Calcium Flux across Disk Membranes

Studies with Intact Rod Photoreceptors and Purified Disks

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ABSTRACT Calcium accumulation by rod disks was studied in excised bullfrog retinas with $^{45}$Ca tracer-exchange methods. Ca uptake by disks is a necessary requirement if light-induced Ca releases from disks mediate photoreceptor excitation. In an hour-long incubation, disks exchanged $\leq 0.01$ mole of Ca per mole of rhodopsin, or $\leq 10\%$ of their total Ca. This corresponds to a unidirectional flux of $\leq 0.01$ fmol/cm$^2$ s, or $\leq 5$ ions/disk-second across the disk membrane. Neither incubation in 10 mM Ca (which increases cytoplasmic activity 10–100-fold) nor photostimulation (which photoactivated up to 50% rhodopsin/h) had measurable effect on exchange rate, though an increase of several orders of magnitude would have been expected according to the hypothesis. The observed exchange could not be explained by: (a) $^{45}$Ca losses from disks before measurement (neither the net efflux nor the Ca-Ca exchange property of disks adequately explains such losses), (b) a limited pool of exchangeable Ca from strongly binding intradiskal sites, or (c) rate-limiting flux across the plasma membrane during incubation. For the study of the Ca efflux properties of disks, separate experiments were performed with $^{45}$Ca-loaded disks. Intradiskal activity could be estimated from the disks’ hyperosmotically sensitive $^{45}$Ca pool and from their intradiskal volume (indirectly assayed by density). Ca-Ca exchange was undetectable ($\leq 0.1$ fmol/cm$^2$ s) in disks whose intradiskal activity was at least 0.3 mM. Net efflux was 0.2 fmol/cm$^2$ s for an intradiskal activity of $\sim 1$ mM and is comparable to published fluxes for phospholipid vesicles. These results seem to exclude the internal space of disks as the source of Ca for photoreceptor excitation.

INTRODUCTION

In response to a light stimulus, photoactivated rhodopsin initiates a set of intracellular reactions that hyperpolarize vertebrate photoreceptors. The nature of these reactions is not known, although it now appears that both calcium and cyclic GMP may be involved. A possible scheme, involving Ca,
was postulated for these reactions some time ago (Yoshikami and Hagins, 1970; Hagins, 1972). According to this Ca hypothesis, dark-adapted rod photoreceptors maintain a low cytoplasmic Ca activity by actively pumping cytoplasmic Ca into the disks, where Ca activity is maintained at a relatively high level. The hypothesis proposes that photoactivated rhodopsin releases intradiskal Ca into the cytoplasm. The increased cytoplasmic Ca activity blocks the Na channels of the plasma membrane, resulting in a hyperpolarization. The voltage response of the cell then decays as the excess cytoplasmic Ca is lowered by reaccumulation into the disks.

Some aspects of the hypothesis have been verified. Light-regulated Na channels are highly sensitive to cytoplasmic Ca (Hagins and Yoshikami, 1974; Wormington and Cone, 1978) and, as shown by several investigators, disks contain more than adequate amounts of Ca. In an extensive study, total Ca (both free and bound) within disks was measured to be about 0.13 mole of Ca per mole of rhodopsin (Ca/rho) (Szuts and Cone, 1977), which is equivalent to a concentration of about 5 mM. Similar results have also been reported by others: ~0.20 Ca/rho (Liebman, 1974), 0.25 Ca/rho (Hess, 1975), and 0.1–0.2 Ca/rho (Noell et al., 1979). In contrast to all these measurements of total Ca, Ca activity within disks has not yet been determined.

Two important aspects of the hypothesis, namely light-induced Ca release from disks and active Ca uptake by disks (a necessary requirement if light-induced releases are to occur) have not yet been verified. Many investigators have tried to measure light-induced Ca releases, but these releases are either undetectable (Sorbi and Cavaggioni, 1975; Szuts and Cone, 1977; Liebman, 1978) or their stoichiometry and rate are two to three orders of magnitude too small (Smith et al., 1977). The absence of the predicted light effect in these experiments is unfortunately difficult to interpret because the results could be attributed to the nonphysiological state of the isolated or fragmented outer segment preparations. Large Ca releases have been observed in some model membrane experiments (review of Montal, 1979; O'Brien, 1979)—which, of course, are also conducted under nonphysiological conditions—but, the results of these experiments are highly variable. For example, Antanavage et al. (1977) observed light-induced proton but no light-induced Ca movements.

Only two reports have been published on the Ca accumulation process of disks (Bownds et al., 1971; Schnetkamp et al., 1977). Although both of them report an ATP-dependent Ca uptake, the reproducibility of these observations has recently been questioned (Schnetkamp, 1979). It is unclear whether this poor reproducibility results from the nonphysiological state of the preparations or from inappropriate assay conditions. Credence is given to the second possibility by the observation of Robinson and Hagins (1979) that light modulates the levels of GTP but not of ATP in isolated outer segments. Indeed, these authors conclude that active Ca uptake by disks is a GTP-rather than an ATP-dependent process. In summary, neither the presence of a light-induced Ca release nor the presence of an active Ca-uptake has yet been verified for rod disks.

The aim of the study reported in this paper was to measure Ca uptake by
disks with tracer-exchange methods. The advantage of this method is that, by incubating frog retinas with saline solutions that contain trace amounts of 45Ca, photoreceptors and their disks are capable of accumulating 45Ca under physiological conditions. Accumulation occurs, because extracellular Ca (40Ca and 45Ca) exchanges with the intracellular Ca in the cytoplasmic and intradiscal compartments. The rate of exchange is set by the magnitude of unidirectional fluxes across the respective membranes. Unidirectional fluxes commonly exist for cell membranes across which a steady activity gradient is maintained; for example, photoreceptors sustain their low cytoplasmic Ca activity, which is three to four orders of magnitude lower than in the external medium, by compensating for the passive unidirectional influx with an equal but opposite energy-dependent unidirectional efflux. To measure intradiscal 45Ca accumulated by the rods during the incubations, the experimental procedure that is selected should (a) involve a sample preparation protocol that causes little or no 45Ca losses from disks before measurement, and (b) utilize a sufficiently sensitive 45Ca detection technique. The procedure employed here apparently meets both of these criteria. It combines an isolation and purification procedure (during which outer segments were sheared off the retina and the disks were separated from contaminants) with liquid scintillation techniques. Although the sample preparation for counting is notably lengthy, intradiscal 45Ca losses (either by net efflux or by Ca-Ca exchange diffusion) were apparently small or negligible, as determined by previously published measurements of the total Ca content of prepared disks, and by separate experiments, performed in this study, in which the efflux properties of 45Ca-loaded disks were measured.

The results of this study imply that unidirectional flux across disk membranes and, consequently, Ca exchange between cytoplasmic and intradiscal compartments is much less than predicted by the 45Ca hypothesis.

**MATERIALS AND METHODS**

Bullfrogs (*Rana catesbeiana*), about 6 inches in body length, were purchased from J. Weil Co. or Acadian Biological Supply Co (both suppliers in Rayne, La.). The animals were kept in a tank through which a steady stream of tap water flowed and were exposed to natural daylight illumination. The frogs were dark-adapted for at least 2 h before decapitation.

The composition and nomenclature of the various saline solutions are listed in Table I. All solutions had a pH between 7.2 and 7.4. Calcium was added to the complete saline after a 95% O2-5% CO2 gas mixture was bubbled through it for at least 5 min (the time needed to achieve pH 7.4 equilibrium) before each experiment. Measurements with a Ca electrode (Model 92-20, Orion Research Inc., Cambridge, Mass.) confirmed that the rate of CaCO3 precipitation was so slow that free Ca did...
not substantially change over several hours at pH 7.4. For an initial 10 mM Ca activity, about a day was needed for the activity to decrease to 5 mM.

**Experiments with 45Ca-incubated Retinas**

Six to eight retinas were dissected for each experiment. Retinas were isolated in stock saline and then transferred to complete saline, in which they were stored as the remaining retinas were being dissected. The tissues were separated into two sets of three to four retinas, and each set was placed into a 20-ml vial that contained 3 ml of complete saline and ~100 μCi of 45CaCl₂. The Ca concentration of the solution was increased by <50 μM upon the addition of 45Ca. Each vial was capped and placed into a light-tight container on top of a wrist shaker. When light effects were studied, one set of retinas served as control (i.e., was kept in total darkness), while the other set (containing the contralateral retinas of the control) was exposed to light flashes. The retinas were incubated for 1 h at room temperature (20–25°C). Throughout the incubation, the retinas were gently agitated by the shaker, and the vials were continuously perfused with the 95% O₂-5% CO₂ mixture (flow rate set at 28 liter/h).

The incubation was terminated by removing the retinas with forceps and transferring them to a test tube containing 2 ml of ice-cold stock saline. All subsequent steps used for the isolation and for the purification of the fragmented rod outer segments (FROS) were performed with solutions and containers that were kept cold on ice. FROS were isolated by mildly vortexing the tube of retinas for 1 min. The vortexing was gentle enough to minimize foaming but was sufficiently vigorous to fragment the outer segments and to make the plasma membranes of all the fragments leaky. The degree of leakiness was measured with the didansylcystine assay (Yoshikami et al., 1974). Although vortexing increased the leakiness of plasma membranes, it is unlikely that it released intradiskal ions; disks retain large cation concentrations (~40 mM Na, 80 mM K, 5 mM Ca, and 8 mM Mg) in spite of a procedure that combines vigorous stirring with a 10-fold hypotonic shock (Szuts, 1975; Szuts and Cone, 1977). The mild vortexing did not dissociate the retinas. The vortexed suspensions were filtered through separate nylon meshes (37-μm opening), yielding two 2-ml filtered samples, which will be referred to as the “unpurified FROS homogenate.” Unless light effects were studied, the two homogenates were pooled.

