J. Physiol. (1985), **368**, *pp.* 641–665
With 2 plates and 8 text-figures
Printed in Great Britain

CALCIUM CONTENT AND CALCIUM EXCHANGE IN DARK-ADAPTED TOAD RODS

BY GORDON L. FAIN* AND WALTER H. SCHRÖDER

*From the Institut für Neurobiologie, Kernforschungsanlage Jülich, Postfach 1913, D-5170 Jülich 1, F.R.G.† and the *Department of Ophthalmology, Jules Stein Eye Institute, UCLA School of Medicine, Los Angeles, CA 90024, U.S.A.*

(*Received 8 January 1985*)

SUMMARY

1. We have used laser-activated micro mass analysis (l.a.m.m.a.) and energy-dispersive X-ray analysis (e.d.x.) to measure Ca content and Ca movements in 'red' rod photoreceptors in the dark-adapted retina of the toad, *Bufo marinus*.

2. Measurements with both l.a.m.m.a. and e.d.x. show that intact rod outer segments contain 4–5 mmol total Ca/l wet tissue volume, or 1–2 Ca per rhodopsin.

3. We could detect no significant variation in the total Ca as a function of distance across or up and down the outer segment. In the inner segment, Ca could be detected only within the mitochondria-rich ellipsoid body, where the total Ca concentration was of the order of 100–400 μmol/l wet tissue volume.

4. To measure the exchange of Ca in outer segments from intact photoreceptors, we exposed the dark-adapted retina to Ringer containing the stable isotope ^{44}Ca. Since l.a.m.m.a. can measure separately the concentrations of each of the isotopes of the elements, and since native rods contain almost exclusively ^{40}Ca, the increase in ^{44}Ca and decrease in ^{40}Ca could be used as a measure of Ca influx and efflux.

5. Ca exchange in intact rod outer segments in darkness is very slow. The rate of accumulation of ^{44}Ca was only 10^5 Ca/rod.s, or about 10% of the total outer segment Ca/h. This slow rate of exchange is apparently not the result of restricted movement of Ca across the plasma membrane.

6. Ca exchange was also measured in outer segments which were either partially or entirely detached from the rest of the photoreceptor. In broken-off outer segments, Ca exchange is faster than in the intact organelles, and in 1 h, half of the ^{44}Ca exchanges for ^{40}Ca.

7. When the retina was incubated in Ringer for which all of the Na was substituted with Li or choline, there was an increase in the rate of ^{44}Ca accumulation in intact outer segments, probably due to an inhibition of Na–Ca counter transport across the plasma membrane.

8. Our measurements indicate that the great majority of the Ca in the rod appears

Authors' names are in alphabetical order.
* To whom reprint requests should be sent.
† Experiments carried out in Jülich.

to be inaccessible to exchange under physiological conditions, probably because it is sequestered within the disks which in intact rods appear to be nearly impermeable to Ca in darkness.

INTRODUCTION

Most of the photopigment in a rod outer segment is contained within closed membraneous sacs called disks which appear to be separated from the plasma membrane and to have no direct contact with the extracellular space (Cohen, 1968; Laties & Liebman, 1970). The bleaching of rhodopsin in a disk is thought to be communicated to other disks and to the plasma membrane by means of one or more internal messengers (see Cone, 1973; Fain, 1985). Yoshikami & Hagins (1971) first suggested that calcium is a messenger substance in rods. Although this hypothesis is now supported by considerable evidence (see Brown, 1979; Gold & Korenbrot, 1981; Kaupp & Schnetkamp, 1982; Korenbrot, 1985), we still know very little about the Ca economy of rods. In spite of considerable experimentation (Hess, 1975; Hagins & Yoshikami, 1975; Hagins, Robinson & Yoshikami, 1975; Szuts & Cone, 1977; Liebman, 1978; Nöll, Stieve & Winterhager, 1979; Schnetkamp, 1979; Szuts, 1980; Walz & Somlyo, 1984; Somlyo & Walz, 1985), it still remains uncertain how much Ca rods contain and where this Ca is located within the photoreceptors. Furthermore, the only evidence we have about the transport and exchange of Ca has been obtained from isolated outer segment preparations (Schnetkamp, 1979, 1980), and these measurements may not be indicative of Ca movements in intact cells.

In order to re-examine Ca content and transport in vertebrate photoreceptors, we have used a new technique called laser micro mass analysis, or l.a.m.m.a. This technique permits the estimation of total Ca content from intact cells in the whole retina with greater sensitivity than standard electron probe microanalysis. It also permits simultaneous measurements of Ca influx and efflux in retinas perfused with the stable isotope ^{44}Ca, since it is possible to measure separately the concentration of the different Ca isotopes in the tissue. Using this method we show that intact rods contain large amounts of Ca concentrated within their outer segments. We also describe some of the properties of Ca transport into and out of the photoreceptors in darkness. Some of these results have been previously published in abbreviated form (Schröder & Fain, 1984*b*; Fain & Schröder, 1985) or were presented at annual meetings of the American Biophysical Society (Schröder & Fain, 1983, 1984*a*).

METHODS

Dissection

Toads (*Bufo marinus*) were obtained from Charles Sullivan (Nashville, TN, U.S.A.), kept in Plexiglas aquaria or in large laboratory sinks, and fed mealworms. We dark-adapted the toads a minimum of 3 h before the dissection. After pithing an animal both rostrally and caudally, we isolated the retina either in dim red light or in darkness, with the aid of an infra-red converter (Find-R-Scope, F.J.W. Industries, Mt. Prospect, IL, U.S.A.). In control experiments, we could determine no significant difference between the Ca content or Ca exchange from retinas isolated under these two conditions (see Results). The eye was hemisected below the ora serrata, and a piece of the eyecup to one side of the optic nerve was cut away with a razor blade and placed into oxygenated Ringer solution. The retina was then carefully removed from the pigment epithelium with fine tweezers, turned over, and placed receptors upwards on a millipore filter. Retinas isolated under these conditions show normal light responses, as demonstrated by the measurement of their electroretinogram.

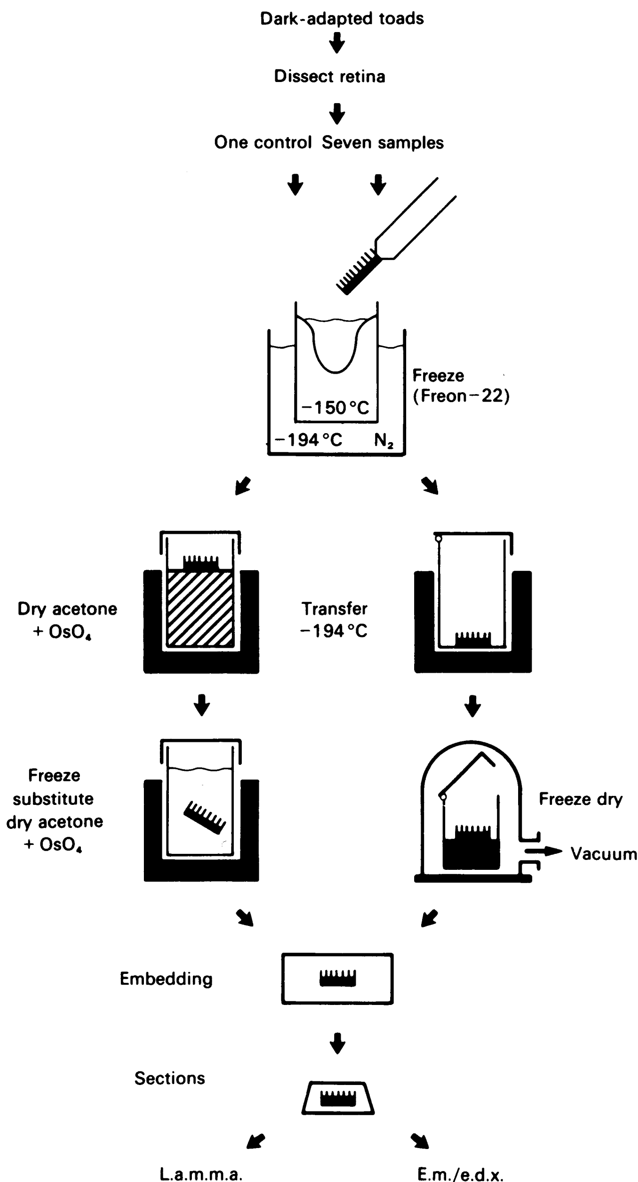


Fig. 1. Flow chart for preparation of tissue for Ca measurements. See text.

