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X-RAY DIFFRACTION STUDIES OF RETINAL RODS
II. LIGHT EFFECT ON THE OSMOTIC PROPERTIES

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SUMMARY

A fast X-ray diffraction technique has been used to study the osmotic reaction of frog rod outer segments to bleaching and to changes in the osmolarity and composition of the outer medium. Dissected excitable retina and isolated outer segments have been used.

Upon bleaching in isotonic Ringer only a small transient diminution of the disc repeat distance is observed in isolated rods. Disorder and slow swelling reactions are also observed in the intact cells. In calcium-free Ringer the light-induced shrinkage is considerably enhanced. In the intact cell this is interpretable as due to the switching off of a large sodium dark current. The persistence of the effect in isolated outer segment suggests the existence of an active ionic efflux from this part of the cell.

Upon hypotonic shock, bleached rods swell more than dark-adapted ones. The difference, however, appears only in a slow component of the osmotic kinetics, a few minutes after the shock.

Upon hypertonic shock, part of the rods, even in the "intact" excitable retina, appear to be leaking. Those cells which are intact are impermeable to all the solutes added to increase the osmolarity: NaCl, KCl, Sucrose, Melezitose. No light dependence of the response to a hyperosmotic NaCl shock is detectable.

The discs are osmotically reactive, even when the outer cell membrane of the rods is leaking. Assuming the discs to be perfect osmometers, a thickness of 20 ± 5 Å is estimated for the liquid layer inside the discs.

INTRODUCTION

In the preceding paper [1], results obtained by the fast X-ray diffraction technique on the structure of the retinal rod disc membrane were presented. Information

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related more to membrane permeability than to structure is also contained in the lamellar diffraction: the spacing of the diffraction peaks gives the disc spacing directly; from the peak intensities one can calculate the autocorrelation function. Its interpretation allows the disc spacing to be analysed into an internal disc thickness and a cytoplasmic thickness. One is then able to measure the thickness of the different compartments within the rod and taking advantage of our fast technique, to study their evolution upon bleaching and upon osmotic changes in the external medium.

There is evidence for a dark ionic current flowing inward at the outer segment in intact rods [2] and this is shut off by light and the cell membrane is hyperpolarised [3]. Osmotic measurements on isolated outer segments, by microscopic observation, have been interpreted as showing that the light-sensitive current is of sodium ions [4].

In an earlier report of X-ray work on the intact retina [5], a shrinkage observed upon bleaching was interpreted to be due to switching off of the Na^+ current. The effect was considerably enhanced in Ca^{++}-free medium. This was in agreement with the calcium transmitter hypothesis of Hagins [6], but does not prove this point. Responses to osmotic shocks, not reported, differed from those observed by microscopy on isolated outer segments [4]. As the difference could be due to the unknown reaction of the inner segment in the intact cell, our X-ray measurements have been extended to a preparation of isolated outer segments oriented in a magnetic field. We present here this study of the osmotic behavior of the outer cell membrane and of the disc membranes of the retinal rods, as observed by X-ray diffraction on intact cells and on isolated outer segments.

MATERIAL AND METHODS

The preparation of frog (*Rana esculenta*) intact retina is described in ref. 5. The preparation of isolated frog rod outer segments, the orientation procedure and the X-ray techniques are described in the preceding paper [1].

RESULTS AND DISCUSSION

Diffraction spectra are shown in the preceding paper [1]. A relative accuracy of 0.5 % on the peaks positions, i.e. on the disc spacing, was obtainable in 10–30 s of counting. In isotonic Ringer at room temperature in the dark, the lattice spacing was 295 ± 3 Å in the intact retina, it varied from 295 to 310 Å, depending on the preparation, for isolated rods. For both kinds of samples this spacing remained stable for up to 1h, if the temperature was kept stable.

The decomposition of the total disc spacing in its intradiscal and cytoplasmic components relies on the interpretation of the Patterson autocorrelation function, which is the Fourier transformation of the diffraction peaks intensity. This interpretation, first proposed by Blaurock and Wilkins [7], is shown in Fig. 1.

Effects of total bleaching in isotonic Ringer

In the intact retina, upon bleaching in isotonic Ringer, an instantaneous shrinkage by about 0.5 % was observed: it was followed by a slow swelling reaching 2 % in the next hour. Furthermore a temporary broadening of the peaks indicated the occurrence of a long range disorder for a few minutes after the bleaching.