In a few experiments, purification of the FROS was kept to a minimum by layering the unpurified FROS homogenate on a continuous metrizamide gradient for isopycnic

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**Table I**

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<td><strong>Nomenclature</strong></td>
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* As described in the text, calcium was sometimes added to these solutions.
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centrifugation. The linear gradient was prepared with a device that mixed 5 ml of 25% (wt/wt) metrizamide (dissolved in water) with 5 ml of 5% (wt/wt) metrizamide (dissolved in stock saline). The gradient was layered over a cushion of 0.5 ml 50% (wt/wt) metrizamide (dissolved in water). The osmolarity of all solutions was measured with a Vapor Pressure Osmometer (Model 5100B, S. Wescor Inc., Logan, Utah). Because the 25 and 5% metrizamide solutions were 250 and 270 mosM, respectively, the linear gradient was nearly isotonic throughout its range and, moreover, was nearly isotonic with the physiological saline solutions, which were measured and found to be ~220 mosM. The gradients were centrifuged for 30 min at 160,000 g (2°C) in a Beckman SW 40 swinging bucket rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). Even though FROS reach their equilibrium density in a few minutes at these forces, the longer centrifugation period was needed to adequately separate mitochondria and lysosomes from disks. The tube bottoms were pierced and 0.7-ml fractions were collected at room temperature. The fractions were then assayed for rhodopsin, protein, and enzyme activity and for radioactivity.

In most of the experiments, the FROS were purified by two successive differential centrifugations, in which the FROS were initially floated and then pelleted. To minimize the hypertonic effect, metrizamide instead of sucrose was used, although improved purity was achieved with the sucrose method, as will be shown later. For the metrizamide procedure, each of the 2-ml unpurified FROS homogenates was mixed with 8 ml of 26.6% (wt/wt) metrizamide (dissolved in water) to yield a final 22% (wt/wt) metrizamide concentration. The mixtures were transferred to centrifuge tubes and 2 ml of 16% (wt/wt) metrizamide (dissolved in stock saline that had been diluted fivefold with water) was layered over them. After centrifugation for 30 min at 111,000 g (SW 40 rotor, 2°C), the carpets of FROS were separately collected in 1-ml aliquots (with a 1-ml syringe with a 6-inch-long 18G needle, blunted tip), and each of the aliquots was separately mixed with 8 ml of 16% metrizamide (final concentration, ~16% metrizamide). The FROS were then pelleted by centrifugation for 20 min at 40,000 g (SW 40 rotor, 2°C). After the supernates were decanted, the two pellets were resuspended in 1 ml of stock saline, and aliquots were removed for the assay of rhodopsin and 45Ca. This same metrizamide procedure was employed in those experiments in which the activity of contaminating enzymes was studied with successive purification.

Experiments with 46Ca-loaded FROS

For each experiment, six to eight retinas were isolated in stock saline, and the outer segments were isolated and filtered as described above. In most of the experiments, the successive differential centrifugations were performed with sucrose solutions because improved purity could be achieved with them. The 2-ml unpurified FROS homogenate was mixed with 8 ml of 38.8% (wt/wt) sucrose (dissolved in stock saline) for a final concentration of 33% (wt/wt) sucrose. 2 ml of 20% (wt/wt) sucrose (dissolved in stock saline) was layered over the mixture, and this step gradient was centrifuged for 30 min at 111,000 g (SW 40 rotor, 2°C). The carpets of FROS were withdrawn in 1-ml aliquots as described above and were mixed with 8 ml of 20% (wt/ wt) sucrose. Centrifugation for 15 min at 18,000 g (SW 40 rotor, 2°C) formed a pellet of FROS, which was resuspended in stock saline. The purified disks were freshly prepared for each experiment. The purification procedure lasted 2–3 h from the vortexing step and provided a yield of ~50 nmol of rhodopsin.

Disks were passively loaded with 46Ca by diluting the purified FROS suspension with stock saline, to which 5 mM CaCl2, 1–5 μCi 46Ca was added. Mild agitation was maintained throughout the incubation, which was at room temperature and lasted 30
min. To ensure identical specific radioactivity inside and outside of disks, 1–10 μM X537A was often added to the loading medium. Loading was terminated by chilling the suspension of loaded disks. If the disks were exposed to the ionophore, 1.5% (wt/wt) bovine serum albumin (BSA) (final pH, 5.5) was added to the suspension at the end of the incubation to remove the ionophore from the disk membranes. Albumin was contaminated with Ca, so that the total Ca concentration of 1.5% albumin solutions was found to be ~0.15 mM, as determined by measurements with the Ca-sensitive dye, Arsenazo III. Albumin addition diluted the Ca concentration of the disk suspension by 6% and decreased 45Ca specific activity <3%.

Two separate procedures were used for measuring 45Ca within 46Ca-loaded disks: gradient centrifugation and filtration. For gradient centrifugation, the chilled suspension of loaded disks was directly placed on a chilled linear density gradient (either sucrose or metrizamide) and centrifuged for 30 min at 111,000 g (SW 40 rotor, 2°C). Fractions were collected as described above, and the distributions of 45Ca and rhodopsin were measured. For the filtration procedure, the chilled suspension of loaded disks was layered over 8 ml of chilled 3% (wt/wt) albumin (dissolved in stock saline, final pH, either 5.4 or 7.0). The FROS were pelleted by centrifugation for 20 min at 18,000 g (SW 40 rotor, 2°C). The 1.5% albumin layer was carefully removed with a pipette, the lower phase was decanted, and the inside of the tube was wiped off before the pelleted FROS were resuspended in 2–3 ml of test solution, usually stock saline with a final pH of 7.0–7.4. Efflux of 46Ca from these artificially loaded disks was then measured by filtration. When incubations were performed without X537A, the loaded FROS were not exposed to albumin. The suspension was layered onto 3% (wt/wt) metrizamide (dissolved in stock saline) for final pelleting.

**Filtrations and Assays**

For the measurement of 46Ca sequestered by disks or by other organelles, aliquots were removed from the FROS suspensions and filtered by suction through MF-Millipore filters (Type HA, 0.45-μm pore size) on a sampling Manifold (Model 1225, Millipore Corp., Bedford, Mass.). The wet filters were rinsed with 10 ml of solution before and after sample addition. Composition and temperature of the rinse solution were identical to the test solution with which the loaded disks were resuspended. The only exception to this was when the suspending medium would have reduced flow rates, as with choline-containing solutions or with concentrated solutions of sucrose, metrizamide, or BSA. If choline was in the medium, Tris saline was used for rinsing. If the medium contained 34% sucrose, 21 or 34% metrizamide, or 25% BSA, the aliquots were first diluted 10-fold with the appropriate saline and then filtered and rinsed with the same saline solution. In these experiments, control samples also went through the same dilution procedures. The filtration time of the samples, from their introduction into the well until the completion of the postrinse, was 15–45 s and depended on the amount of sample material.

With the filtration protocol described above, filters were tested for their ability to retain disks but not 46Ca. For measuring disk retention, material on the filters was solubilized with 2% Triton X-100. Virtually all the FROS, or >96% of the initial rhodopsin, was retained by the filters. When 46Ca solutions were filtered, as much as 0.02% of the initial radioactivity was found on the filters. So that this radioactivity remained <20 cpm, which was the background count on the scintillation counter, the initial activity of the samples that were to be filtered was always adjusted to be <105 cpm. The adjustment was usually made by varying the filtration volumes.

The effect of filtration on the 46Ca content of disks was tested by comparing the contents of filtered and centrifuged disks. Centrifugation was in the presence of
tritiated polyethylene glycol (4,000 mol wt). Because membranes are impermeable to this molecule, it was used to measure extradiskal space so that extradiskal $^{45}\text{Ca}$ could be subtracted from the total $^{45}\text{Ca}$ of the pellet. Assuming that neither extradiskal $^{45}\text{Ca}$ nor polyethylene glycol was bound by the membranes, the $^{45}\text{Ca}$ content of centrifuged disks was found to be only 30–50% greater than that of filtered disks. Given the assumptions, the two measurements yield comparable results. Thus, filtration per se does not appear to cause Ca losses from disks.

Aliquots from gradient fractions were mixed with water and Aquasol (New England Nuclear, Boston, Mass.) for the measurement of radioactivity. Aquasol was also used for Millipore filters, which were initially dissolved in 1 ml of 2-ethoxyethanol. $^{45}\text{Ca}$ activity was measured in a liquid scintillation counter (Model 3330, Packard Instrument Co., Inc., Downers Grove, Ill.), with an efficiency of $\sim 93\%$.

Rhodopsin was determined spectroscopically, as described previously (Szuts and Cone, 1977), by measuring $\Delta O D_{360}$. The $\beta-N$-acetylglicosaminidase assay procedure of Baggionini (1974) was modified by raising the substrate solution pH to 5.0, which is the pH$_{\text{max}}$ of pigment epithelial lysosomes (Berman, 1971). Cytochrome oxidase activity was measured as described by Yonetani (1967) and was expressed as micromoles of cytochrome oxidized per minute. Cytochrome oxidase activity was measured within a day of an experiment. Control experiments have shown that this mitochondrial enzyme is inactivated over several days, even in the absence of sucrose. The loss was especially severe in the pellet of contaminants prepared by the differential centrifugation of unpurified FROS homogenate. A modified Lowry procedure was used for total protein determination (Schacterle and Pollack, 1973), with BSA as the standard. Interference from metrizamide was eliminated by initially precipitating the proteins with trichloroacetic acid (Bensadoun and Weinstein, 1976). Sucrose and metrizamide distributions on the density gradients were measured at room temperature with an Abbe-type refractometer (Model Abbe-3L, Bausch & Lomb Inc., Rochester, N. Y.).

Metrizamide, p-nitrophenyl-2-acetamido-2-deoxy-\(\alpha\)-glucopyranoside, horse heart cytochrome c, Triton X-100, Arsenazo III, and BSA (fraction V powder) were purchased from Sigma Chemical Co. (St. Louis, Mo.). New England Nuclear (Boston, Mass.) was the supplier of $^{45}\text{CaCl}_2$ and polyethylene glycol (1,2-\(\alpha\)-H). X537A was a gift from Dr. W. E. Scott of Hoffmann-La Roche Inc., Nutley, N. J.