In most experiments in this paper, the tissue was processed as outlined in the flow chart in Fig. 1. The retina on the millipore filter was sliced into eight wedge-shaped pieces by a series of radial cuts (as for a pie). One piece was immediately shock-frozen and processed as a control. We then impaled the filter paper at the rims of each of the remaining pieces with insect pins and secured each piece to a 30 mm diameter disk of dental wax. The dental wax was then placed in a 35 mm diameter plastic Petri dish containing an oxygenated Ringer solution. The Ringer was kept oxygenated during the course of an experiment by blowing moistened O_2 at a high rate over the surface of the liquid. Normal Ringer had the following composition (in mM): NaCl, 106; KCl, 2·5; $NaHCO_3$, 0·13; $MgCl_2$, 1·5; $CaCl_2$, 1·8; glucose, 5·6; and *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid (HEPES), 3·0. In most experiments, the normal $CaCl_2$ in the Ringer

(consisting of 97 % ^{40}Ca) was replaced with $^{44}CaCl_2$, which was taken from a stock solution prepared by dissolving $^{44}CaCO_3$ (99 % pure, Rohstoffeinfuhr, Dusseldorf, F.R.G.) in HCl. In the experiments of Fig. 11, the NaCl in the Ringer was substituted (mole for mole) with LiCl or choline chloride. Solutions containing low Ca (for Figs. 3*D* and 4*D*) were made by adding EGTA as in Bastian & Fain (1982). High Ca solutions (for Figs. 3*B*, *C*, and 4*C*) were made simply by adding a given amount of $^{40}CaCl_2$ or $^{44}CaCl_2$ to the Ringer without correcting for the increase in ionic strength or tonicity. All Ringer solutions were brought to pH 7·8, either by the addition of 1–2 ml per litre of 1 M-NaOH or (for Na-substituted Ringer) by adding a measured amount of 1 M-KOH and reducing the KCl accordingly, so that the total K^+ concentration remained 2·5 mM.

Shock-freezing, dehydration, and embedding

Samples of retinas supported by millipore filters were picked up by tweezers, and excess liquid was removed by briefly touching the bottom of the preparation with absorbant tissue. The sample was then quickly immersed into freezing Freon-22 at a temperature of −150 °C. The Freon-22 was contained in a brass cylinder cooled by liquid nitrogen. After the initial freezing step, samples were dipped into liquid nitrogen (−194 °C) as a safety precaution for the following transfer to the cryosubstitution tubes.

In most experiments, the retina was dehydrated by cryosubstitution. Plastic scintillation vials (Packard No. 600817), filled with 15 ml dry acetone containing 0·5 % OsO_4, were placed in tightly fitting holes in a 10 kg brass block. This metal block was temperature-isolated using a wooden case with a 1 cm thick layer of two component polyurethane foam (80 kg/m^3, supplied by Voss Chemie, Ratingen, F.R.G.). A 1 cm gap between the foam layer and the metal block allowed for cooling of the block with liquid nitrogen during sample collection. At the end of the experiment, leftover nitrogen was decanted and the metal block transferred to a −75 °C freezer.

The temperature-rise kinetics of our samples during the cryosubstitution procedure depended mainly on the mass of the metal block and the characteristics of the isolating foam. We selected these parameters so that the temperature in the vials reached −90 °C (the freezing point of acetone) within 3–6 h and then, within the next two days, slowly increased to −75 °C (the critical temperature of recrystallization of vitreous ice into other forms of ice with large, damaging crystals – see Rebhuhn, 1972). After this two-day period, the container was transferred to a −20 °C freezer for one day and then to room temperature for processing.

Samples were immersed three times (15 min each) in dry acetone, transferred to a mixture of 50 % acetone and 50 % Epon-araldite for 8 h, and then placed into Epon-araldite for 24 h. Samples were carefully aligned, and the embedding medium was cured at 70 °C for two days (Luft, 1961).

In some experiments, the tissue was dehydrated according to a procedure which we shall refer to as 'freeze drying'. Retinas, shock frozen as described above, were stored in closed vials without acetone or osmium at −194 °C in the same metal blocks as used for cryosubstitution. At the end of the experiment, the retinas were transferred to an evacuated chamber (at 10^{-7} Torr) and placed on top of a metal disk 7 cm in diameter and 5 cm high, whose temperature was gradually elevated from −194 °C to room temperature over the course of two days. The partial pressure of water was lowered well below 10^{-7} Torr by using a cold trap (−194 °C) close to the retina. The retinas still in the vacuum were then immersed directly into Epon-araldite embedding medium and cured and sectioned as for cryosubstitution.

Sectioning

Sections 0·5 μm in thickness were obtained at room temperature using a Reichert Ultracut microtome and a diamond knife. Sections were floated on distilled water and picked up onto 150-mesh electron microscope (e.m.) grids without support film within 2–3 s to minimize exposure to water. In some experiments we picked up the sections in air, without floating on liquid. We could detect no significant difference in the Ca signals from sections obtained by these two methods. For example, in one series of measurements from a dark-adapted control retina, the outer segment ^{40}Ca signal in sections floated on water was 814 ± 227 (mean ± S.D., $n = 250$), while that from sections picked up in air was 783 ± 194 ($n = 50$). This is in accord with previously published observations (see Schröder, Frings & Stieve, 1980; Ungar, Piscopo, Letizia & Holtzman, 1984). Substantial Ca can be lost, however, if the sections are floated for longer periods of time or with much thinner sections (see Schröder, Frings & Stieve, 1980; Ornberg & Reese, 1980).

We measured section thickness in the following way. The section was first mounted on an e.m.

grid. After analysing the tissue for Ca content with l.a.m.m.a. or energy-dispersive X-ray analysis (e.d.x.), we vacuum-deposited a thick coating of platinum carbon (PtC) onto both of its sides. Grid and section were precisely aligned, re-embedded, and sectioned at an angle perpendicular to the plane of the section. The thickness of the original section was measured by determining the width between the two PtC lines from electron micrographs. We found that with fixed embedding and sectioning parameters, the actual thickness of our sections, though quite constant (within plus or minus 10%) from section to section, could vary as much as 30% from the settings on the microtome. We monitored the section thickness routinely and made individual measurements of thickness for each of the sections used in the calibration of the absolute concentration of Ca in the outer segment (see Results).

L.a.m.m.a. analysis

Ca content was analysed with a LAMMA-500 (Leybold-Heraeus, Cologne, F.R.G.). A complete description of this instrument is given in Wechsung, Hillenkamp, Kaufmann, Nitsche & Vogt (1979) and in Vogt, Heinen, Meier & Wechsung (1980). In brief, the LAMMA-500 uses two lasers, a low-power He–Ne laser and a high-power Nd:YAG pulse laser, which are co-linear. The tissue sections (supported on an e.m. grid) were placed in a vacuum chamber on the stage of a light microscope. The stage was moved to position the low-power laser to a region of interest, and the high-power laser was then activated to vaporize a small area 1–5 μm in diameter. Ions formed during vaporization were accelerated into the field of an electromagnetic lens and detected with a time-of-flight mass spectrometer. Spectra were recorded with a Biomation model 8000 transient recorder with 2000 channels, using a time resolution of 10 ns per channel. Spectra were transferred to a Hewlett Packard 1000 computer (Hewlett Packard, Inc., Palo Alto, CA, U.S.A.) and stored on Hewlett Packard 7906H magnetic disks. The positions of the calcium isotopes were plotted according to their mass in a.m.u. divided by their charge (m/e) and were calibrated with standards of known composition. The values of the ^{40}Ca and ^{44}Ca peaks were determined by means of a peak recognition program in the computer. This program first calibrates the positions of the peaks by comparison to known standards, whose positions are entered into the computer memory at the beginning of each series of measurements. The calcium signals are calculated by integrating within the limits of 39·5 and 40·5 (for ^{40}Ca) and 43·5 and 44·5 (for ^{44}Ca), such that all of the counts of the channels within these limits are summed. The computer also evaluates the amplitudes of the signals of each of the limits (that is, at 39·5, 40·5, 43·5, and 44·5). If these signals are greater than some predetermined value, an error message is printed and the spectrum is rejected. This procedure controls for the possibility of significant overlap between Ca peaks and neighbouring peaks (in particular between the Ca peak at $m/e = 40$ and the K peaks at 39 and 41).

The value of the ^{40}Ca and ^{44}Ca signals can be used to determine the absolute Ca content of the cell by means of a calibration procedure, which we describe below. Whenever this calibration was performed, we have expressed the rod Ca content in units of mmol Ca/l wet tissue volume. When this calibration was not performed on the particular retinal piece from which Ca measurements were made, we express this integral in arbitrary units which are nevertheless consistent from one measurement to the next, to permit comparison of individual measurements within experiments.

Energy-dispersive X-ray analysis

Energy-dispersive X-ray analysis (e.d.x.) was performed with an EDAX 9100 (Phillips, Einhoven, the Netherlands) fitted to a Phillips EM 400 (twin lens) electron microscope. In all e.d.x. measurements we used the cold trap in the column. Sections to be analysed were coated with a thin carbon film (approximately 3 nm thick) to improve mechanical stability. For improved reproducibility of measurements, all conditions such as the high voltage setting, beam current, and spot size were kept as constant as possible. Beam current was determined by measuring the current of the electron beam as it fell on a Faraday cup. This Faraday cup was connected through a 1 MΩ resistor to a high gain, FET differential amplifier in the current-measuring mode. As the beam current cannot be adjusted with high precision, we corrected for beam-current deviation using an empirical formula. Under these conditions, reproducibility of measurements from the same sample was $< \pm 1$%. In most measurements we used a high voltage setting of 120 kV, a beam current of approximately 100 pA, a spot size of 0·23 μm diameter, a model 1EM400T–154–10 detector with an active area of 30 mm^2, and a sampling time of 60 min. The specimen holder was a Philips low-background holder.