In isolated rods, only the transient shrinkage subsisted, and it was at the limit

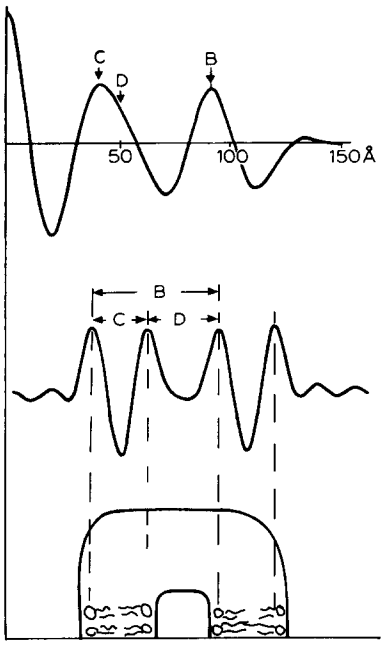


Fig. 1. Patterson autocorrelation function and its interpretation. The upper curve is the Patterson function calculated from the intensities of the 10 first orders of lamellar diffraction. The lower curve is the corresponding electron density profile of a disc, which is sketched below. Arrows B, C and D on the Patterson correspond to the vectors indicated on the electron density profile. When a disc swells, B and D shift and C remains constant. The contributions of C and D are not resolved in the first peak of the Patterson, but D contributes only about 20 % of the amplitude of this peak at its maximum at 42 Å, in isotonic conditions.

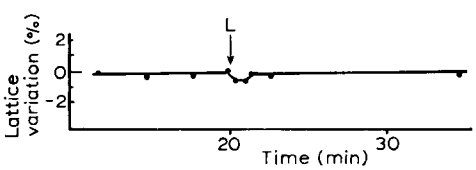


Fig. 2. Effect of a strong flash on the disc spacing in isolated rods in isotonic Ringer. The transient shrinkage observed is at the limit of the sensitivity for one measurement, but is reproducible. Contrary to what is observed on the intact retina [5], there is no slow swelling. The origin of the time scale is the instant of separation of the rods from the retina.

of our sensitivity (Fig. 2). The broadening of the peaks was much less important, sometimes barely noticeable and the disc spacing remained stable for the following hour. This indicated that long range disorder and slow swelling resulted from osmotic readjustments with the inner segment of the cell after the large perturbation caused by the simultaneous bleaching of the 10^9 rhodopsin molecules within one cell. The only reaction directly related to the photic excitation is probably the small transient shrinkage.

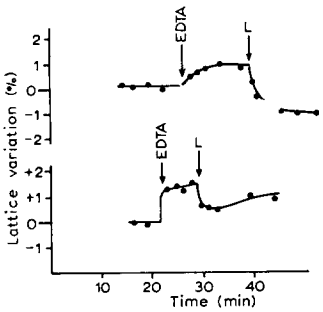


Fig. 3. Effect of illumination on isolated rods in the absence of calcium. EDTA indicates the time of replacement of the Ringer solution by a solution of the same composition except for the absence of calcium and the addition of 0.5 mM of EDTA. L indicates the instant of illuminations. Due to absorption in the samples, the amount of bleaching was not measurable on these preparation. It was close to 100 % for the two cases shown here. Two curves are shown to demonstrate the variability of the response.

Effects of illumination in low-calcium isotonic Ringer

We have shown earlier that the total removal of calcium from the external solution and the addition of EDTA induced a swelling of the rods in the intact retina. Subsequent illumination triggered a shrinkage of the cell toward its original length, the extent of the effect being proportional to the amount of light; it was still detectable when bleaching only 1 % of the rhodopsin [5].

Quite similar although smaller effects are observed on isolated rods (Fig. 3). It is not clear whether the swelling observed when changing from the normal Ringer to the calcium-free solution, was instantaneous or progressive: changing the liquid perturbed the diffraction for 1 to 2 min. After a few minutes the lattice seemed to stabilise at a value about 1 % larger than initially measured. The light-triggered shrinkage in some cases overcompensated the swelling. The phenomenon did not require total bleaching, but no quantification was attempted as for the intact retina, where repeated measurements could be made on a given sample. On the isolated rods, the effect was observed up to 40 min after the rods had been detached from the retina.

Our interpretation of the effect observed in the intact retina [5] did not provide an explanation for its persistence in the isolated rods. It seems too large to be a residual effect due to the few rods which still carry a small part of the inner segment. The simplest expanation would be the existence of a small active efflux of sodium, or another ion from the outer segment itself. But disc spacing depends probably also on their net charge, which may vary upon bleaching. The regularity of the structure might also be controlled by intracellular polysaccharides or even longitudinal fibrillar structures sometimes observed in the rim of the discs [8]. These structures might be sensitive to perturbations of the intracellular medium following the bleaching, or to the calcium concentration.