The dissections, purification or artificial loading procedures, and efflux measurements were all performed under dim red light (60-W bulb, $\lambda > 680$ nm), which indirectly illuminated the working area. When light effects were studied on isolated retinas, infrared light was used from the beginning of the dissection until the preparation of the unpurified FROS homogenate. Subsequent handling was performed under red light, since control experiments have shown that red light had no effect on such FROS. The infrared converter was a head-mounted viewer (Find-RScope, Model 80301, F JW Industries, Mt. Prospect, Ill.), and its source bleached $< 0.01$ rhodopsin molecule per disk during the course of the experiment. The bleaching rate was estimated psychophysically (Szuts and Cone, 1977) and by radiometer measurements (Model 111A, United Detector Technology, Inc., Santa Monica, Calif.). During the incubation with $^{45}\text{Ca}$, test retinas were repetitively illuminated every 10 s with a constant intensity light flash (Model 470, AutoStrobonar, Honeywell Inc., Photographic Products Div. Littleton, Colo.). Illumination was through the bottom of the vial, which was placed directly on the flash unit. A Schott KG3 infrared-absorbing filter (Fish-Schurman, Inc., New Rochelle, N. Y.), a Wratten 23 filter (Eastman Kodak Co., Rochester, N. Y.), and a diffuser were placed in the light path to provide diffused light at 620 nm. With neutral density filters, the flash intensity
was varied between experiments so that a predetermined amount of rhodopsin could be photoactivated.

**RESULTS**

*Intradiskal Ca Exchange in Isolated Retinas*

Intradiskal $^{45}$Ca was measured in the shortest interval after outer segment isolation by centrifuging the chilled unpurified FROS homogenate on a cold metrizamide gradient (Fig. 1). $^{45}$Ca is introduced into the layers of such a gradient because dense vesicles (disks and other organelles) either sequestered $^{40}$Ca in their intravesicular space, bound $^{45}$Ca on their membrane surface, or physically trapped $^{46}$Ca in the adhering solution. There is a very poor match between $^{45}$Ca and rhodopsin distribution on the gradient, even though rhodopsin, the membrane protein of disks, comprises most of the protein of the sample. The high $^{45}$Ca levels associated with contaminating organelles nearly mask the relatively small amount of $^{45}$Ca associated with the disks. The distribution of two marker enzymes, besides rhodopsin, was also assayed on the gradient. The activity of cytochrome oxidase (an integral protein of the inner membrane of mitochondria) was monitored because inner segment mitochondria are contaminants in outer segment suspensions, and because mitochondria are known to sequester Ca. A lysosomal enzyme marker, $\beta$-$N$-acylglucosaminidase, was measured because the density of some lysosomal fractions are reported to be nearly the same as that of fragmented outer segments (Tolbert, 1974), making lysosomes a serious source of contamination for purified outer segments. Reflecting the activity of its phagosomes, the pigment epithelium possesses a large $\beta$-$N$-acylglucosaminidase activity (Berman, 1971). However, it is not known what portion of the total lysosomal enzyme activity observed in the homogenate originates from the pigment epithelium. Surprisingly, the $^{45}$Ca distribution matched the lysosomal enzyme distribution more closely than that of rhodopsin or that of cytochrome oxidase, which was undetectable in the fractions shown in Fig. 1.

The total amount of rhodopsin on the gradient of Fig. 1 was 9.20 nmol, and the fraction of total $^{45}$Ca that comigrated with it was $2.8 \times 10^{-6}$. Since some $^{45}$Ca may be external to disks, the amount of $^{45}$Ca associated with the disks gives an upper limit for intradiskal $^{45}$Ca: 170 cpm/nmol of rhodopsin, which in this experiment would be equivalent to an exchange of 0.004 Ca/rho. Because the Ca content of frog disks is 0.13 Ca/rho, an exchange of $\leq 0.004$ Ca/rho is equivalent to an exchange of $\leq 10\%$.

The observed intradiskal $^{45}$Ca content is unlikely to have been modified by prior $^{45}$Ca losses by either net efflux or by Ca-Ca exchange. Net efflux of $^{45}$Ca is unlikely, since previously published data indicate that the total content of disks does not fall below 0.10 Ca/rho, even under favorable conditions, such as a lengthy purification with excess EGTA (Liebman, 1974) or a hypotonic shock with excess EDTA (Szuts and Cone, 1977). Loss by Ca-Ca exchange diffusion is unlikely, because this process is undetectably small for $^{45}$Ca-loaded disks, as will be shown later. The actual time resolution for $^{45}$Ca measurement in the experiment of Fig. 1 is set by the elapsed time between isolation of the
outer segments and the arrival of the disks at their equilibrium position. Provided that it is not more than several hours, the interval between equilibrium attainment and fractionation of the gradient is of no consequence, since

![Diagram of rhodopsin and 45Ca distribution](image)

**Figure 1.** The distribution of rhodopsin and 45Ca on a metrizamide density gradient after isopycnic centrifugation of unpurified FROS homogenate at 0°C. The distribution of protein, β-N-acetylglucosaminidase (a lysosomal enzyme marker), and metrizamide (expressed as percent total solids because the variable contribution of ions was not subtracted) is also shown. The homogenate consisted of freshly isolated and fragmented outer segments from two retinas that were incubated with 3 mM Ca for 1 h (specific activity of 45Ca during incubation was 27.7 × 10^{12} cpm/mol Ca). The homogenate volume was 2 ml and its initial activities were: 2.5 × 10^7 cpm of 45Ca; 9.20 nmol of rhodopsin; 0.22 μmol of p-nitrophenol produced/h for β-N-acetylglucosaminidase; 0.96 mg of protein; and 6.8 nmol cytochrome c oxidized/min for cytochrome oxidase (a mitochondrial enzyme). The initial activity of cytochrome oxidase was so low that its activity could not be detected in the fractions. Intradiskal 45Ca comigrates with the disks so that not only its location but also its spread should match that of rhodopsin. (A) Total solids. (B) 45Ca. (C) β-N-Acetylglucosaminidase. (D) Rhodopsin. (E) Protein.
diffusion is so slow over several millimeters that only a negligible amount of Ca could diffuse out of the disk fraction during that time. For the experiment of Fig. 1, time resolution was about 40 min. This interval is much shorter than the purification procedure of Liebman (1974), which uses EGTA. It is also short enough that no major losses by Ca-Ca exchange diffusion could have occurred. Thus, it appears that the low intradiskal $^{45}$Ca content shown in Fig. 1 actually represents a small Ca exchange within the disks of $^{45}$Ca-incubated retinas.

Results similar to those of Fig. 1 were obtained irrespective of photostimulation during retinal incubations and irrespective of the ionic composition (high K or choline saline) of the solutions during and after the isolation step. Because on linear density gradients contaminants can often mask intradiskal $^{45}$Ca, sometimes even more so than in Fig. 1, disks need to be purified for any quantitative measurements of their $^{45}$Ca content. The purification procedure, using two successive differential centrifugations, is about 2 h long and so is much longer than the procedure illustrated in Fig. 1. Given that comparably handled disks do not lose all their Ca by net efflux (as noted above, they retain at least 0.1 Ca/rho) and that Ca-Ca exchange diffusion is too slow, it is unlikely that disks lost a substantial amount of their $^{45}$Ca content during these purifications.

Figure 2 shows the effect of successive purification on enzymatic activities and on sequestered $^{45}$Ca for experiments in which either sucrose or metrizamide density solutions were used in the purification procedure. Sequestered $^{48}$Ca was measured with the filtration technique and represents $^{45}$Ca retained by vesicles on the filter. Because the density difference between lysosomes and outer segments is greater in sucrose than in metrizamide solutions, improved purity was achieved with the sucrose method (cf. Fig. 2 A and 2 B). Indeed, FROS purity with the sucrose method was such that no further improvement could be achieved by additional differential centrifugations. For purified disks, rhodopsin was found to be about 15 nmol/mg of protein. As Fig. 2 demonstrates, mitochondrial and lysosomal enzyme activities and sequestered $^{45}$Ca decreased as the purity of disks increased. This decrease in sequestered $^{45}$Ca is not due to Ca losses from disks but rather to the removal of $^{45}$Ca-containing contaminating organelles. This correlation can be seen when, at the end of the first differential centrifugation, the enzyme distributions are measured in the three fractions into which centrifuge tube contents are divided: pellet of contaminants, carpet of FROS, and supernate of pooled upper and lower phases. Enzyme activities and sequestered Ca are presented on a frequency distribution histogram in Fig. 3. The data in both Figs. 2 and 3 were obtained from the same experiments. As shown in Fig. 3 B, >90% of all the sequestered Ca but <10% of disks was found with the contaminating organelles. Thus, these organelles appear to contain more than an order of magnitude more $^{45}$Ca than the disks, if Ca content is normalized to milligrams of protein. This raises the possibility that even for purified disks, some of the $^{45}$Ca may originate from residual contamination. Neither ionic substitutions (choline or K for Na) nor the addition of 10 μM LaCl₃, 1 mM dithiothreitol, or 10 μM
Figure 2. The effect of successive purification in metrizamide (A) and in sucrose (B) on the specific activities of rhodopsin, cytochrome oxidase, β-N-acetylglucosaminidase and sequestered 45Ca. Three bars are presented for each of these categories. The first bar represents the initial specific activity observed in the unpurified FROS homogenate. The height of the bar is normalized to 100%, and the measured specific activity is written within it. Successive bar heights indicate the percent of initial specific activity found in the disks after the first and second steps of purification. As contamination was removed, the relative specific activity of rhodopsin increased and that of mitochondrial and lysosomal enzymes decreased. The specific activity of rhodopsin is operationally expressed here as moles of rhodopsin per milligram of protein. In analogy with enzyme nomenclature, sequestered 45Ca is expressed as counts per minute per milligram of protein and represents the radioactivity retained by the Millipore filter per milligram of protein before filtration. The error bars represent SD and are set by the variability between three filtered samples. (A) Purification performed with metrizamide, using stock saline. FROS were obtained from retinas that were incubated with 10 mM Ca for 1 h. The initial specific activities were: 5.0 nmol rhodopsin/mg protein; 139 nmol cytochrome c oxidized/min-mg protein; 199 nmol p-nitrophenol produced/h-mg protein; and 11,300 cpm/mg protein. Exchanged Ca within the purified disks was 0.006 Ca/rho. (B) Purification performed with sucrose, using choline saline. The retinas of this experiment were also incubated with 10 mM Ca. Specific activities are in the same units as described above. The exchanged Ca within the purified disks was 0.001 Ca/rho.
FIGURE 3. Distribution of enzymatic activities and sequestered $^{45}$Ca after the first differential centrifugation. (A) Distribution observed in the experiment of Fig. 2 A. (B) Distribution observed in the experiment of Fig. 2 B. After the differential centrifugation, the centrifuge content was divided into three fractions: carpet of disks, pellet of contaminants, and supernate (mixture of dense and light phases). Protein content and enzymatic activities of each of these fractions were measured. Enzymatic distribution among the three fractions is plotted as relative specific activity vs. percent of total recovered protein. Relative specific activity is the percentage of activity in the fraction divided by the percentage of protein in same fraction. The abscissa presents the percent of protein for each fraction, shown cumulatively. For example, in A, 9% of the total protein was found with the pellet, 35% with the supernate, and 56% with the carpet of disks. In this way the surface area of the rectangle for each fraction is equal to the percent of enzyme associated with it. Thus, in A, only ~20% of the sequestered $^{45}$Ca was with the carpet of disks, even though it contained 80% of the rhodopsin. Similarly, in B, the pellet contained over 50% of the sequestered $^{45}$Ca, yet it contained negligible amounts of rhodopsin. These distributions demonstrate that much more $^{45}$Ca is associated with the contaminants than with the disks.
diltiazem (an inhibitor of voltage-sensitive Ca fluxes) to the media during the purification procedure resulted in significantly greater $^{45}\text{Ca}$ contents for purified disks. In all cases, $<10\%$ of the intradiskal Ca exchanged during the incubations.