The K_α peak for Ca cannot be measured directly from the e.d.x. spectrum from the outer segment, since this peak largely overlaps the larger K_β peak for K. In order to isolate the Ca K_α peak, we first subtracted the background from the spectrum using a linear extrapolation routine for the region of the spectrum including the K and Ca peaks. We then fitted the K_α K peak in the spectrum with the corresponding peak from a K standard using a least squares procedure (as in Shuman, Somlyo & Somlyo, 1976). The fit of the K_α peak in the standard provided an estimate of the amplitudes of both the K_α and K_β K peaks, which were then subtracted from the spectrum. This left the K_α peak for Ca. No attempt was made to correct our spectra for shifts in the resolution or position of the K peak centroids, since the error introduced by such shifts is small by comparison to the Ca signals we typically measure (Kitazawa, Shuman & Somlyo, 1983).

Calibration of Ca concentration: thin film standards

We calibrated the absolute concentration of Ca in our specimens, both for l.a.m.m.a. and for e.d.x., with thin films, vacuum deposited onto the sections (Schröder, 1981). Sections to be analysed were masked with a 1000-mesh grid, and a thin film of CaF_2 was vacuum deposited onto the sections (Schröder & Fain, 1984*b*). At the same time, CaF_2 was deposited onto standards consisting of SiO_2 substrates 50 nm thick, placed next to the tissue sections. The amount of Ca on the standards was used as a measure of Ca deposition onto the sections. This was more accurate and more convenient than directly measuring the Ca deposited onto the sections themselves, because of the lower background of the SiO_2 substrates.

The Ca content of the standards deposited onto the SiO_2 substrates was determined with the e.d.x. system as described in the preceding section and was compared directly to master standards, produced from 1 cm^2 CaF_2 films whose weight was determined on a microbalance. The Ca content of the standards which were produced during CaF_2 deposition onto the sections could then be calculated, and this value could be used to calculate the Ca deposition onto the tissue section. The mask through which the Ca was deposited onto the tissue produced a checkerboard-like array of 25 μm by 25 μm squares, with alternate areas containing the CaF_2 film (see Fig. 1*c* of Schröder & Fain, 1984*b*). Comparative analysis of sample areas in the section with and without the film allowed computation of the absolute amount of Ca present within the samples. The calculated Ca concentration in the rods was corrected for the volume loss during polymerization of the embedding medium, which we measured for each individual batch of resin used (typically 10–15%). This correction was confirmed by comparing the diameters of rods in our sections with the diameters of outer segments in freshly dissected retinas, viewed in the light microscope.

Electron microscopy

Electron micrographs were taken with a Phillips EM 400 electron microscope (Phillips, Einhoven, the Netherlands), fitted with a twin lens and a LaB6 cathode. For the thick sections used in these experiments, we used an acceleration voltage of 120 kV. Sections were mounted onto grids without any support film to reduce background for subsequent e.d.x. or l.a.m.m.a. analysis.

Nomenclature: photoreceptor types in toad

The retina of *Bufo marinus*, as of most amphibians (Denton & Wyllie, 1955), contains two kinds of rods, one with a pigment having a λ_{max} (peak absorption) at 502 nm and commonly called the red rod, and another with a pigment at 433 nm and commonly called the green rod (Harosi, 1975). The red rod is by far the more abundant of the two and has a thinner but longer outer segment. Examples of red rods are given in Pl. 1 (labelled r.r.). Most of the rods in Pl. 1 (including those containing the holes produced by the l.a.m.m.a.) are red rods. The green rods (labelled g.r. in Pl. 1) represent only 17% of the total rod population (Fain, 1976). They have a much shorter outer segment, which is broader than that of the red rods and slightly conical in shape. Their outer segments usually protrude slightly above those of the red rods (Denton & Wyllie, 1955; Fain, 1976).

All of the results of this paper have been taken from red rods, which we refer to in the text simply as 'rods'.

RESULTS

Plate 1 illustrates the typical appearance of the retina after shock freezing, sectioning, and l.a.m.m.a. analysis. The retina in Pl. 1 was removed from a dark-adapted toad and was frozen directly after the dissection as a control. It was dehydrated by freeze substitution (see Methods). The preservation of the tissue is clearly best in the distal outer segment (o.s. in Pl. 1) of the receptors, which was the part of the rod most immediately exposed to the freezing medium and therefore most rapidly frozen by our technique. This part of the rod showed no apparent morphological deformation nor any evidence of ice crystal formation. Higher magnification electron micrographs of the distal outer segment (Schröder & Fain, 1984*b*; Fain & Schröder, 1985) show that the disk and plasma membranes are intact and well preserved, and that the disks are stacked regularly with approximately the same repeat distance as in chemically fixed (Cohen, 1973) or fresh (Chabre, 1975) material.

The proximal half of the outer segment and the inner segment, which were more slowly frozen by our procedure, are less well preserved. The proximal outer segment shows gross striations and other evidence of tissue damage. The striations appear to lie along the lines of the incisures and may be formed by preferential ice crystal formation in these regions. There are also larger gashes in the tissue and obvious structural disorganization, probably also caused by ice crystal formation. In the inner segment, the mitochondria of the ellipsoid region (e. in Pl. 1) are clearly visible though somewhat more disorganized and less well preserved than in chemically fixed tissue. The nucleus (n. in Pl. 1) can also still be observed, though the chromatin is sparse and irregular. Below the nucleus, there is obvious damage to the membranes of the cells, and it was usually not possible to trace the rest of the photoreceptor down to its synaptic terminal.

Ca content of rod outer segment: measurements with l.a.m.m.a.

In order to determine the concentration of total Ca in the outer segment, we made measurements with both l.a.m.m.a. and e.d.x. on dark-adapted rods in retinas prepared according to two alternative protocols (see Methods). Fig. 2 shows a typical spectrum for low atomic weight, positively charged ions obtained with the l.a.m.m.a. technique on a shock-frozen retina prepared by freeze substitution in acetone and 0·5 % OsO_4. Similar results were obtained from retinas preserved by shock freezing and freeze drying (see Fig. 2 of Schröder & Fain, 1984*b*). Since the microplasma produced by the high-energy laser of the l.a.m.m.a. has been shown to consist almost exclusively of singly charged particles (Wechsung *et al.* 1979; Vogt *et al.* 1981), the m/e ratios in the spectrum give the atomic weights (in a.m.u.) of each of the analysed ions directly. In Fig. 2, we have tentatively identified the peaks which correspond to the principal isotopes of several cations presumed to be present in the photoreceptor. These include ^{23}Na, ^{39}K and ^{41}K, ^{24}Mg, and the two stable isotopes of Ca.

There are two identifiable peaks for Ca, the larger of which is that of ^{40}Ca, which has a natural abundancy of about 97 %. There is some overlap between the ^{40}Ca peak and the adjacent peak for ^{41}K, but this was less than 1 % of the total Ca signal and was below the threshold for rejection by the peak recognition program (see Methods).

A small but significant signal can also be obtained from the less common ^{44}Ca. Although the amplitude of the ^{44}Ca peak is difficult to determine in single spectra, averages of many spectra show it to be 2–3 % of the amplitude of the ^{40}Ca peak. For example, in one series of measurements from fifty outer segments from the same control piece of dark-adapted retina, the mean ^{44}Ca signal was $2{\cdot}8 \pm 0{\cdot}4$ % of the mean ^{40}Ca signal. This is only slightly higher than the ratio predicted by the natural abundancies of these isotopes (2·2 %).

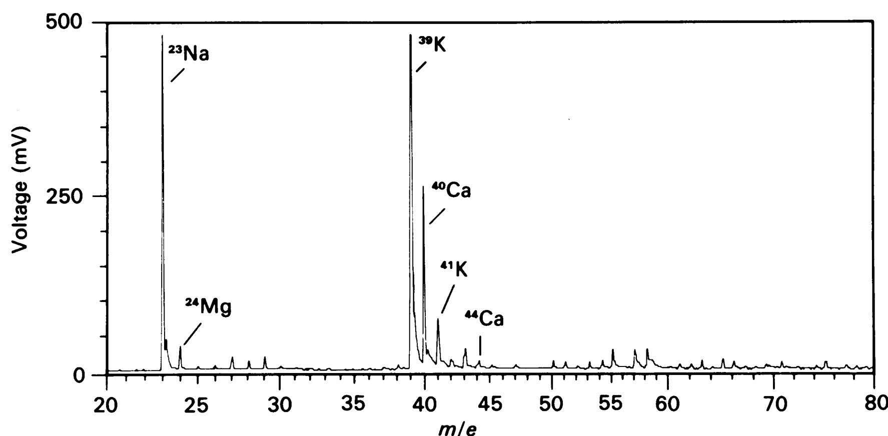


Fig. 2. L.a.m.m.a. spectrum from 'red' rod outer segment of dark-adapted retina, dissected under infra-red illumination and frozen immediately after the dissection as a control. Retina was dehydrated by freeze substitution in acetone and 0·5 % OsO_4. Spectrum shows only low atomic weight, positively charged species. ^{40}Ca peak corresponds to approximately 5 mmol total Ca/l wet tissue volume (see text). Concentrations of other elements cannot be estimated directly from this spectrum, since the efficiency of ion production and sensitivity of the l.a.m.m.a. vary for different elements (Vogt *et al.* 1981). Ordinate gives the voltage signal from the secondary electron multiplier tube after amplification. Since the gain of this amplification can be varied, the amplitudes of the voltage signals in the spectra in this paper (and in Schröder & Fain, 1984*b*) are not necessarily comparable.

