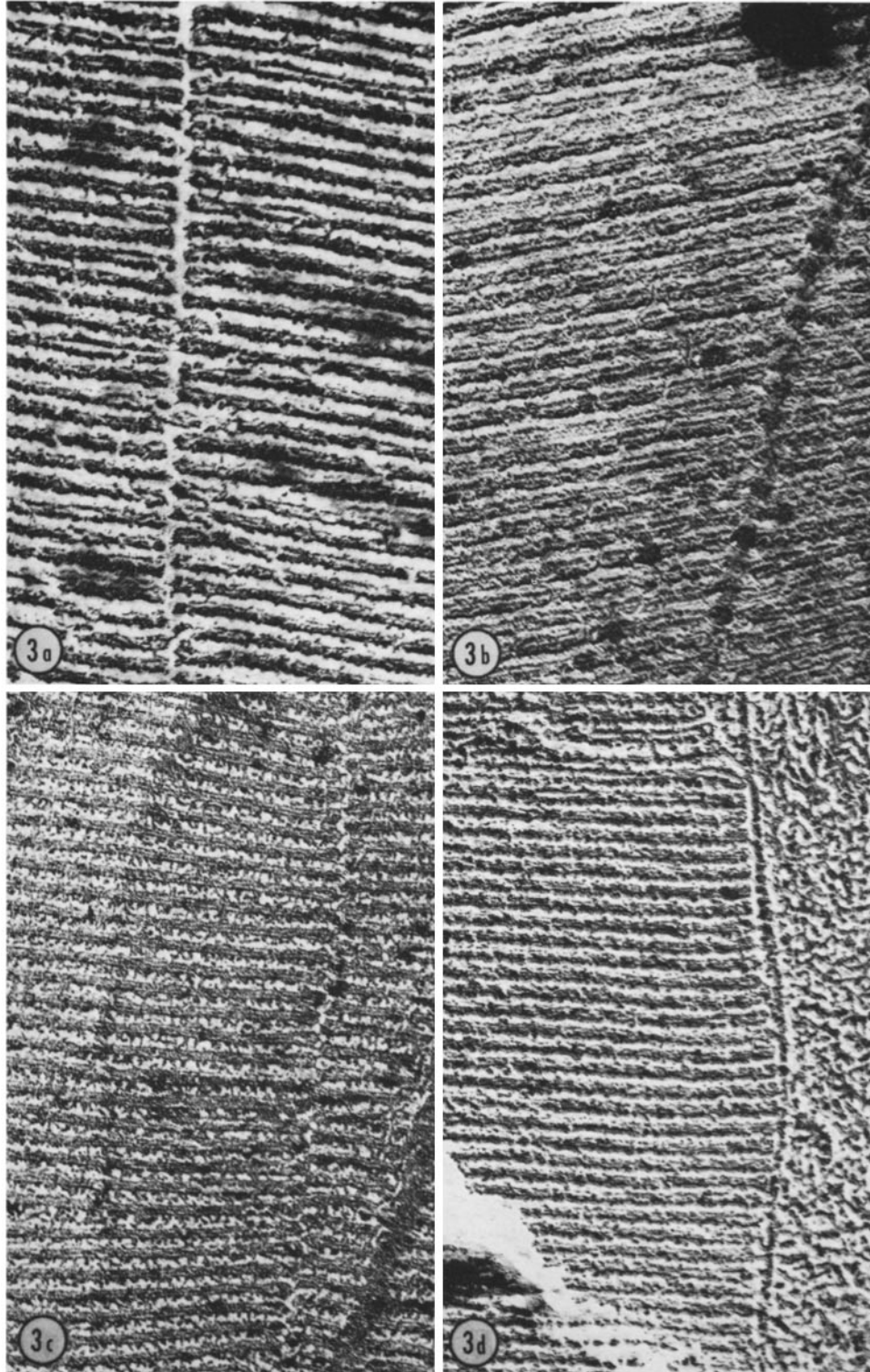


TABLE III
Analysis of Freeze-Etch Images

	Repeat distance ± SEM \AA	Disc thickness ± SEM \AA	Repeat to disc ± SEM (ratio)	n*
1 Is	308 ± 1	154 ± 2	1.99 ± 0.01	48
	300 ± 3	150 ± 2	1.99 ± 0.03	37
	308 ± 1	155 ± 2	1.99 ± 0.03	38
3 Is NaCl (dark)	308 ± 4	154 ± 2	1.99 ± 0.04	32
	299 ± 4	151 ± 3	1.97 ± 0.06	30
	308 ± 2	153 ± 2	1.99 ± 0.02	48
3 Is KCl	227 ± 1	147 ± 2	1.55 ± 0.02	56
	228 ± 1	150 ± 1	1.52 ± 0.01	47
	232 ± 2	150 ± 1	1.53 ± 0.01	39
3 Is NaCl (bleach)	231 ± 1	147 ± 1	1.55 ± 0.01	51
	232 ± 1	151 ± 1	1.52 ± 0.01	44
	232 ± 1	150 ± 1	1.53 ± 0.02	51

The errors presented arise from statistical uncertainty. Systematic errors have been estimated to be no larger than ± 10%.

* Number of individual measurements per image.