Effect of Light and Extracellular Ca on Intradiskal Exchange

The experimental conditions were advantageous for testing light effects on intradiskal exchange, because retinas are known to be in a physiologically active state under these incubation conditions (Sickel, 1973). This was confirmed in this study. When the electroretinogram of isolated bullfrog retinas was monitored, the photoreceptors retained their scotopic sensitivity for at least 2 h as judged by a criterion b-wave response. To measure light effects of Ca exchange, both control and test retinas were dissected and handled under infrared illumination. Test retinas were exposed to repetitive light flashes every 10 s during the hour-long incubation. Both sets of retinas were then chilled and their fragmented outer segments isolated while still under infrared exposure. The subsequent purification procedure was performed in red light. The results of such experiments are given in Table II. Test retinas were exposed to flashes that photoactivated $5 \times 10^4$ rhodopsin molecules per flash, equivalent to activating $7 \times 10^3$ rhodopsin molecules per disk by the end of incubation. If the stoichiometry of release is $10^3 \text{ Ca/rho}^*$ (i.e., Ca ions per photoactivated rhodopsin molecule) as suggested by Cone (1973), all Ca within the disks, or $0.1 \text{ Ca/rho}$, should have exchanged. However, the results show that if there was any increased exchange it was $<0.01 \text{ Ca/rho}$. Similar results were obtained in other experiments, whether the flashes collectively bleached as much as $50\%$ of the pigment in 1 h of incubation or as little as $0.8 \text{ rho}^*/\text{disk-hour} (5 \text{ rho}^*/\text{rod-flash})$.

$^{45}\text{Ca}$ exchange was also insensitive to the Ca concentration of the incubating solutions (Figs. 2 and 3 and Table II). Such increased extracellular Ca is known to increase cytoplasmic Ca activity within the receptor. For example, by measuring the Na permeability of isolated frog outer segments, Wormington and Cone (1978) have shown that cytoplasmic Ca increases 10–100-fold (to $\sim 10 \mu\text{M}$), with an extracellular concentration of 10 mM. Such an increase in cytoplasmic Ca was expected to facilitate $^{45}\text{Ca}$ accumulation by the disks. However, as is shown in Table II, no such increase was detected.

Passive Loading of Disks with $^{45}\text{Ca}$

To test for possible intradiskal $^{45}\text{Ca}$ losses during the isolation and purification procedure described above, separate experiments were performed with $^{45}\text{Ca}$-loaded disks. The ultimate aim was to measure Ca-Ca exchange diffusion and net Ca efflux, mechanisms whereby $^{45}\text{Ca}$ losses could have occurred, under intradiskal and extradiskal conditions similar to those described above. As will be discussed later, previously published measurements on intradiskal Ca content indicate that the observed results could not have been affected by net efflux. Nevertheless, both net efflux and Ca-Ca exchange were studied in $^{45}\text{Ca}$-loaded disks so that they could be characterized under similar conditions.
Purified disks were freshly prepared for each experiment using the same procedure as in Fig. 2 B, except nonradioactive retinas were the source. $^{45}$Ca could not be incorporated into these disks by an ATP-dependent process. Active uptake by purified disks was not observed, even in media that resembled the cytoplasmic environment (high K saline with 2 mM ATP, 2 mM GTP, 20 µM cGMP, 96 µM vitamin E, and 20 mM reduced glutathione). However, disks could passively accumulate $^{45}$Ca. They were, thus, routinely loaded by suspension in a solution containing trace amounts of $^{45}$Ca and 5 mM CaCl$_2$. This Ca concentration was chosen for loading, because it approximates the maximal activity that disks could ordinarily possess. To ensure that accumulation was maximal and that specific radioactivity remained the same in both intradiskal and extradiskal spaces, loading was often performed in the presence of the ionophore X537A. The ionophore was removed from the membranes of these disks with serum albumin. Control disks, loaded in the absence of the ionophore, also accumulated some $^{45}$Ca passively.

Fig. 4 demonstrates the close correlation between radioactivity and rhodopsin when disks, which were loaded using the ionophore, were centrifuged on a sucrose gradient. This close correlation should be compared with the poorer correspondence when freshly isolated outer segments from $^{45}$Ca-incubated retinas were centrifuged on a similar density gradient (Fig. 1). The total amount of rhodopsin between fractions 3 and 10 of Fig. 4 was 19.4 nmol and the fraction of total $^{45}$Ca that comigrated with it was $4.7 \times 10^{-3}$. Comparison with Fig. 1 shows that the amount of $^{45}$Ca with the disks could not have been due to external binding or solvent drag. The measured Ca content for the disks of Fig. 4 is about 20 times greater than the expected 0.11 Ca/rho

| TABLE II |
| EFFECT OF ILLUMINATION AND EXTRACELLULAR CALCIUM ON $^{45}$CA EXCHANGE IN THE DISKS OF ISOLATED RETINAS |
|---|---|---|---|
| Extracellular Ca activity during incubation | Density material used for purification | Ca exchange per rhodopsin-hour |
| mM | | Dark | Light | Light - Dark |
| Control | | | | |
| Experiments with mock illumination | 3 | Sucrose | 0.0072 | 0.0062 |
| | 3 | Sucrose | 0.0134 | 0.0076 |
| | 3 | Sucrose | 0.0050 | 0.0080 |
| | 3 | Sucrose | 0.0119 | 0.0108 |
| | 3 | Metrizamide | 0.0031 | 0.0027 |
| Test | | | | |
| Experiments with flashes that photoactivated $7 \times 10^9$ rhodopsin* per disk per hour | 3 | Sucrose | 0.0059 | 0.0114 |
| | 10 | Sucrose | 0.0099 | 0.0091 |
| | 3 | Metrizamide | 0.0040 | 0.0080 |
| | 3 | Metrizamide | 0.0030 | 0.0033 |
| | 3 | Metrizamide | 0.0226 | 0.0075 |

‡ Mean ± SD.
calculated for an intradiskal concentration of 5 mM with negligible binding. An intradiskal volume of 22 liter/mol rho was used for this calculation. It was derived for a 7-μm diameter disk, which contains $2 \times 10^9$ rhodopsin molecules and a 20-Å-wide osmotically active space (Korenbrot et al., 1973; Chabre and Cavaggioni, 1975) that is equivalent to an intradiskal volume of 0.077 flter. Clearly, the large Ca content that was observed in Fig. 4 could arise from: (a) intradiskal binding, (b) increased intradiskal volume, or (c) membrane potential. The last alternative is unlikely, since X537A is a nonspecific ionophore.

Figure 4. Distribution of rhodopsin and $^{45}$Ca on a sucrose density gradient after isopycnic centrifugation at 0°C for disks that were loaded in the presence of the ionophore. The sucrose gradient provided a hypertonic shock to the disks as they traversed the gradient. The distribution of protein, β-N-acetylglucosaminidase, cytochrome oxidase, and sucrose is also shown. The error bars for the mitochondrial and lysosomal enzymes represent estimated errors. Specific activity of $^{45}$Ca during loading was 616 cpm/nmol Ca. The initial volume of the disk suspension was 2.2 ml, and its initial activities were: $6.8 \times 10^6$ cpm of $^{45}$Ca; 26.2 nmol rhodopsin; 24 nmol $p$-nitrophenol produced/h for β-N-acetylglucosaminidase; 1.5 mg of protein (this was assayed before BSA was added to the disks for the removal of the ionophore); and 13 nmol cytochrome c oxidized/min for cytochrome oxidase. (A) Sucrose. (B) Cytochrome oxidase. (C) $^{45}$Ca++. (D) β-N-Acetylglucosaminidase. (E) Rhodopsin. (F) Protein.
and most likely abolished the membrane potential during the incubation. Because bovine disks bind Ca (Hendriks et al., 1977), intradiskal binding would appear to be a likely explanation. However, the extent of binding is unknown, because the distribution of the reported binding sites across the two sides of the disk membrane has not yet been measured. Although Ca binding may be partly responsible for the large Ca content in Fig. 4, experimental evidence obtained in the course of this study indicates that an increase in intradiskal volume may also be an important factor.