Although the isotope ratios for Ca and for the other elements, as well as the results of experiments on other preparations (Schröder *et al.* 1980) tend to confirm the element identifications for the m/e values we have given in Fig. 2, it seemed useful to provide independent evidence for these identifications for the rod outer segments. In this paper we present such evidence only for Ca, using experiments in which we have changed the extracellular Ca concentration. We shall give only a brief description of these experiments in this paper and shall reserve a more detailed presentation for a later publication (G. L. Fain & W. H. Schröder, in preparation).

In Fig. 3*A*, we show a control spectrum from the same retinal piece as that of Fig. 2, expanded to show only the part of the spectrum for positively charged ions from $m/e = 40$ to $m/e = 44$. This should be compared to the spectrum in Fig. 3*B*, which shows the results of an experiment in which a piece of retina was incubated for 30 min in a Ringer containing 20 mM-$^{40}CaCl_2$. In four separate experiments,

incubation of the tissue for 30 min in 20 mM-^{40}Ca produced a large increase in the signal at $m/e = 40$, corresponding to 223 ± 95 % (mean $\pm$S.D. from forty outer segments, ten from each retina) of its mean amplitude measured in outer segments ($n = 40$, ten each from four pieces) from these same retinas, shock-frozen at the

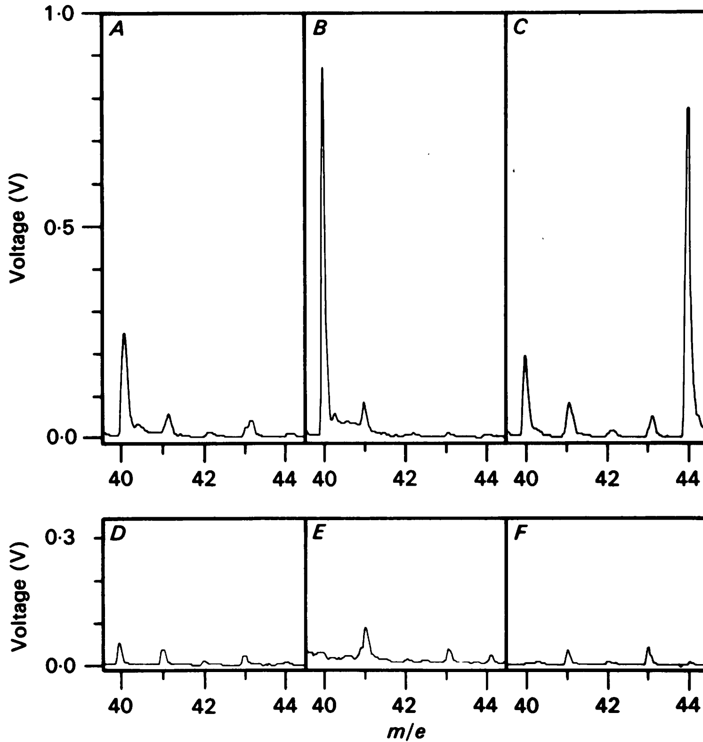


Fig. 3. L.a.m.m.a. spectra for identification of Ca peaks. All spectra are from measurements made from the outer segments of 'red' rods from dark-adapted retinas. Ordinates for spectra as in Fig. 2. *A*, control spectrum from retina frozen immediately after dissection. Same retinal piece as for Fig. 2. *B*, spectrum from retina incubated in darkness for 30 min in Ringer containing 20 mM-^{40}Ca. Note that peak at $m/e = 40$ is much larger than in control spectrum. *C*, spectrum from retina incubated in darkness for 30 min in Ringer containing 20 mM-^{44}Ca. Here peak at $m/e = 40$ is approximately the same as in the control, but peak at $m/e = 44$ is much larger. *D*, spectrum from retina incubated in darkness for 30 min in Ringer for which the Ca was buffered with EGTA to produce a free-Ca concentration of 10^{-9} M. Note reduction in peak at $m/e = 40$. *E*, spectrum from *same rod outer segment* as in *B*, after section has been floated for 30 min (15 min on each side) on a solution of 50 mM-EGTA at pH 7·0. Large peak at $m/e = 40$ in *B* is absent in *E*, ^{41}K peak is unchanged. Peak at $m/e = 39$ (^{39}K) is also unchanged (not shown). *F*, spectrum from same section as for *A*, but with laser aimed to one side of the photoreceptors into the embedding medium. All spectra are at same amplifier gain.

beginning of the experiments as controls. In Fig. 3*C*, we show a spectrum from a similar experiment, but in this case the retina was exposed for 30 min to 20 mM-$^{44}CaCl_2$. Here the peak at $m/e = 40$ is the same as in the controls (89 ± 20 %, mean$\pm$S.D. from thirty outer segments, ten each from three retinas, as a percentage of mean of thirty control outer segments, ten each from different pieces of same three

retinas), but the signal at $m/e = 44$ is much larger ($232 \pm 29\%$). These experiments indicate that large increases can occur in the signals at $m/e = 40$ and at $m/e = 44$, corresponding to accumulations of ^{40}Ca and ^{44}Ca in the photoreceptors.

When the retina was exposed to low Ca solutions, there was a progressive decrease in the amplitude of the Ca signals, most easily seen for the larger ^{40}Ca peak (Schröder & Fain, 1983). Fig. 3*D* is a representative spectrum from an outer segment of a piece of retina incubated for 30 min in a Ringer having a free-Ca concentration of 10^{-9} M (buffered with EGTA, see Methods). The mean amplitude of the ^{40}Ca peak is $10.1 \pm 5.6\%$ (mean $\pm$ S.D., thirty outer segments, ten each from three retinas) of that of the mean Ca signal from thirty outer segments from control pieces of the same retinas, fast frozen immediately after the dissection. The decrease in the amplitude of the $m/e = 40$ signal again confirms our identification of this signal as coming from ^{40}Ca. The amplitude of the $m/e = 40$ peak after this 30 min EGTA incubation provides an upper limit for the contribution to this peak of organic ions or other sources of extraneous background from the cytosol of the rod outer segment.

This upper limit is probably an over-estimate, since incubation of the thin sections themselves in EGTA gave no detectable signal at $m/e = 40$. This can be seen in the experiment of Fig. 3*E*, where the same section of retina used for the analysis of Fig. 3*B* was floated on a solution containing 50 mM-EGTA at pH 7.0. Each side of the section was exposed for 15 min to this solution. The section was then rinsed in distilled water, dried, and re-analysed. The spectrum which we show in Fig. 3*E* was taken from the same photoreceptor used for the spectrum of Fig. 3*B*, with the laser spot positioned approximately 10 μm further along the outer segment. Although this photoreceptor showed a large peak at $m/e = 40$ before the EGTA incubation, no detectable signal could be measured afterward. The disappearance of the Ca signal was not the result of some non-specific disruption of fixation or wash-out of ionic contents during the EGTA exposure, since the structure of the photoreceptor was not visibly altered by this procedure, and the amplitude of the ^{41}K peak (as well as that of the ^{39}K peak, not shown) remained nearly unchanged.

As a final control, we measured the contribution to the Ca peaks of the embedding medium used to prepare the tissue. L.a.m.m.a. measurements were made from the same section as that used for Fig. 3*A* but with the laser spot positioned so that it was distal to the tips of the photoreceptors. A typical result is given in Fig. 3*F*. The Ca content is below the limit of detection of the l.a.m.m.a. device (40 μm/l wet tissue volume under these conditions).

In order to relate the amplitude of the ^{40}Ca signal to the actual concentration of Ca in the outer segment, we evaporated a thin film, CaF_2 standard directly onto the outer segment through a mask, as described in the Methods (see also Schröder & Fain, 1984*b*). We performed this calibration on two pieces of tissue, both from dark-adapted retinas dissected in dim red light and fast frozen immediately after the dissection. Measurements were made only from well-preserved, well-oriented rods, and one spectrum was taken from each of several outer segments in the two retinas. The placement of the laser within the outer segments was random, in some cases at the tip, in others in the middle or at the base. However, as we shall show, there is no significant variation of Ca content within the outer segment (see Table 1).

The Ca content of rods which we calculated from these measurements was

$4{\cdot}4 \pm 1{\cdot}8$ mmol Ca/l wet tissue volume (mean ±S.D., $n = 14$) for one retina and $4{\cdot}7 \pm 1{\cdot}8$ mmol/l wet tissue volume ($n = 21$) for the other. Using a volume for the rod outer segment of 10^{-12} l and a rhodopsin concentration of 3 mM (Fein & Szuts, 1982), we calculate that the Ca concentration we measure is equivalent to $3\text{–}5 \times 10^{9}$ Ca atoms per rod outer segment, or 1–2 Ca per rhodopsin molecule.

As controls for these determinations, we made a direct comparison of the Ca content of rod outer segments from two retinas dissected in dim red light and three dissected in darkness, with the aid of an infra-red converter. Ten outer segments were measured from each of the retinas. The amplitudes of the ^{40}Ca signals (in arbitrary units – see Methods) from the recorded spectra were 816 ± 181 (mean ±S.D., red light) and 818 ± 181 (infra-red). These are clearly not significantly different.