Freeze-Etch Observations of Osmotic Behavior

Images of the outer segments in the various solutions tested are presented in Fig. 3 and analysis of these and other images is presented in Table III. It can be seen in Table III that the repeat to disc ratio decreases markedly when the outer segments shrink after hyperosmotic shocks in KCl, and also in NaCl after flash bleaching. In contrast, after dark-adapted outer segments have recovered in length after NaCl shocks, the repeat to disc ratio is the same as that in the isosmotic solution. Despite the many factors which mitigate against making direct comparisons, it can be seen in Table III that the disc thickness is nearly the same in all solutions, but the repeat distance undergoes marked changes. Furthermore, the variability of the measurements is the same under all conditions tested. Since the discs always appear collapsed, the marked changes in the repeat to disc ratio imply that it is the repeat distance which must change. Moreover, on assuming that changes in disc thickness are negligibly small, the magnitude of the change in the repeat to disc ratio observed in the freeze-etch images closely agrees with the changes in outer segment length observed with the light microscope (Table IV).

These observations strongly imply that the changes in length arise primarily from change in the extradisc space. It appears, therefore, that the plasma membrane is the semipermeable barrier responsible for the osmotic behavior described here.

TABLE IV
Equilibrium Length after Osmotic Shock

	Freeze-etch images	Light microscopy
	%*	%*
1 Is	100	100
3 Is NaCl (dark)	100 ± 4	99 ± 1
3 Is KCl	76 ± 3†	74.2 ± 1
3 Is NaCl (bleach)	75 ± 3†	74.0 ± 1

* Results are expressed as percentage units relative to the dimensions in the standard isosmotic solution.

† These values are calculated assuming that disc thickness remains constant. If the discs are assumed instead to contain an osmotically active space 5–10 \AA in thickness, the freeze-etch observations yield the same value observed with light microscopy.

The disc membrane could be responsible for this behavior only if the discs were in some way continuous with the plasma membrane and not free floating. As discussed above this is highly unlikely.

The light-sensitive Na influx and the water flux must therefore occur through the plasma membrane. Since the magnitude of the Na influx and the area of the plasma membrane are known, it is possible to calculate from the data published by Korenbrot and Cone (1972) that the plasma membrane has a Na permeability constant of about 2.8×10^{-6} cm/s. This value is comparable to the permeability constant for cations in other excitable

membranes (Hodgkin and Horowicz, 1959; Hurlbut, 1970). In addition, an upper bound for the osmotic (hydraulic) permeability coefficient of the plasma membrane can be calculated from the rate at which the outer segments shrink when rapidly shocked with hyperosmotic KCl solutions (Korenbrodt and Cone, 1972). For a 3 Is shock the outer segments shrink at a rate in excess of $155 \mu\text{m}^3/\text{s}$, a rate which yields a value of $2 \times 10^{-3} \text{ cm/s}$ for the osmotic permeability coefficient. This value is necessarily a lower bound since in making the calculation the reflection coefficient (σ) for KCl is assumed to be 1, and since no correction is made for unstirred layers.

Thickness of the Disc Membrane

Three methods can now be used to determine the thickness of the disc membrane: X-ray diffraction, freeze-etch images, and osmotic behavior. Analysis of X-ray diffraction patterns may provide a reliable measure of the disc membrane thickness, however at present there is agreement only on the repeat distance being within 10% of 300 \AA , and the thickness of each disc being about 150 \AA —Gras and Worthington (1969) have proposed an asymmetrical membrane which is 75 \AA thick, while Blaurock and Wilkins (1969) have proposed a membrane which is symmetrical and 55 \AA thick. Osmotic swelling and shrinking of intact outer segment (Corless, 1972; Blaurock and Wilkins, 1972) has recently been used to corroborate the original phase assignment made by Blaurock and Wilkins in their interpretation of the diffraction pattern. However, Blaurock and Wilkins based their thickness estimate of 55 \AA on the percent solid content of the outer segments. The estimate is thus for an equivalent "dry" membrane which should be somewhat too thin since the entire water content of the outer segment is taken to be outside the structure of the membrane.

The osmotic response of intact outer segments to various hyperosmotic sucrose solutions has been studied by changes in the X-ray diffraction pattern (Corless, 1972; Blaurock and Wilkins, 1972). The plasma membrane appears permeable to sucrose since only small changes are detected in the repeat period. On the other hand, the disc membrane appears impermeable to sucrose since the thickness of the disc decreases with increasing sucrose concentration. The decrease in disc thickness suggests a maximum intradisc space of $15\text{--}20 \text{ \AA}$.

Korenbrodt and Cone (1972) reported measure-

ments of length, width, and volume of isolated outer segments as a function of osmotic pressure. As the osmotic pressure increases, the length of the outer segment decreases, and finally reaches a minimum at $52 \pm 2\%$ of the original length. Beyond this point, large increments in osmotic pressure fail to further shorten the outer segment. This result implies that $52 \pm 2\%$ of the repeat period is effectively occupied by the disc structure. Therefore, since the normal repeat period is $300 \pm 10 \text{ \AA}$, each disc can be no thicker than $156 \pm 5 \text{ \AA}$, and the thickness of the disc membrane cannot exceed $78 \pm 3 \text{ \AA}$. This should be somewhat too thick since the measurement includes osmotically inactive cytoplasmic matrix.

In our freeze-etch studies we found the repeat to disc ratio in the standard isosmotic solution to be 1.99 ± 0.19 . For a normal repeat period of $300 \pm 10 \text{ \AA}$ this implies that each disc is $151 \pm 15 \text{ \AA}$, and, if we account for an intradisc space of $15\text{--}20 \text{ \AA}$, the disc membrane is $67 \pm 7 \text{ \AA}$ thick. Therefore, the electron density profile, the freeze-fracture surfaces, and the osmotic compression of outer segments all yield nearly the same dimension for the thickness of the disc membrane.

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