**Intradiskal Volume of Purified Disks**

For the calculation of intradiskal concentrations and activities, the intradiskal volume of the $^{40}$Ca-loaded disks must be known. Intradiskal volume was indirectly obtained from density measurements. Fig. 5 presents the result of an experiment in which the density of both freshly isolated and sucrose-purified disks was measured. Disks, which were loaded with the ionophore, were simply mixed with fresh material, and the mixture was centrifuged on a continuous metrizamide gradient until isopycnic conditions were reached. The relative positions of the two disk populations could be unequivocally determined because the purified disks were tagged with $^{40}$Ca. Although density varied from 1.16 to 1.01 g/cm$^3$ (values at 20°C) in the linear portion of the gradient, osmolarity was nearly constant (250–230 mosM). The constant osmolarity ensured that disk volume, and therefore disk density, remained essentially unaltered as the disks traversed the gradient. The densities of the purified disks and of the freshly isolated disks were 1.03 and 1.10 g/cm$^3$, respectively. The 6% decrease in the density of purified disks is highly reproducible and is insensitive to previous exposure to X537A. Although sucrose-purified disks show an especially marked decrease in their density, similar changes were also observed for freshly isolated disks. A fraction of the fresh material illustrated in Fig. 5 already exhibits such a partial density reduction, and a similar light fraction is illustrated in Fig. 1.

A 6% decrease in the density of sucrose-purified disks is equivalent either to a 7% loss of solid content or to a 20-fold increase in intradiskal volume. The factor ($n$) by which intradiskal volume increased, was calculated from $n = v_1(d_1 - d_2)/v_0(d_2 - d_{H_2O})$, where $d_1$ = initial density = 1.10 g/cm$^3$, $d_2$ = final density = 1.03, $d_{H_2O}$ = aqueous density = 1.00, $v_1$ = total disk volume = 0.65 liter, and $v_0$ = intradiskal osmotic volume = 0.077 liter. Whether the density decrease is due to loss of membrane components, to volume increase, or to a combination of both, cannot be determined with certainty because no published information is currently available. However, in unpublished experiments, a similar 20-fold increase in intradiskal volume was measured when the intradiskal space of sucrose-purified disks was directly assayed with radioactive markers. Thus, it may be that most, if not all, of the density change can be attributed to an intradiskal volume increase. If so, the intradiskal volume of sucrose-purified disks may be as much as 440 liter/mol rho. This final conclusion is supported by subsequent calculations that yield reasonable intradiskal Ca activities only with such large intradiskal volumes.

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2 Thacher, S. Personal communication.
FIGURE 5. Distribution of rhodopsin and 45Ca on a metrizamide density gradient after isopycnic centrifugation at 0°C of 45Ca-loaded disks that were mixed either with saline (A) or with unpurified FROS homogenate (B). The purified disks were loaded with 45Ca in the presence of the ionophore. This experiment demonstrates: (a) the density difference between sucrose-purified and freshly isolated disks, and (b) the lack of effect of unpurified FROS homogenate on the 45Ca content of loaded disks. The rhodopsin content of the fractions is given as the optical density of a threefold diluted 0.2-ml sample (in previous distributions, rhodopsin was determined by ΔOD502). Metrizamide distribution is expressed as percent total solids because the variable contribution of ions was not subtracted. In this experiment, all solutions (including the metrizamide solutions) contained 10 μM Ca-EGTA to minimize lipid peroxidation in disks (Farnsworth and Dratz, 1976), although the same results are obtained in the absence of this precaution. The 45Ca-loaded disks were resuspended with stock saline (in the absence of BSA) and were divided into 2 1-ml volumes (~22 nmol rhodopsin/ml and 40,000 cpm/ml), to which either 1 ml stock saline or 1 ml unpurified FROS homogenate (~10 nmol rhodopsin/ml) were added. These mixtures were then placed on the density gradient for centrifugation. Specific activity during loading was 609 cpm/nmol Ca.
Net Efflux from $^{45}$Ca-loaded Disks

For the measurement of efflux kinetics, $^{45}$Ca-loaded disks were separated from the loading medium by centrifugation and collected as a pellet. The pellet of disks was resuspended and efflux was monitored by measuring intradiskal $^{45}$Ca content with the filtration technique. Fig. 6 compares the efflux rates from disks that were loaded either in the presence or absence of the ionophore. The $^{45}$Ca that is observed in association with the disks is located within the intradiskal space rather than bound to the outer surface, since it can be rapidly released by X537A but not by excess EGTA. Because the solvent in the ionophore stock solutions was 95% ethanol, the addition of $10^{-5}$ M X537A to the disk suspensions illustrated in Fig. 6 also introduced ethanol to a final concentration of 0.04%. At this concentration, however, ethanol by itself showed no effect on $^{45}$Ca content.

At room temperature and in an isotonic solution with a Ca activity of $\lesssim 10^{-8}$ M, Ca efflux was 0.3 fmol/cm$^2$ s (half-time $\sim 100$ min) from the disks of Fig. 6A that were loaded using the ionophore. This was one of the fastest effluxes observed for disks in isotonic solution. Under similar conditions, the “apparent” efflux rate was $\lesssim 0.1$ fmol/cm$^2$ s for control disks that were loaded in the absence of the ionophore (Fig. 6B). The low efflux from the disks of Fig. 6A, and the even lower effluxes seen in Figs. 7 and 8, confirm that the albumin procedure adequately removed the ionophore from the disk membranes at the termination of loading. Of course, if removal were less than complete, the observed rates would overestimate the true efflux rates from disks.

Although efflux was slow in isotonic solutions, disks rapidly released 80–90% of their initial Ca when suspended in a hypertonic solution that was made 9.1 times isotonic by sucrose addition (Fig. 6). Efflux was similarly increased when solutions were made hypertonic with metrizamide. As seen in Fig. 7, increased efflux could be elicited with a 2.5 times isotonic 34% metrizamide solution, but none could be observed with a nearly isotonic 21% solution. Since disk membranes are impermeable to sucrose (Chabre and Cavaggioni, 1975) and to metrizamide (789 mol wt), the observed increase in efflux is most likely from increased intradiskal Ca activity caused by osmotic compression of the disks. It was on the basis of these experiments that metrizamide, rather than sucrose, was chosen as the density material for centrifugation in the experiments of Figs. 1–3; even the most concentrated 21% metrizamide solution was sufficiently isotonic not to cause any release of Ca. In contrast to the osmolarity effect, efflux appears to be insensitive to the ionic strength of the suspending medium (Fig. 7B). Efflux was also found to be unaffected by 25% serum albumin. Thus, disks that were loaded with the ionophore did not lose their Ca while the ionophore was removed from them at the termination of $^{45}$Ca loading.

Whether or not they were loaded in the presence or absence of the ionophore, disks seemed to possess comparable efflux properties, as is shown in Fig. 6. In both cases, the time-course for shock-induced efflux was comparable and efflux was insensitive to excess EGTA. Thus, the flux properties of disks do not appear to have been modified by prior exposure to X537A.
Shock-induced efflux was insensitive to the ionic composition of the suspending medium, to pH 6, to 100 μM LaCl₃, and to 10⁻⁴ M diltiazem. Lowering the temperature, however, decreased the half-time of the efflux.

**Figure 6.** ⁴⁵Ca efflux from loaded disks at room temperature. (A) Disks loaded in the presence of the ionophore. After centrifugation, the pellet of disks was resuspended with stock saline that contained 1% BSA and 1 mM EGTA (final pH = 7 and Ca activity ≤ 10⁻⁶ M) and was divided into two equal aliquots. One of the aliquots was mixed with hypertonic sucrose (dissolved in stock saline) (○), while the other was mixed with only saline (●). Aliquots of 0.4 ml, containing 1.3 nmol rhodopsin, were removed from the mixtures and filtered at increasing time intervals after the onset of mixing. Both efflux curves were drawn by eye. The Ca content of the disks before and after hypertonic shock is shown on the right. Specific activity of ⁴⁵Ca during loading was 1,000 cpm/nmol Ca. (B) Control disks loaded in the absence of the ionophore. After centrifugation, the pellet of disks was resuspended with stock saline. The rest of the procedure is the same as in A. The 0.4-ml aliquots contained 3.2 nmol rhodopsin. The efflux curve for the hypertonically shocked sample was drawn by eye. For the control sample, a straight line was drawn at the average of the data points. The error bar gives ±SD. The apparent Ca in the disks before and after hypertonic shock is shown on the right. The specific activity of ⁴⁵Ca during loading was 909 cpm/nmol Ca.
from ~3 min at 25°C to 30 min at 0°C. In both isotonic and hypertonic media, $^{45}\text{Ca}$ efflux was relatively insensitive to external Ca activities that ranged from $\sim 10^{-8}$ to $10^{-3}$ M (Figs. 6–8). Therefore, shock-induced efflux appears to be a net release process.

Rapid Ca efflux at increased intradiskal activities may explain why the Ca contents in Figs. 6 and 7 were less than the observed 2.1 Ca/rho in Fig. 4.

**Figure 7.** Effect of concentrated solutions of serum albumin, metrizamide, and sucrose on $^{45}\text{Ca}$ content of loaded disks at room temperature. Disks were loaded in the presence of the ionophore. (A) Resuspended disks were divided into three aliquots, which were mixed with: (O) control solution (stock saline with 1% BSA, 0.1 mM Ca, pH 7, 0.22 osM); (●) 34% wt/wt metrizamide (dissolved in control solution, 0.57 osM); (□) 34% wt/wt sucrose (dissolved in control solution, 2.0 osM). (3.6 nmol rhodopsin/0.4-ml aliquot for filtration; specific activity = 653 cpm $^{45}\text{Ca}$/nmol Ca). Ca content of disks at last data point is shown on the right. (B) Resuspended discs were divided into three aliquots, which were mixed with: (O) 25% wt/wt BSA (dissolved in stock saline, 2.5 mM Ca, pH 5.3, 0.31 osM); (●) 21% wt/wt metrizamide (dissolved in water with 1% BSA, 0.1 mM Ca, pH 7, 0.25 osM); (□) 21% wt/wt metrizamide (dissolved in stock saline, 1% BSA, 0.1 mM Ca, pH 7, 0.43 osM). Straight line was drawn at the average of the data points. The error bars give ± SD (2.1 nmol rhodopsin/0.4-ml aliquot for filtration; specific activity = 698 cpm $^{45}\text{Ca}$/nmol Ca).

Initial Ca content in Fig. 6 A was 0.69 Ca/rho, or three times less than that in Fig. 4. Similarly, initial content was 0.50 ± 0.25 Ca/rho (mean ± SD) for all the 15 filtration experiments performed with disks loaded with the ionophore. Control disks, loaded in the absence of the ionophore, showed an “apparent” initial content of 0.12 ± 0.05 Ca/rho (mean ± SD for seven experiments).
confirms that Ca losses must have occurred from loaded disks before their measurement with the filtration procedure.