In order to investigate the effects of our fixation methods on the amplitude of the Ca signal, we incubated two pieces of retina in each of three experiments for 1 h in ^{44}Ca Ringer in darkness and then treated the two pieces identically, except that one of the two pieces in each experiment was dehydrated by freeze substitution and the other by freeze drying. When the Ca measurements were pooled, the pieces dehydrated by freeze substitution gave a ^{40}Ca signal of 604 ± 147 and a ^{44}Ca signal of 128 ± 45 (mean ±S.D., $n = 61$), whereas the pieces dehydrated by freeze drying gave a ^{40}Ca signal of 666 ± 147 and a ^{44}Ca signal of 152 ± 37 (mean ±S.D., $n = 61$). Comparison of these measurements by the Student's t test indicated that both the ^{40}Ca and the ^{44}Ca levels were significantly higher in the retinas dehydrated by freeze drying ($P > 98\,\%$), suggesting that freeze drying preserves the Ca in the tissue better than freeze substitution. However, the total Ca signal in the freeze-dried rods was only about 10 % larger than that in the freeze-substituted tissue, so that the effect of fixation method on our Ca measurement was rather small. We also compared the Ca contents of rods from retinas prepared by freeze substitution in the normal acetone–OsO_4 medium (see Methods) and in a similar medium containing 2 mM-oxalic acid (Ornberg & Reese, 1980), but in this case no significant difference could be detected.

Ca content of rod outer segment: measurements with e.d.x.

In order to test the reliability of the l.a.m.m.a. technique, we measured rod Ca levels from the same specimens used for the l.a.m.m.a. measurements with e.d.x. To do this, we modified the Phillips EM400 in order to measure and correct for alterations in beam current (see Methods), and we collected data for long sampling times, typically 60 min. Fig. 4*A* shows a representative e.d.x. spectrum before background subtraction from a dark-adapted rod outer segment dehydrated by freeze substitution. The K_α peak for Ca is largely obscured by the larger K_β peak for K. To isolate the Ca K_α peak, we removed the K_β K peak and the background as described in the Methods. The isolated Ca K_α peak for the spectrum in part *A* of this Figure is shown below in *B*.

In order to confirm our method of isolation of the Ca peak, we applied the same methods in Fig. 4*A* and *B* to retinas which had been exposed to high and low Ca solutions. Two representative spectra are given in Fig. 4*C* and *D*. In *C*, we show the K_α Ca peak isolated from a spectrum taken from an outer segment in a retina exposed for 30 min in darkness to a Ringer containing 20 mM-^{44}Ca. The increase in the amplitude of the Ca signal is similar to that previously described for the

l.a.m.m.a. measurements (see Fig. 3*B* and *C*). Note that e.d.x. cannot distinguish the different Ca isotopes. In *D*, we show the K_α Ca peak for an outer segment from a retina exposed for 30 min in darkness to 10^{-9} M-free-Ca Ringer. This should be compared to the corresponding l.a.m.m.a. measurement in Fig. 3*D*.

We calibrated the amplitude of the K_α Ca signals in control retinas (as in Fig. 4*B*) using a thin film standard similar to those used for the l.a.m.m.a.

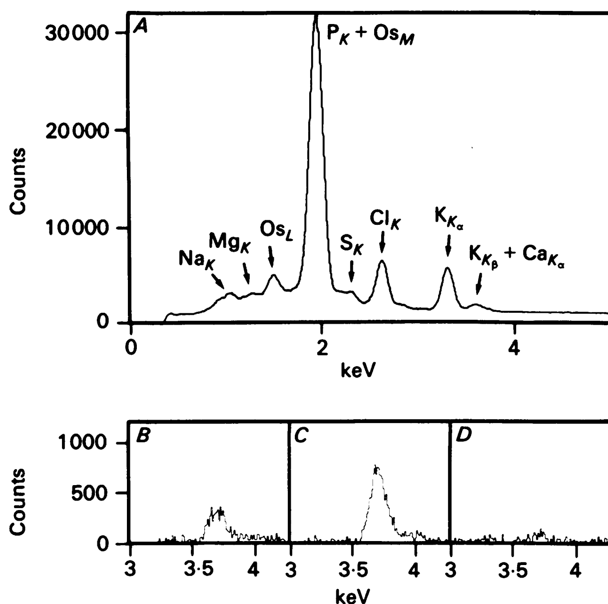


Fig. 4. E.d.x. spectra from dark-adapted rod outer segments. All retinas were dehydrated by freeze substitution in acetone and 0·5 % OsO_4. *A*, complete spectrum for control retina dissected under infra-red illumination. Same retina as for Figs. 2 and 3*A*. The location of the major peaks for ions of physiological interest are indicated on the spectrum. The *K* line for phosphorus is largely obscured by the *M* line for osmium (Os). The K_α peak for Ca is also obscured by the larger K_β peak for K. *B*, K_α peak for Ca from the spectrum of *A* after subtraction of background and the K K_α and K_β peaks. See Methods. *C*, K_α peak for Ca after background and K subtraction from a retina incubated for 30 min in darkness in a Ringer containing 20 mM-^{44}Ca. *D*, same as in *C* but from a retina incubated for 30 min in darkness in a Ringer having a free-Ca concentration of 10^{-9} M. K_α, K_β, *L*, and *M* refer to the characteristic energies of the X-ray emissions from the elements of the sample.

measurements. Five determinations were made in different outer segments from each of three dark-adapted retinas. These gave a mean outer segment Ca content of $5{\cdot}18 \pm 1{\cdot}31$ mmol/l wet tissue volume (mean ±S.D., $n = 15$). This is not significantly different from the values obtained with l.a.m.m.a.

Sources of error

The l.a.m.m.a. method is intrinsically quite precise, with repeated measurements from thin film Ca standards varying by less than ±5 %. However, our estimates of rod Ca content are less accurate, principally because of sample variability, that is,

intrinsic differences in Ca content among rods. In order to estimate the variability in outer segment ^{40}Ca content, we made measurements from a large number of rods in the same retinal piece. In the most extensive series, 250 outer segments were analysed from the same dark-adapted control in a retina dissected in darkness with the aid of an infra-red converter and dehydrated by freeze substitution. These measurements gave a mean ^{40}Ca signal of 814 (arbitrary units), with a standard deviation of 227 or 28 % of the mean. Less extensive measurements from other dark-adapted retinas gave values of the sample S.D. which ranged between 10 and 30 % of the mean. The large S.D. of our measurements may indicate physiological differences in total Ca among rods, or it may be the result of an artifactual loss of Ca from the outer segments which, for some reason, was greater from some rods than from others.

In addition to sample variability, our measurements are subject to error in the absolute calibration of our Ca signals. The largest source of error in the Ca standard film calibration is the weighing of the master standards on the microbalance, which we have estimated to be no greater than ± 10 % of the mean by repeated weighings of the same samples. E.d.x. measurements of the thin film standards were extremely precise ($< \pm 1$ %), since the background of the SiO_2 substrate is low. Measurement of the thickness of the retinal sections from which Ca measurements were made is also probably accurate to within ± 10 %, and variations in thickness within the section were negligible. In sum, the error in the calibration is probably no greater than ± 30 %. This is approximately as large as the largest values for the S.D. of our measurements. Since these two sources of error are multiplicative in our determination of the absolute Ca content of the outer segment, our mean values of Ca content can be considered to be accurate to within a factor of 1·5–2.

Distribution of Ca within the rod

In order to investigate the distribution of Ca within the rod, we systematically positioned the laser spot in different parts of the cell. We first examined possible differences up and down the length of the outer segment. Seven equally spaced holes were made in thirty well-aligned rods. The first was made at the tip of the outer segment and the last at the base. The measurements at the tip were then averaged and set to 100 %. The means and S.D.S at the other positions, normalized to the mean Ca signal at the tip, are given in Table 1. These measurements reveal no significant variation in the outer segment Ca content along the length of the rod.

We also attempted to investigate possible differences in Ca from one side of the outer segment to the other. We placed holes 3–4 μm in diameter at three positions, one at either edge of the outer segment and one in the middle. Since the outer segment is only 7–8 μm in diameter, these holes overlapped somewhat and had to be placed at different distances along the long axis of the cell. These measurements also showed no significant variation in Ca content.

In the inner segment, we could detect significant Ca only in the mitochondrial-rich ellipsoid region. Ca levels in the cytoplasm between the ellipsoid and nucleus or in the nucleus itself were below the limit of detection of the l.a.m.m.a. technique (about 40 μm Ca/l tissue volume). Even in the ellipsoid, the Ca content was much lower than in the outer segment. Note the tenfold difference in the voltage scales in Fig. 5, which compares spectra taken from the outer segment and ellipsoid of the same

TABLE 1. Distribution of Ca in rod

Position	Mean (%)	S.D. (%)
	Outer segment	
1: tip	100	11·7
2	107·3	8·3
3	101·7	11·7
4: middle	93·4	9·6
5	105·0	6·0
6	103·1	10·5
7: base	108·2	10·0
	Inner segment	
Ellipsoid	7·7	4·7
Cytoplasm	< 1	
Nucleus	< 1	