Response to hypotonic shocks

Hypotonic shocks have been studied only on the intact retina. When changing the perfusing Ringer for Ringer diluted with distilled water, the shock induced a fast lattice change which was completed in the first minute (Fig. 4). This fast component

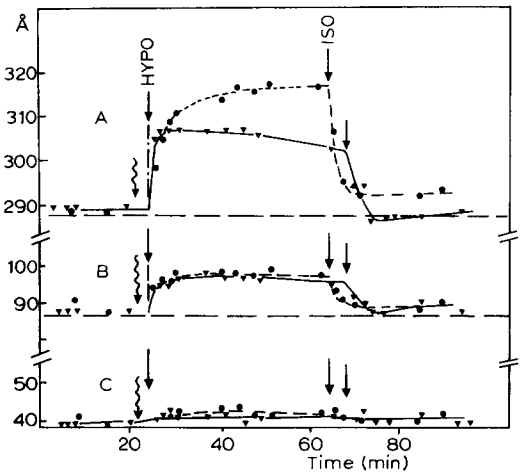


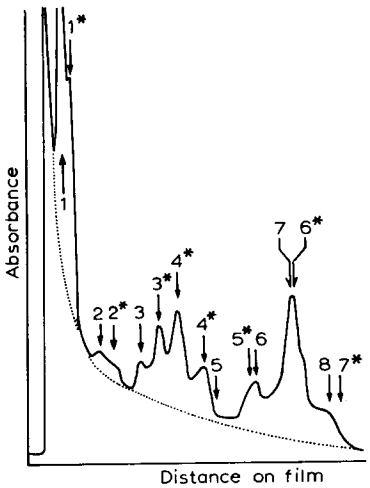
Fig. 4. Hypotonic shock on isolated retina. —, Dark-adapted retina; - - -, bleached retina. The bleaching is performed just before the shock (wavy arrow). The hypotonic solution is a standard Ringer diluted by the addition of 33 % distilled water. The two shocks have been performed successively on the same retina and the curve shifted to compare them better. Checks have been made that two successive shocks on a dark-adapted retina give reproducible results. (A) Lattice change. (B) Variation of the disc thickness as given by the variation of the second peak of the Patterson (see Fig. 1). (C) Bilayer thickness as given by the position of the first peak of the Patterson. The difference observed upon bleaching in (A) does not appear in (B) indicating that this slow component of swelling is extradiscal. The slight shifts observed in (C) are due to the small contribution of intermembrane correlation (Vector D in Fig. 1), to the first peak of the Patterson.

was identical for a dark-adapted retina and for a retina which had been strongly illuminated just before the shock; the difference only became apparent later. The lattice of the dark-adapted rods had a plateau followed by a slow return and that of the bleached ones a slow swelling for the next 10–20 min. With dilution to 33 % water, the shock was harmless for the dark-adapted retina, which recovered their original disc spacing and electroretinogram when the perfusion was switched back to Ringer. The different swelling of dark-adapted and bleached rods is entirely confined to the cytoplasmic space, the discs swelling by the same amount in both cases. It may be due to a difference in the outer cell membrane permeability. But the fact that it appeared only slowly and under unrealistic illumination makes it very improbable that it could be directly related to the photic excitation. It is probably of the same nature as the slow swelling observed after bleaching in isotonic Ringer. No effect is observed with low intensity flashes which, however, give saturating electroretinograms.

The swelling of the discs, independent of their bleaching, is quite significant: assuming them to be perfect osmometers one calculates a thickness of 20 ± 5 Å for the liquid layer inside the disc.

Response to hypertonic shocks

On the isolated retina, the response of the lamellar diffraction to a hyperosmotic shock was complex: within a few minutes after the shock a composite pattern which could be analysed as the superposition of two compressed lamellar patterns with different lattices could be observed (Figs 5 and 6). The two sets of diffraction



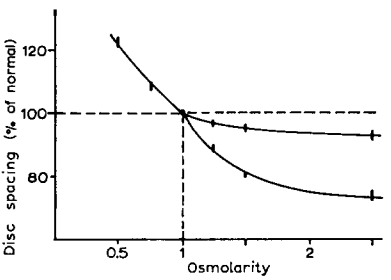


Fig. 5. Lamellar diffraction of isolated retina in hypertonic solution. The osmolarity has been increased to 150 % of normal by the addition of sucrose to the Ringer. Densitometer tracing of a film, exposure time 100 min. A film has been chosen to demonstrate the stability of the shrinkage. Similar spectra are obtained when adding NaCl, KCl or Melezitose in comparable amounts to the Ringer solution, and the smaller lattice remains stable over long periods even when adding NaCl on a dark-adapted retina.