**Estimation of Intradiskal Ca Activity**

The shock-induced efflux measurements are especially useful for calculating intradiskal activities. With such a calculation, an approximate relationship between efflux and intradiskal activity can easily be obtained. The calculation is based on the properties of unshocked and shocked disks and involves the comparison of their efflux rates and the estimation of their bound intradiskal Ca. The following reasonable assumptions are required: (a) efflux rate uniquely depends on intradiskal activity, so that disks with identical activities show identical efflux rates and (b) translocation across the disk membrane, rather than dissociation from intradiskal binding sites, is the rate-limiting process for efflux. The latter assumption is supported by the data of Fig. 6: efflux rates were much faster in response to X537A exposure than to osmotic shocks, implying that dissociation half-times from chelators were much shorter than the measured half-time of 3 min for the overall shock-induced efflux. With these assumptions, the intradiskal activities within shocked and unshocked disks are equal whenever their efflux rates are identical. Since their activities are the same, bound Ca within them must also be identical and must be equal to, or less than, the Ca content of the shocked disks at that moment. Consider the data of Fig. 7 A as an example of such a calculation. About 20 min after the onset of the shock, efflux from sucrose-shocked disks achieved the same rate as that from control disks. At this point the intradiskal activity within both sets of disks is identical, and their bound Ca is <0.04 Ca/rho, the total Ca content of the shocked disks. Thus, for an intradiskal volume of 440 liter/mol rho, as discussed previously, intradiskal activity within the unshocked disks of Fig. 7 A is estimated to be \((0.18 - 0.04)/440 = 0.32 \times 10^{-3}\) M. By similar calculations, the initial activity for the unshocked disks of Fig. 6 A is estimated to be \((0.69 - 0.11)/440 = 1.3 \times 10^{-3}\) M. Note that these activities are underestimates, based on the assumption that at the referred time all Ca within shocked disks is bound and that intradiskal volume is 440 liter/mol rho. The estimated activities can, in turn, be used to calculate the initial activities within hypertonically shocked disks. Disks were assumed to be perfect osmometers for this calculation, meaning that a hypertonic shock of \(n\) times isotonic decreased their intradiskal volume \(n\)-fold.

By such calculations, the relationship between estimated intradiskal activity and measured efflux rate can be derived from the data of Figs. 6 and 7. The derived values are tabulated in Table III. A more extensive determination of this relationship was not undertaken in the absence of any accurate data on intradiskal binding and volume. The data of Table III support the notion that due to an initial 5 mM intradiskal activity, rapid efflux reduced the Ca content of 45Ca-loaded disks by the time they were measured with the filtration procedure. Moreover, based on the data of Table III, the observations that Ca content remained at a level of 0.50 ± 0.25 Ca/rho for disks loaded using the ionophore, and the observation that 9.1 times isotonic shocks—whenever tested—always released about 80% of total Ca, it can also be concluded that
intradiskal activity does not fall below 0.3 mM in isotonic solutions. Thus, with the procedures employed, disks that were loaded with the ionophore routinely contain an intradiskal activity of at least 0.3 mM.

**Ca-Ca Exchange in 45Ca-loaded Disks**

As shown previously, 45Ca release, and hence Ca-Ca exchange, was undetectable in solutions whose Ca concentrations were 10-100 μM. Specifically, the exchange rate in Fig. 7 A was ≤0.1 fmol/cm² s (half-time, ≥2 h) at room temperature. Note that the disks of this experiment were comparable to native disks in their total Ca content (0.18 Ca/rho for the loaded disks and 0.13 Ca/rho for native disks) and possibly in their intradiskal Ca activity (which was at least 0.3 mM for the loaded disks). Assuming that purification does not alter disk properties, native disks would also be expected to exchange Ca with a half-time of ≥2 h. Half-time may even be >10 h at near 0°C if exchange is as temperature sensitive as net efflux. At such rates, Ca-Ca exchange is unlikely to have caused the release of significant amounts of 45Ca from the disks of 45Ca-incubated retinas.

To increase the exchange rate and its possible detection, the effect of increased extradiskal concentration (1.4 mM) was tested (Fig. 8 A). Ca-Ca exchange however, still remained undetectable at ≤0.4 fmol/cm² s. This slow rate was surprising since a much faster exchange process had been previously reported for bovine disks (Schnetkamp et al., 1977). To test whether exchange was sensitive to experimental conditions, an experiment similar to that reported by Schnetkamp et al. (1977; Fig. 5 in their report) was performed. Efflux was monitored from 45Ca-loaded disks that were incubated, centrifuged, and resuspended in Tris saline, whose composition was similar to that used by the previous investigators. Neither 1.4 mM Ca nor 105 mM Na caused any significant increase in efflux rate over an interval of 15 min, as is shown in Fig. 8 B, even though these concentrations were reported to have released 50% of the Ca in <1 min. In spite of an intradiskal Ca activity of at least 0.3 mM,
$^{45}$Ca efflux was always found to be insensitive to external Ca and Na. The rate of Ca-Ca exchange in Fig. 8 B is $\leq 0.4$ fmol/cm$^2$ s.

**Effect of Unpurified FROS Homogenate on Efflux**

The effect of unpurified FROS homogenate on Ca efflux was measured to check whether enzymes or cofactors present in the initial homogenate could

![Fig. 8](image)

**Figure 8.** Effect of increased extradiskal Ca and Na concentrations on $^{45}$Ca content of disks that were loaded using the ionophore. (A) Experiment in which initial suspending medium was the stock saline of Table I. BSA was absent in the final resuspension. Aliquots for filtration had a volume of 0.2 ml and contained 2.20 nmol rhodopsin. Specific activity during loading was 597 cpm/nmol Ca. The straight line was drawn at the average of the data points before Ca addition. The error bar gives ± SD. The Ca content of the disks was 0.46 Ca/rho. (B) Experiment in which initial suspending medium was the Tris saline of Table I. Data points: (○) control Tris saline; (□) 105 mM NaCl; (○) 1.4 mM CaCl$_2$; (▲) data of Schnetkamp et al. (1977) after the addition of 100 mM NaCl or 1 mM CaCl$_2$. The experimental conditions of Schnetkamp et al. (1977) were reproduced because under their conditions Na-Ca and Ca-Ca exchange was observed in bovine disks. Tris saline was used throughout loading, centrifugation, and subsequent resuspension of the disks. BSA was absent in the final resuspension. Three aliquots were filtered before the suspension was divided into three 0.7-ml volumes, to which either 105 mM NaCl (6.3 ml of stock saline to dilute initial volume 10-fold) or 1.4 mM CaCl$_2$ (without significant dilution of initial volume) was added. The third volume remained as control. Aliquots for filtration were either 0.2 ml (control and Ca) or 2 ml (Na) and, irrespective of the volume, contained 2.9 nmol rhodopsin. Specific activity during loading was 614 cpm/nmol Ca. For the control sample, a straight line was drawn at the average of the data points. The error bar gives ± SD. The Ca content of control disks was 0.68 Ca/rho.
have induced $^{45}$Ca release. $^{45}$Ca-loaded disks were handled according to the filtration procedure. The suspensions were kept on ice to reproduce the conditions of the experiments of Figs. 1–3. The low temperature also reduced Ca efflux and maintained a high intradiskal activity in the loaded disks. The suspension of loaded disks was divided into two equal aliquots: the test aliquot was mixed with unpurified FROS homogenate that had been freshly prepared about 15 min before mixing, and the control aliquot was mixed only with saline. The Ca concentration in the mixtures was 10–100 $\mu$M, similar to the concentration when outer segments of $^{45}$Ca-incubated retinas were isolated in the experiments of Figs. 1–3. Both mixtures were placed on separate gradients and were centrifuged simultaneously. The results of this experiment are shown in Fig. 4. Rhodopsin and $^{45}$Ca peaks were the same for both control and test mixtures, indicating that no significant $^{45}$Ca release occurred even though intradiskal Ca activities exceeded 0.3 mM, as judged by the large intradiskal Ca content. Thus, it is unlikely that $^{45}$Ca was released from the disks of $^{45}$Ca-incubated retinas by factors present during the isolation of the outer segments and of the disks.

**Light Effect on Efflux**

By the filtration procedure, light effect on Ca efflux was also measured. No light-induced Ca release was observed, even in experiments that were performed completely under infrared illumination. The absence of a light effect is not surprising in view of the results already shown in Figs. 6–8. All these experiments were performed in dim red light that continuously photoactivated $\sim$0.01% rhodopsin every minute. Yet Ca efflux was undetectable, even though intradiskal Ca activity was at least 0.3 mM and extradiskal activity was as low a 10$^{-8}$ M. For example, the stoichiometry of release was <25 Ca/rho* in Fig. 7 A. Therefore, photoactivated rhodopsin does not seem to release Ca either transiently or continuously from $^{45}$Ca-loaded disks.

**DISCUSSION**

**Exchange in the Disks of Intact Receptors**

The purpose of this study was to characterize the Ca uptake of disks within intact rod photoreceptors. The $^{45}$Ca exchange technique was well suited for this purpose because retinal receptors could easily be kept under physiological conditions throughout the incubation. Surprisingly small amounts of $^{45}$Ca were found within disks after they were isolated from the $^{45}$Ca-incubated retinas. Some, if not all, of this $^{45}$Ca may originate from residual contamination because contaminating organelles contained large amounts of $^{45}$Ca. Most of the $^{45}$Ca comigrated with $\beta$-N-acetylglucosaminidase, a lysosomal enzyme marker. Although the pigment epithelium contains more Ca than the retina (Hess, 1975) and exhibits large $\beta$-N-acetylglucosaminidase activity (Berman, 1971), the $^{45}$Ca-containing lysosome cannot be ascribed with certainty to the pigment layer, since neither the relative turnover of the Ca pool nor the distribution of the lysosomal enzyme in these two tissue is known. If all the
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$^{45}$Ca associated with the disks resided within the disks, observed exchanged Ca would be $<0.01 \text{Ca/rho}$. For an intradiskal content of 0.13 Ca/rho, an exchange of $<0.01 \text{Ca/rho}$ represents a turnover of $<10\%$.