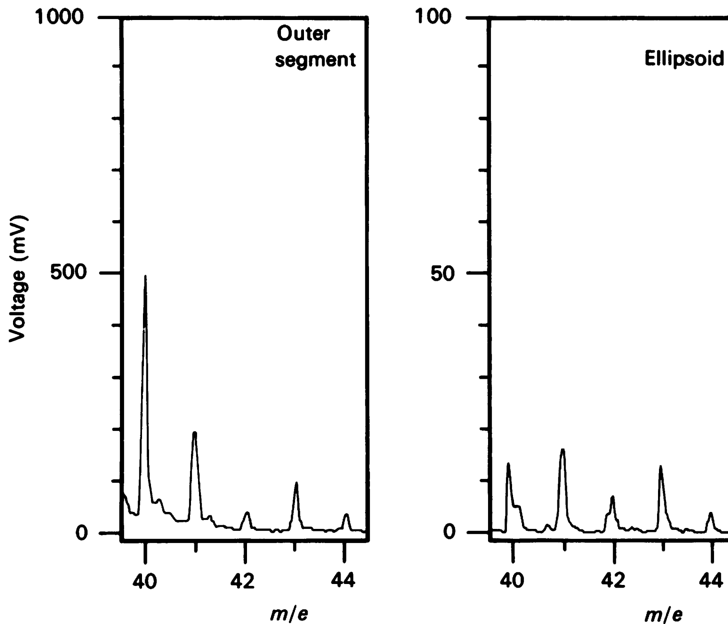


Fig. 5. Comparison of l.a.m.m.a. spectra from outer segment and ellipsoid of same rod in dark-adapted retina. Retina dissected under infra-red illumination and dehydrated by freeze substitution. Same retinal piece as for Figs. 2 and 3*A*. Note difference in voltage scales between two parts of this Figure.

dark-adapted rod. In a series of measurements from thirteen dark-adapted receptors (seven from freeze-substituted retinas and six from freeze-dried), the Ca content in the ellipsoid was only $7{\cdot}7 \pm 4{\cdot}7$ % of that in the outer segment. A second series of measurements from twenty dark-adapted rods all from retinas dehydrated by freeze substitution gave $4{\cdot}7 \pm 2{\cdot}9$ %. The Ca concentration of the ellipsoid of a dark-adapted rod is therefore of the order of 100–400 μmol/l tissue volume.

Fluxes in darkness

In order to examine exchange of Ca in darkness, we placed five pieces of a dark-adapted retina into a Ringer which was normal in its composition except that all of the Ca (which is normally 97 % ^{40}Ca) had been replaced with the stable isotope ^{44}Ca (see Methods). Since rods before immersion into this solution contained almost

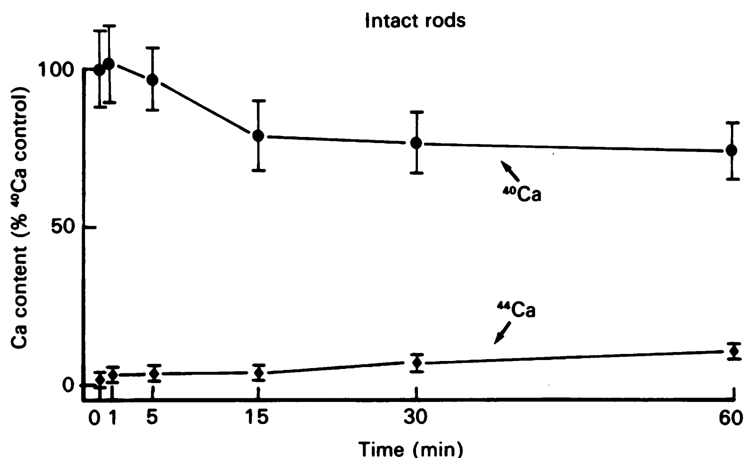


Fig. 6. Ca exchange in intact rod outer segments in darkness. Data points give the means and one standard deviation for ten measurements each from four to six retinas, processed according to Fig. 1 and dehydrated by freeze substitution. The number of retinas was variable, since pieces for some of the time points were unusable, either because the retina was folded over or poorly fixed, or because it was lost during the fixation or dehydration. Data points at time zero give the Ca content for the control pieces, which for each retina were frozen directly after the dissection. The other pieces of retina were incubated in darkness in Ringer containing the normal (1·8 mM) Ca concentration, but with all of the ^{40}Ca substituted with ^{44}Ca. These pieces were removed from this solution at 1, 5, 15, 30 and 60 min and immediately frozen. Data points give the ^{40}Ca (●) and ^{44}Ca (◆) content, normalized to the mean ^{40}Ca content in the control pieces. The mean ^{40}Ca signal for the intact outer segments in the controls was 817 ± 179 ($\pm$S.D., arbitrary units).

exclusively ^{40}Ca in their outer segments (see Figs. 2 and 3*A*), the increase in ^{44}Ca in the cells could be used as a measure of Ca accumulation. Similarly, the decrease in ^{40}Ca could be used as a measure of the quantity of Ca leaving the cell.

The results of these experiments are given in Fig. 6. The data points give the means and one standard deviation, based upon measurements taken from ten rods each from between four and six retinas. The data plotted at time zero were taken from control pieces, which in each of the retinas were frozen immediately after the dissection. The decline in ^{40}Ca in the rods (circles) is about 20 % of the total Ca/h and is best fitted by linear regression with a slope of about 0·5 %/min, or 3×10^5 Ca/rod.s, assuming a dark Ca concentration of 4·5 mmol/l tissue volume. The increase in ^{44}Ca (triangles) is less than this, amounting to about 10 % of the total rod Ca/h. It is best fitted with a slope of about 0·15 %/min, or 1×10^5 Ca/rod.s. The low rate of ^{44}Ca entry into the rods demonstrates that Ca exchange in intact, dark-adapted rods

occurs very slowly in normal Ringer. Since ^{40}Ca leaves the rod at a greater rate than ^{44}Ca accumulates inside, the total rod Ca content declines with time in our preparations, at the rate of about 10% of the total Ca/h. We presume that this is the result of a slow deterioration of the isolated retina preparation.

Ca fluxes: broken-off outer segments

In many of our specimens, we observed rod outer segments partially or completely broken off from the rest of the photoreceptor. These broken-off outer segments had

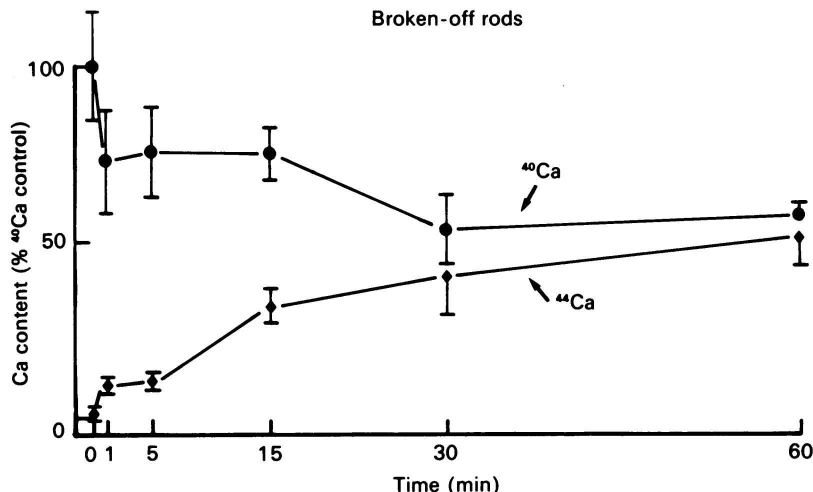


Fig. 7. Ca exchange in broken-off rod outer segments in darkness. Measurements taken from the same retinal pieces as for Fig. 6. All measurements were made from outer segments which were partially detached from the rest of the receptor (as in Pl. 2) or which were completely detached and resting above the tips of the intact photoreceptors. Measurements were made from at least two retinas at each time point, and the total numbers of outer segments used in each measurement were as follows: control, 25; 1 min, 24; 5 min, 24; 15 min, 16; 30 min, 22; and 60 min, 11. Data points give the ^{40}Ca (●) and ^{44}Ca (◆) content, normalized to the mean ^{40}Ca content of the broken-off outer segments in the control pieces. The mean ^{40}Ca signal for the broken-off outer segments of the controls was 773 ± 240 ($\pm$S.D., arbitrary units) and was not significantly different from that for the intact outer segments.

about the same total Ca concentration as intact ones, but they showed a much higher rate of Ca exchange. We give an example of such a rod in Pl. 2. This rod is from a preparation which had been incubated for 5 min in ^{44}Ca Ringer in darkness. Even though the upper part of the outer segment remains attached to the lower part, the two halves of the cell behave independently. The lower half shows high ^{40}Ca and low ^{44}Ca, typical of intact rods (see Fig. 6). The upper half shows much less ^{40}Ca and much more ^{44}Ca, indicating higher rates of Ca exchange. The total amount of Ca in the two halves of the rod is about the same.

In Fig. 7 we plot the ^{40}Ca and ^{44}Ca levels as a function of time for broken-off outer segments, for the same retinas as in Fig. 6. Most of the outer segments used for these measurements were completely broken off with no apparent connexion to the inner

segment. In the majority of cases, the outer segments were lying above the rest of the retina, with their long axes perpendicular to those of the outer segments. Not all pieces of retina contained broken-off outer segments, and the number of them suitable for analysis was rather small and variable from retina to retina. However, each of the means and S.D.S in Fig. 7 are based on measurements from at least eleven outer segments from a minimum of two retinas.

The data in Fig. 7 confirm for a large population of broken-off outer segments the general impression given by the cell in Pl. 2. The rates of ^{40}Ca exit and ^{44}Ca entry are much greater than for intact cells, with about half of the total Ca exchanging per hour. However, the total Ca concentration does not change with time and is comparable to that in control, intact outer segments: the Ca signal in control intact outer segments was 817 ± 179 (mean $\pm$S.D., arbitrary units), while that in the broken-off outer segments of the control retinas was 773 ± 240. These are not significantly different.