Fig. 6. Variation of disc spacing with osmolarity of the external solution, in isolated retina. Osmolarity units are relative to the standard Ringer solution. For the hyperosmotic solutions, the two curves correspond to the two lattices observed.

peaks were sharp, indicative of two distinct but homogeneous classes of diffracting elements. This spectrum remained stable and converted back to the initial spectrum when the perfusion was switched back to Ringer. The phenomenon seemed qualitatively independent of the type of solute added to the Ringer to increase the osmolarity: NaCl, KCl, sucrose and melezitose, when added in corresponding amounts, gave the same two lattices, although with a certain variability as to the relative contributions of the two lattices to the composite pattern. No difference was ever observed between dark-adapted and illuminated retinas. The overlap of orders 5 and 6 of the smaller lattice with orders 6 and 7 of the other makes the Fourier analysis unreliable. But the fact that on the total spectrum, the diffraction intensity seemed systematically minimal at reciprocal distances where the Fourier transform of a normal disc has zeros, indicated that in both lattices the disc had the same structure, which was very close to the normal one. Our interpretation is that part of the cells had their outer membrane leaking and the small compression was entirely due to the shrinkage of the discs that have remained intact. Those cells which are impermeable reacted like an osmometer and shrank by a greater amount.

These observations on the intact retina do not agree with the results of Korenbrot and Cone [4] on isolated outer segments. They have observed that illuminated outer segments remain contracted for long periods after a hyperosmotic shock induced by an excess of NaCl, but that dark-adapted ones rapidly recover their length. Our experiments were, therefore, repeated on isolated outer segments. The

same two-lattice system was observed as in the intact retina and again no recovery in the dark and no effect of light on the osmotic properties were observed. To make sure that the X-rays were not stimulating the rods, NaCl hyperosmotic shocks were induced in the dark before exposing the rods to any X-rays, and the X-rays were switched on only 5 min after the shock. This would have left enough time for the dark-adapted rods to recover their initial length, but the diffraction still showed the persistence of the compressed lattice.

It is possible that our technique is not adequate, the intracellular volume is not directly measured. After an initial shrinkage, the cells might have swelled back without showing any change in the lattice. This would imply that the discs remain regularly compressed and that the additional water that has entered the cell has been inserted irregularly at one end or at few places between stacks of discs. This is, however, not in agreement with the electron microscopic observation of Korenbrot et al. [9].

Systematic controls by optical microscopy showed that, in our hands, dark-adapted rods do not recover their length after a hyperosmotic shock, even when the shock is made within 30 s after the detachment of the rods from the retina. Recently Cobbs and Hagins [10] have also failed to detect any recovery after a hypertonic NaCl shock in the dark. By a fluorescence probe technique [11], the presence of leaking rods in isolated rod preparations has been detected. Using this technique, around 30 % of the rods in our preparations are found to be leaking.

CONCLUSIONS

By working on isolated outer segments, the osmotic response of the rod to illumination has been isolated from the reactions due to the interaction with the inner segment. The only remaining response is a small transient diminution of the disc repeat distance. After removal of external calcium, a more substantial light-induced shinkage occurs, the phenomenon remaining, however, smaller than in the intact retina. This might imply the existence of an active, light-stimulated, ionic efflux from the outer segment itself. This is not considered in Hagins' scheme of the ionic fluxes in the rods [6]. (See Note Added in Proof.)

The osmotic shock studies suggested that in our "intact" retina, which elicit electroretinograms of reasonable amplitude, a sizeable part of the rods are leaking, although they are not detached from the retina, or even disoriented: the two sets of diffraction lines corresponding to impermeable and leaking cells have the same degree of orientation. This occurs even when the retina is covered with the pigment epithelium layer. Impermeable and leaking rods have exactly the same disc spacing in isoosmotic medium.

The outer cell membrane of the rods is found to be impermeable to all the solute tried: NaCl, KCl, sucrose, melezitose. No light-dependence of the response to hyperosmotic NaCl shocks has been observed, contrary to the finding of Korenbrot and Cone [4], but in agreement with Cobbs and Hagins [10]. The origin of the discrepancy is not understood, but our results do not contradict the hypothesis of a light-modulated Na^+ current, since it could be explained by the impermeability of the membrane to Cl^- [11].

The disc membrane, whether it is dark-adapted or bleached, is impermeable

to NaCl, sucrose and melezitose. The osmotically active intradiscal volume corresponds to a liquid thickness of 20 ± 5 Å. Our fast X-ray diffraction method seems to be the best technique to study the osmotic properties of such small compartments, not accessible to light microscopy.

NOTE ADDED IN PROOF (received January 29th, 1975)

From an analysis of the external currents, R. Zuckerman [12] has also reached the conclusion that part of the electrogenic sodium pump is localised in the base of the outher segment.

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