Before such small exchange can even be considered to be caused by small fluxes across disk membranes, alternative explanation of the measured data must be considered. These include: (a) effluxes from disks during their isolation and purification, (b) intradiskal Ca binding, which would reduce the size of the exchangeable Ca pool, and (c) rate-limiting Ca flux across the plasma membrane that would reduce the availability of cytoplasmic $^{45}$Ca for uptake by disks. None of these alternative explanations, which will now be discussed in sequence, appear to be supported by experimental observations.

(a) The major potential limitation of the tracer exchange procedure is that after incubation is terminated, disks could lose part of their initial $^{45}$Ca while they are prepared for counting. Such potential losses were separately investigated in this study with $^{45}$Ca-loaded disks. These experiments were performed before, and concurrently with, the set of experiments with $^{45}$Ca-incubated retinas so that the least perturbing conditions could be picked for disk isolation and purification. For example, the choice of metrizamide over sucrose for density gradient material was based on these studies. At the concentration used for density gradients, sucrose but not metrizamide released $^{45}$Ca by osmotically shrinking the disks (Fig. 7 A).

$^{45}$Ca losses from disks, if present, could occur either by net Ca efflux or by Ca-Ca exchange. The former mechanism includes Na-Ca exchange diffusion, other cation-Ca countertransport and Ca-anion cotransport. Based on published values for total Ca content, $^{45}$Ca losses by net efflux are not likely. Even under conditions that are favorable for net efflux, disks do not lose all their Ca—they always retain at least 0.1 Ca/rho. For example, total content in frog disks remained at about 0.20 Ca/rho after a lengthy purification procedure with sucrose and EGTA (Liebman, 1974). Similarly, about 0.13 Ca/rho was found within bullfrog disks after a hypotonic shock with a solution containing EDTA (Szuts and Cone, 1977)—a much harsher treatment than the mild vortexing used in this study. Thus, if net loss ever occurs, it does not decrease Ca content to $<0.1 \text{Ca/rho}$. In view of this, it is hard to sustain the notion that disks of $^{45}$Ca-incubated retinas released $>90\%$ of their contents and retained $<0.01 \text{Ca/rho}$ under the isolation and purification conditions of this study.

In this regard, the observed relationship between efflux and intradiskal activity (Table III) is not useful for calculating $^{45}$Ca losses from the disks of incubated retinas. This is because intradiskal activity has not yet been independently measured for native disks. This activity, nonetheless, can be estimated from the previously cited Ca content data and from the data of Table III. Such a calculation predicts an activity of $<0.3 \text{mM}$ within native disks.

In spite of the many conditions under which it was tested, Ca-Ca exchange was not observed for $^{45}$Ca-loaded disks. Its rate was always less than the experimental variability. In the experiment of Fig. 7 A, in which the loaded
disks were comparable to native disks in total Ca content (0.18 and 0.13 Ca/ 
 rho for the loaded and native disks, respectively) and possibly in intradiskal 
 activity (which was at least 0.3 mM for the loaded disks), Ca exchange at 
 room temperature was \( \leq 0.1 \text{ fmol/cm}^2 \text{ s} \), with a half-time of \( >2 \text{ h} \). Because of 
 the comparable conditions, the exchange rate for native disks would be 
 expected to be the same as in Fig. 7 A, if it is assumed that the membranes of 
 the \( ^{45}\text{Ca} \)-loaded disks were not altered by the purification procedure. With an 
 exchange of \( \leq 0.1 \text{ fmol/cm}^2 \text{ s} \), \(<20\% \) of the initial intradiskal \( ^{45}\text{Ca} \) content 
 could have been lost before the measurement shown in Fig. 1. Even smaller 
 losses (reduced by almost an order of magnitude) may have actually occurred 
 if the near \( 0^\circ \text{C} \) temperatures at which the isolations and purifications of Figs. 
 1–3 were performed decreased the exchange rate as much as the net efflux 
 rate. Thus, based on these measurements, it appears that major \( ^{45}\text{Ca} \) losses 
 from disks could not have occurred by exchange diffusion.

The effect of unpurified FROS homogenate on Ca efflux was also tested, 
 since such homogenates (consisting of fragmented outer segments and retinal 
 cells sheared off the retinas) could conceivably contain Ca-releasing enzymes 
 or cofactors. However, Ca release by such factors was undetectable when a 
 fresh homogenate was mixed with a suspension of \( ^{45}\text{Ca} \)-loaded disks (Fig. 4).

In summary, experimental observations do not support the notion that 
 significant \( ^{45}\text{Ca} \) losses occurred from disks while they were isolated and 
 purified for counting.

(b) If Ca binding were to reduce the size of the exchangeable pool within 
 disks, the half-time of these complexes must be comparable to, or longer than, 
 the hour-long incubation periods of the retinas. Such half-times would be 
 three to four orders of magnitude longer than those for other Ca-binding 
 biological molecules (e.g., troponin [Johnson et al., 1979]) and would reflect 
 an extraordinary affinity for Ca. For example, if the half-time of the complex 
 were \( 1 \text{ h} \), the affinity of the intradiskal site would be \( >10^{13} \text{ M}^{-1} \) (diffusion- 
 limited association rate was taken to be \( 2.5 \times 10^8 \text{ M}^{-1} \text{ s}^{-1} \) [Burgen, 1966]). 
 This predicted affinity is much higher than the measured \( 10^4–10^6 \text{ M}^{-1} \) for the 
 strongest sites in bovine disks (Hendricks et al., 1977). In addition, the data 
 obtained during this study do not support the existence of such strongly 
 binding Ca sites. Purified disks were routinely loaded with \( ^{45}\text{Ca} \) for periods of 
 30 min, an interval sufficiently long to exchange Ca at as many as 30\% of the 
 sites characterized by a dissociation half-time of \( 1 \text{ h} \). When X537A was added 
 to a suspension of such disks, all intradiskal \( ^{45}\text{Ca} \) was rapidly released, with no 
 detectable amounts chelated for times as long as a few minutes (Fig. 6). Thus, 
 Ca binding does not seem to have limited intradiskal Ca turnover.

(c) Based on its exchange property and permeability, the plasma membrane 
 is not a rate-limiting barrier for intradiskal uptake of \( ^{45}\text{Ca} \). Ca exchange flux 
 across the plasma membrane of bovine outer segments was recently measured 
 to be 5 pmol/cm\(^2\) s (Schnetkamp, 1979) and is on the order of magnitude 
 observed for most cells (e.g., it is 1 pmol/cm\(^2\) s for frog toe muscle [Kirby et 
 al., 1975]). If Ca exchange across the plasma membrane of frog and bovine 
 outer segments are similar, calculations show that an exchange flux of 1 pmol/ 
 cm\(^2\) s would rapidly exchange cytoplasmic and extracellular Ca.
tion involves solving the equivalent mathematical model for the outer segment: two compartments (intradiskal and cytoplasmic space) in series with an extracellular reservoir. The equivalent model and its solutions in graphical form are presented in Fig. 9. For a flux of 1 pmol/cm² s across the plasma membrane (\( \phi_p \)) and across the disk membrane (\( \phi_d \)), these are the least established. For a bullfrog outer segment with a dimension of 7 x 70 \( \mu \)m, the other parameters are: total surface area of disks, 1.8 x 10⁻³ cm²; surface area of plasma membrane, 1.6 x 10⁻⁵ cm²; total intradiskal Ca content, 0.75 fmol (equivalent to a content of 0.1 Ca/\( \rho \)); Szuts and Cone, 1977), total cytoplasmic Ca content, 4.5 fmol (equivalent to a content of 0.6 Ca/\( \rho \) and is the difference between the total Ca contents of disks and of the entire outer segment; the latter was measured to be ~2 mM or ~0.7 Ca/\( \rho \) by electron microprobe analysis; Yoshikami and Hagins, 1976). (A) Schematic representation of the compartments with their approximate total Ca concentration when extracellular concentration is ~1 mM. Because the resting cytoplasmic Ca activity (or free Ca) is ~10⁻⁶ M, most of the cytoplasmic Ca is bound. (B) Fraction of exchanged cytoplasmic Ca as a function of time and \( \phi_p \) (10⁻¹² and 10⁻¹³ mol/cm² s). \( \phi_d \) = 1 pmol/cm² s for both curves. (C) Fraction of exchanged intradiskal Ca as a function of time and \( \phi_d \) (10⁻¹², 10⁻¹⁵, and 10⁻¹⁷ mol/cm² s). For all three curves, \( \phi_p \) = 1 pmol/cm² s.
membrane, half of the cytoplasmic Ca exchanges within the first 4 min of incubation. This rate is relatively insensitive to Ca flux across the disk membrane because (based on the cited measurements) Ca content in the cytoplasm is greater than in disks. Because incubation periods were 1 h, or 15 times longer than the half-time for cytoplasmic exchange, \(^{45}\text{Ca}\) accumulation by disks could not have been limited by Ca flux across the plasma membrane.

An additional observation in support of this conclusion is the apparent insensitivity of intradiskal exchange to 10 mM extracellular Ca, which leads to a net influx of Ca into the receptors (Yoshikami and Hagins, 1973; Wormington and Cone, 1978) that would have partially overcome the effect of any rate-limiting exchange flux across the plasma membrane.

In view of the above discussion, the small intradiskal \(^{45}\text{Ca}\) content that was observed in the disks of \(^{46}\text{Ca}\)-incubated retinas remains to be attributed to a small unidirectional flux across the disk membrane. Such a small flux appears to be a true property of disks. As shown in Fig. 9 C, an exchange of \(<10\%\) of intradiskal Ca is equivalent to a flux \(<0.01\ \text{fmol/cm}^2\ \text{s}\), which corresponds to \(<5\ \text{ions/disk-second}\) or \(<0.07\ \text{ions/\mu m}^2\ \text{s}\).