Ca fluxes: Na-substituted Ringer

Since previous experiments have suggested that much of the Ca movement across the rod plasma membrane occurs by way of Na–Ca counter transport (see Schnetkamp, 1980; Fain & Lisman, 1981; Kaupp & Schnetkamp, 1982), we examined the effect of Na substitution on Ca fluxes in intact rods in darkness. We substituted all of the Na in the Ringer with either lithium or choline, since neither lithium nor choline would be expected to replace Na in Na–Ca exchange (Schnetkamp, 1980; Yau & Nakatani, 1984; see also Requena, 1983). As in previous experiments, the $CaCl_2$ in the Ringer contained exclusively ^{44}Ca.

The results of these experiments are given in Fig. 8. All of the data in this Figure are from intact outer segments of 'red' rods, measured from pieces of dark-adapted retinas incubated in darkness. In both graphs the data for the measurements in Na-substituted Ringer are given as the squares (^{40}Ca) and triangles (^{44}Ca), with their associated error bars. Each data point and bar gives the mean and one standard deviation for thirty outer segments, ten each from three retinas. These are compared to the ^{40}Ca and ^{44}Ca levels from outer segments in Na, taken directly from Fig. 6. These results show that substitution for Na causes a clear increase in ^{44}Ca accumulation. Similar effects have been demonstrated for isolated outer segments (Schnetkamp, 1980) and in squid giant axon (see Blaustein, 1974; Mullins, 1979; Requena, 1983). The effects of Na substitution on Ca efflux are less clear. In the choline-substituted Ringer, there appeared to be a decrease in the rate of decline of ^{40}Ca in the tissue. Such an effect was not observed in the Li substitution experiment. The reason for this difference is unknown.

DISCUSSION

The Ca content of dark-adapted rods

Our results show that a dark-adapted toad rod contains large amounts of Ca concentrated within its outer segment. This conclusion is based upon measurements made with two different methods of microanalysis (l.a.m.m.a. and e.d.x.) upon

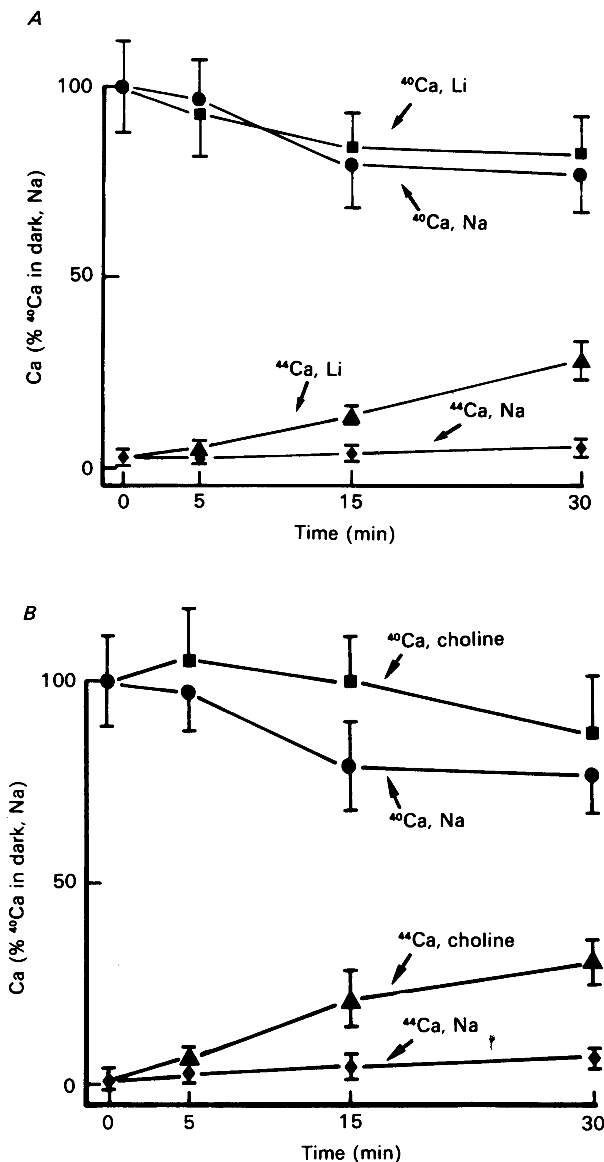


Fig. 8. Ca exchange in Na-substituted Ringer. Retinas were incubated in ^{44}Ca in darkness as in Figs. 6 and 7, but with all of the Na in the Ringer substituted with Li (*A*) or choline (*B*). Data points in Na-substituted solutions give the mean and one standard deviation for the ^{40}Ca (■) and ^{44}Ca (▲) content, averaged from ten measurements each in three retinas. All measurements are from intact 'red' rod outer segments, and all of the retinas were dehydrated by freeze substitution. Results in Na-substituted solutions are compared to the ^{40}Ca (●) and ^{44}Ca (◆) content from normal (Na) Ringer, taken directly from Fig. 6. All data were normalized to the ^{40}Ca content of control pieces of retina, frozen directly after each dissection. The controls produced for each of the three series of experiments in Fig. 8 (in Na, Li, and choline) were used to normalize the data in each of these series individually. The control data plotted in Fig. 8 include only the measurements taken from the Na experiments, but there was no significant difference in the mean ^{40}Ca content of the controls produced during the three sets of experiments.

retinas which have been fast-frozen and dehydrated by two alternative protocols. Since our measurements have been made from intact rods in whole retina, we believe they represent the true concentration of Ca in the photoreceptors under physiological conditions. Although we cannot exclude the possibility that some Ca may have been lost from the rods during tissue preparation, we have attempted to minimize this loss at least for the outer segment by using a method of rapid freezing which faithfully preserves the morphology of the majority of this part of the cell (Schröder & Fain, 1984*b*; Fain & Schröder, 1985). Since the total outer segment Ca concentration we have measured is nearly as large as the largest values obtained from isolated outer segments (Schnetkamp, 1979, 1980; U. B. Kaupp, personal communication), we believe loss of Ca from this part of the rod to have been small.

In the inner segment, on the other hand, there is a greater likelihood of Ca loss, since the morphology of the tissue in our preparations is much more poorly preserved. However, it is unclear what effect this might have had upon the preservation of Ca in the cell, since the concentration of the Ca appeared to be uniform within the outer segment (see Table 1), in spite of large differences in structure preservation between the distal and proximal halves. Furthermore, in a sample of over 100 rods from dark-adapted and light-exposed retinas (G. L. Fain & W. H. Schröder, unpublished), we have without exception observed a large difference in Ca content between the most proximal part of the outer segment (position 7 in Table 1) and the most distal part of the inner segment containing the mitochondria, even though there was no apparent difference in preservation between these two regions. We believe that our measurements in the proximal outer segment and ellipsoid reflect their Ca concentrations under physiological conditions, but we cannot be certain of this. We are even less certain of our measurements from the myoid, nucleus, and more proximal regions of the cell, where the damage to the ultrastructure of the tissue is extensive.

Though we may have underestimated the Ca content, especially of the inner segment, we do not think that we could have *over-estimated* this content as the result of an artifactual leakage into the rod from the medium (Somlyo & Walz, 1985). This conclusion is based upon two observations. First, in Fig. 6 we show that even after bathing the retina for 1 h in darkness in a Ringer containing 99 % $^{44}CaCl_2$, the concentration of ^{40}Ca in the outer segments was still about 85 % of the control value, or about 4 mmol/l of wet tissue. This observation is difficult to reconcile with the claim that much of the Ca we normally measure is due to artifactual influx from the Ringer. Secondly, though long exposures to low Ca media produce a substantial depletion of outer segment Ca content (see Fig. 3*D*), short (5 min) exposures to free-Ca concentrations of 10^{-6} or 3×10^{-8} M produce a loss of only 10–20 % of the ^{40}Ca (Schröder & Fain, 1983). The free-Ca concentration in the medium during exposure to low Ca solutions and the subsequent freezing and preparation of the tissue could not have exceeded a few micromolar, and it is difficult to imagine a significant Ca influx into the rod interior. Nevertheless, outer segments after short exposures to such low Ca solutions still contained 80–90 % of the ^{40}Ca content of the outer segments of cells in control retinas.

A significant Ca influx is also unlikely from the fixation and embedding media, since these contained levels of Ca below the resolution of the e.d.x. and l.a.m.m.a.

techniques (see Fig. 3*F*). Although ^{40}Ca could conceivably have leaked from other cells in the retina into the rods, we find it difficult to understand how this could have occurred preferentially into the outer segments in retinas which had been bathed for 1 h in ^{44}Ca Ringer, shock frozen and evacuated to dryness at low temperature, and embedded in a large volume excess of Epon-araldite which itself contained no detectable Ca. Although in most experiments we have sectioned our tissue over distilled water, through which a redistribution of Ca could possibly have occurred, the volume of the water bath was many orders of magnitude larger than that of the section, and it is again difficult to imagine how Ca could have been preferentially sequestered in the outer segment. Nevertheless, as a control for this possibility, we have also sectioned the retina in air, with no significant difference in our results (see Methods).