**Absence of Effect with Light and Extracellular Ca**

If disks were to accumulate Ca, as predicted by the Ca hypothesis, Ca uptake would be stimulated by illumination or by increased cytoplasmic activity. Such an effect was not observed in intact receptors. In spite of repetitive flashes that collectively photoactivated \(7 \times 10^3\ \text{rho}^*\)/disk (or 0.4% of the pigment), Ca exchange was still \(<0.01\ \text{Ca/rho}\) (Table III). This exchange rate sets an upper limit for active uptake and hence for light-induced release. The rate corresponds to a release of \(<3\ \text{Ca ions/rho}^*\). It is two to three orders of magnitude lower than what the hypothesis predicts if the stoichiometry of release is \(10^2\)–\(10^3\ \text{Ca/rho}^*\) (Cone, 1973; Yoshikami and Hagins 1973).

Intradiskal exchange was also found to be insensitive to cytoplasmic Ca activity. Unidirectional flux, which was measured to be \(<0.01\ \text{fmol/cm}^2\ \text{s}\) (or 5 ions/disk-second, or 0.07 ions/\mu m\(^2\) s) across disk membranes, was not significantly affected by 10 mM extracellular Ca, which is known to hyperpolarize photoreceptors and to increase 10–100 times the cytoplasmic Ca activity (Hagins and Yoshikami, 1974; Wormington and Cone, 1978). The measured rate of \(<5\ \text{ions/disk-second}\) is several orders of magnitude too small for the predicted transport process that can accumulate \(\sim 10^3\ \text{ions/disk-second}\) (or 200 ions/\mu m\(^2\) s) in rat rods. (Calculation was based on the time-course of rat photorepponse [Penn and Hagins, 1972] and on the photorepponse dependence on relative cytoplasmic activity [Hagins and Yoshikami, 1974].) If each disk contains the minimum of one pump, the observed maximum velocity of this putative enzyme would be \(\sim 5\ \text{ions/s}\), an unusually low rate.

Based on the results of the tests described above, this study does not support the hypothesis that light-induced Ca release from disks occurs during either phototransduction or light adaptation within intact receptors. Similar conclusions were drawn by several investigators who studied phototransduction in cones by electrophysiological techniques (Bertrand et al., 1978; Arden and Low, 1978).
Increase in Intradiskal Volume

The decrease in disk density after the isolation of outer segments was an unexpected finding. As far as it is known, such an observation has not been reported. The predominant, if not the sole, cause of this density change appears to be increased intradiskal volume, as discussed in Results. If this interpretation is correct, the intradiskal volume of sucrose-purified disks may be as much as twenty times greater than that of intact disks. The apparent volume increase for freshly isolated disks was not as marked as for sucrose-purified material (Fig. 4). This difference may have been caused either by the absence of sucrose or by the shorter time interval after isolation. The experiments of Figs. 1 and 4 indicate that disks do not swell simultaneously; only a subset of the disk population is initially involved, although all disks eventually achieve a swollen state. (Experiments are currently in progress to determine the magnitude and the ionic mechanism of the swelling process.)

Although very little is known about disk swelling, it has been cited as the source of the well-known refractile change that appears with time in intact albeit isolated outer segments (Falk and Fatt, 1972). In view of this observation, the swelling observed in this study is unlikely to have been an artifact of the employed isolation and purification procedure. It appears that all experiments with disk preparations so far reported in the literature may unknowingly have been performed with swollen material.

The effect of swelling on Ca permeability of disk membranes is unknown. From geometric considerations, the effect would be expected to be minimal because initial surface to volume ratio of disks is so large that a greater than 800-fold increase in intradiskal volume could occur without membrane stretching. On the other hand, disk swelling proceeds by irregular expansions at localized sites (Falk and Fatt, 1973). Because the membrane may be stretched at these sites, the permeability barrier may have been partially disrupted. Thus, Ca permeability for purified disks may be, in fact, greater than that for intact disks.

Absence of Ca-Ca Exchange and Active Ca Uptake

Ca-Ca exchange was undetectable in this study with purified disks. This observation is in conflict with previous studies using bovine disks (Schnetkamp et al., 1977; Schnetkamp, 1979). Schnetkamp et al. (1977) performed their experiments with sucrose-purified disks, which were most likely as swollen as the sucrose-purified material of this study. According to their observation, 1 mM Ca in the suspending medium rapidly released 45Ca from their disks. The time-course of their reported release is shown in Fig. 8 A. Intradiskal Ca activity or Ca content of the bovine disks, however, was not specified in their experiments. When the same experiment was repeated in this study with bullfrog disks, Ca-Ca exchange was undetectable (≤0.4 fmol/cm² s), even though intradiskal Ca activity was at least 0.3 mM (Fig. 8 A). Aside from possible interspecific difference, the use of fresher disks in this study (bullfrog disks were isolated from the retinas only 3–4 h before the onset of the efflux measurements; see Materials and Methods) may explain the difference in results. Because 45Ca efflux from control disks (loaded in the absence of the
ionophore) was also insensitive to extradiskal Ca, it is unlikely that previous exposure to the ionophore could have affected the exchange process shown in Fig. 8A.

Besides Ca-Ca exchange, active uptake by disks was also undetectable in this study. Purified disks were unable to actively sequester Ca, even under conditions that presumably resembled the composition of the cytoplasm (high K saline with 10 μM Ca activity, 2 mM ATP, 2 mM GTP, 20 μM cGMP, 96 μM vitamin E, and 20 mM reduced glutathione). Although this lack of uptake is consistent with the small unidirectional fluxes observed in this study for the disks of intact receptors, it is in conflict with two reports that claimed an ATP-dependent Ca uptake by disks. However, the accuracy of these published observations is now doubtful. The uptake reported by Bownds et al. (1971) was subsequently found to be from mitochondria contamination, whereas the uptake reported by Schmetkamp et al. (1977) was later claimed to be nonreproducible (Schmetkamp, 1979).

Although no other reports have been published on energy-dependent Ca uptake by disks, several studies have focused on the other aspect of this process: Ca-dependent nucleotide triphosphatase activity. A Ca-dependent ATPase had been reported in disk preparations by several investigators (Ostwald and Heller, 1972; Sack and Harris, 1977), but it now appears that the measured activity was probably due to contaminants (Berman et al., 1977). The presence of a Ca-dependent GTPase in frog rod outer segments was only recently reported (Biernbaum and Bownds, 1979). Whether this enzyme is the same light-sensitive GTPase that had been described by others is unknown (Wheeler et al., 1977; Robinson and Hagins, 1979). Although Robinson and Hagins (1979) have not shown that their GTPase is Ca dependent or that it is involved in Ca translocation, they interpret their light-sensitive GTPase activity as the mechanism that "removes transmitter particles previously released in a photon response." Based on the observations of this study, it is unlikely that the GTPase activity observed by Biernbaum and Bownds and by Robinson and Hagins is involved in Ca translocation across disk membranes. Assuming that the GTPase is not associated with some other enzymatic process, the inferred translocation may occur across the plasma membrane.

Ca Permeability Compared with Other Membranes

Ca-loaded disks could rapidly release part of their Ca content whenever their intradiskal activity was increased above 1 mM. As shown in Table III, a 1 mM intradiskal activity leads to an efflux of about 0.2 fmol/cm² s at room temperature. For the same concentration gradient, net efflux is 0.1 fmol/cm² s from ATP-depleted erythrocytes (Lew and Ferreira, 1978) and 0.001-0.1 fmol/cm² s from single-walled phospholipid vesicles (de Blond et al., 1975). Thus, when compared with phospholipid vesicles, Ca permeability across disks is relatively low.

3 Bownds, D. Personal communication.
Summary

Nearly all published reports on Ca flux across disk membranes have been performed with either isolated or fragmented outer segments. Because of the nonphysiological conditions, the reported absence of predicted fluxes in these experiments has been difficult to interpret in terms of the Ca hypothesis. To circumvent this limitation, Ca uptake by disks was tested in this study by a tracer-exchange technique that permits $^{45}$Ca fluxes to occur under physiological conditions as excised frog retinas are incubated in solutions containing trace amounts of $^{45}$Ca. After the termination of the hour-long incubation, the amount of $^{45}$Ca within the disks of isolated and fragmented outer segments was surprisingly small and indicated an exchange of $<0.01$ Ca/rho or $<10\%$ of the total intradiskal Ca.

The tracer-exchange technique has its own-potential limitation: $^{45}$Ca may be lost while disks are isolated and purified for counting. $^{45}$Ca losses, if they occur, could occur either by net efflux or by Ca-Ca exchange diffusion. Net efflux is unlikely to have caused $^{45}$Ca losses because, even under conditions favorable for net losses, intradiskal content remains at least 0.1 Ca/rho (Liebman, 1974; Schnetkamp et al., 1977; Szuts and Cone, 1977), at a level that is more than ten times greater than the exchangeable pool. Similarly, Ca-Ca exchange diffusion is unlikely to have caused significant decrease in intradiskal $^{45}$Ca contents, since it is too slow a process, as was measured here with $^{45}$Ca-loaded disks.

It appears that the low intradiskal $^{45}$Ca content of $^{45}$Ca-incubated retinas indeed reflects a rather low exchange rate between cytoplasmic and intradiskal spaces. This exchange rate corresponds to a unidirectional flux of $<0.01$ fmol/cm$^2$ s or $<5$ ions/disk-second across disk membranes. This low exchange rate within intact receptors is consistent with related disk properties observed for isolated preparations. These properties include the relative impermeability of disks to Ca and their inability to actively accumulate Ca. That intradiskal exchange within intact receptors is insensitive to photostimulation and increased cytoplasmic Ca, conditions that are expected to increase the rate by two to three orders of magnitude, is inconsistent with some aspects of the Ca hypothesis. Thus, if Ca is the intracellular transmitter of phototransduction, this study suggests that it does not originate from the internal space of disks.

The two membranes of the rod outer segment appear to possess contrasting Ca flux properties. As measured in this study, active Ca translocation across disk membranes (from cytoplasm into disks) is less than the measured 5 ions/disk-second, while active transport across the plasma membrane (from cytoplasm to outside) is several orders of magnitude greater (Yoshikami and Hagins, 1978). More interestingly, photostimulation does not seem to alter the active process of disks, whereas recent measurements indicate that it transiently increases net efflux across the plasma membrane (Gold and Korenbrot, 1980). Although Ca may yet be shown to be the intracellular transmitter of phototransduction, the results of this study imply that this is performed not by calcium from the internal space of disks but rather by calcium originating solely from the cytoplasm.
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