The amount of Ca we measured in the rod outer segment is in substantial agreement with the previous electron microprobe measurements of Hagins and his collaborators from sections of frozen frog retina (Hagins & Yoshikami, 1975; Hagins *et al.* 1975). Similar values have also been obtained by atomic absorption spectroscopy of frog and bovine rod outer segments, isolated in low or normal free-Ca solutions in the absence of NaCl (Schnetkamp & Kaupp, 1982; U. B. Kaupp, personal communication; M.D. Bownds, personal communication). Much lower levels (0·3–0·5 mmol/l wet tissue volume) have been measured from rods isolated in low Ca solutions in the presence of Na (Hess, 1975; Szuts & Cone, 1977; Liebman, 1978; Nöll *et al.* 1979; Szuts, 1980), probably because the rods lose Ca under these conditions (see Fig. 3*D* and Schnetkamp, 1980; Schröder & Fain, 1983; M.D. Bownds, personal communication).

Lower values have also been obtained by Somlyo & Walz (1985) in their recent electron microprobe study of frog rods. They report an outer segment Ca concentration of 0·4 mmol/kg dry weight. This can be converted to a concentration in terms of tissue wet weight from their value for the phosphorus content of the outer segment (597 ± 11 mmol/kg dry weight), the number of phospholipid molecules per molecule of rhodopsin (61 – see Drenthe, 1981), and the concentration of rhodopsin in a frog rod (3 mM – see Fein & Szuts, 1982). By this means (suggested to us by P. P. M. Schnetkamp), we estimate Somlyo's and Walz's data to give 0·1–0·2 mmol Ca/l wet tissue volume. This is, to our knowledge, the lowest value for the Ca content of outer segments ever obtained by any method (see Fain & Schröder, 1985).

Somlyo & Walz (1985) found somewhat higher Ca concentrations in the inner segment. Although they report no detectable Ca in the mitochondrial-rich ellipsoid region, where we find 100–400 μmol/l, they report considerable amounts in the endoplasmic reticulum of the myoid body ($3{\cdot}8 \pm 0{\cdot}4$ mmol/kg dry weight, equivalent to about 1 mmol/l wet tissue volume), where we were unable to detect significant Ca levels. They suggest as an explanation for the difference between our results and theirs that the Ca which they have measured in the endoplasmic reticulum may have become liberated in our preparations during our fixation procedure and then somehow sequestered within the outer segments and mitochondria of our cells. However, this explanation could not apply to the broken-off outer segments, which in our preparations contain nearly as much Ca as intact outer segments but which have no inner segments from which to have acquired it. Furthermore, the total

amount of Ca Somlyo and Walz find in the photoreceptor (integrated over the total volume of the cell) is between 20 and 100 times less than the total amount we find in our preparations. There is simply too little Ca in the endoplasmic reticulum of their rods to account for our results, no matter how one imagines the Ca to have been redistributed. We suggest as a more likely alternative for the discrepancy between our results and theirs that they have somehow lost the Ca from their outer segments during the dissection or preparation of their tissue, or during the long (1 h) interval between the dissection and cryofixation, and that some of this Ca has accumulated in the endoplasmic reticulum of their cells as a result of a Ca sequestration mechanism known to be localized to this part of the receptor (Ungar, Piscopo, Letizia, & Holtzman, 1984).

Ca exchange in rods in darkness

When the retina is bathed in darkness in Ringer containing the normal complement of ions and ^{44}Ca at a concentration of 1·8 mM, the increase in ^{44}Ca and the decrease in ^{40}Ca are both very slow (see Fig. 6). There are two possible explanations for this observation. On the one hand, it is possible that Ca moves very slowly across the plasma membrane. This seems unlikely, since when the Ca concentration is raised to 20 mM (see Fig. 3*B* and *C*), large amounts of Ca accumulate within the rod. Furthermore, if rods are preloaded with Ca (as in Fig. 3*B* and *C*), this Ca can leave the rod in exchange for external Ca at rates as high as 1 mmol/l wet tissue volume . min, even under conditions where there is no driving force for net Ca movement across the rod plasmalemma (G. L. Fain & W. H. Schröder, in preparation). These results indicate that Ca movement across the rod plasma membrane can be quite rapid. A similar conclusion has been reached by Schnetkamp (1979, 1980) from studies of isolated bovine rod outer segments, as well as by Yau & Nakatani (1984), who have measured an electrogenic component of Na–Ca exchange across the outer segments of toad rods using suction electrodes.

If the movement of Ca across the plasma membrane is not limiting, then the slow rate of exchange in Fig. 6 would indicate that extracellular Ca coming into the cell is as quickly pumped out, with little net accumulation. This could occur if the intracellular Ca were tightly bound to some cytosolic buffer, but the properties of this hypothetical buffer would have to be unlike those of any known Ca-binding protein. Some of the properties that this buffer would have to have can be inferred from our data, under the assumption that Ca equilibrates rapidly across the plasma membrane. In this case, the free Ca in the rod cytosol would consist almost entirely of ^{44}Ca, and the rate constant for the dissociation of the Ca-buffer complex (k_{off}) can be estimated from the rate at which ^{44}Ca replaces ^{40}Ca in the outer segment, provided that the increase in ^{44}Ca is small and the rate is linear. Since these conditions are fulfilled in the data of Fig. 6, we can obtain the rate directly from the best-fitting slope of the ^{44}Ca increase. This gives 0·15%/min, for a k_{off} of $2{\cdot}5 \times 10^{-4}$/s and a mean lifetime for the Ca-buffer complex of 4000 s. This lifetime is orders of magnitude larger than the measured lifetimes for Ca-binding proteins such as calmodulin, parvalbumin, and troponin (Seaman & Kretsinger, 1983). Since the association rate constant (k_{on}) for the Ca-buffer complex is likely to be diffusion limited (Weston & Schwartz, 1972), the equilibrium association constant (K_a) of

our hypothetical buffer would have to be of the order of 10^{10}/mol. While it is possible that such a buffer could exist in outer segments, it would have to be about as abundant as Ca itself, which is present in the ratio of 1–2 per rhodopsin. Biochemical investigations have not so far revealed any such protein (Kühn, 1984).

We consider it more likely that the Ca in the outer segments exchanges slowly because it is sequestered within an intracellular compartment, probably the disks. Since our exchange measurements provide no evidence for more than one rate constant of ^{44}Ca accumulation within the outer segment, we believe that nearly all of the outer segment Ca is contained within the disks of dark-adapted photoreceptors. A similar conclusion was previously reached by Schnetkamp (1979), on the basis of osmotic lysis experiments on bovine rod outer segments.

If the Ca of dark-adapted rods is mostly within the disks, then the slow rate of exchange which we observe would indicate that the disks are largely impermeable to Ca in darkness. We interpret the large increase in the rate of exchange in broken-off outer segments (Pl. 2 and Fig. 7) to be the result of some change in the permeability or transport of Ca across the disk membrane, due perhaps to a change in the concentration of some ion or metabolite in the isolated organelle. However, a different explanation must be given for the increase in the rate of ^{44}Ca accumulation in high Ca media (Fig. 3*C*) or in Na-substituted Ringer (Fig. 8), since here the increase in ^{44}Ca is not accompanied by a commensurate decrease in ^{40}Ca. We suggest that, in these cases, ^{44}Ca may not be exchanging with the ^{40}Ca in the disks but may rather be accumulating in the cytosol, as the result of an inability of the rod in these circumstances to extrude Ca as quickly as it enters.

When the retina is exposed to illumination, the Ca content of the outer segment decreases (Schröder & Fain, 1984). If the Ca in darkness is sequestered within the disks, light must release a part of this pool of Ca. Our results suggest that this may occur as the result of an increase in Ca permeability or transport across the disk membrane. We shall describe the Ca economy of illuminated rods in more detail in a subsequent publication (G. L. Fain & W. H. Schröder, in preparation).

We are grateful to M. L. Woodruff for his help at the beginning of these experiments; A. Einerhand and J. Lauer for technical assistance; H. Kühn, H. Stieve, and N. Farahbakhsh for useful discussion; and H. Stieve and the staff of the Kernforschungsanlage for their hospitality to G.L.F. during the course of these experiments. This research was supported in part by NIH grants EY 01844 and EY 00331 to G.L.F. and SFB grant 160 to W.H.S.

REFERENCES

BASTIAN, B. L. & FAIN, G. L. (1982). The effects of low calcium and background light on the sensitivity of toad rods. *Journal of Physiology* **330**, 307–329.

BLAUSTEIN, M. P. (1974). The interrelationship between sodium and calcium fluxes across cell membranes. *Review of Physiological and Biochemical Pharmacology* **70**, 33–82.

BROWN, J. E. (1979). Excitation in vertebrate retinal rods. In *Membrane Transduction Mechanisms*, ed. CONE, R. A. & DOWLING, J. E., pp. 117–121. New York: Raven Press.

CHABRE, M. (1975). X-ray diffraction studies of retinal rods. I. Structure of the disc-membrane, effect of illumination. *Biochimica et biophysica acta* **382**, 322–335.

COHEN, A. I. (1968). New evidence supporting the linkage to extracellular space of outer segment saccules of frog cones but not rods. *Journal of Cell Biology* **37**, 424–444.

COHEN, A. I. (1973). Rods and cones. In *Handbook of Sensory Physiology, Physiology of Photoreceptor Organs*, ed. FOURTES, M. G. F. Berlin: Springer-Verlag.

CONE, R. A. (1973). The internal transmitter model for visual excitation: some quantitative implications. In *Biochemistry and Physiology of Visual Pigments*, ed. LANGER, H., pp. 275–282. Berlin: Springer-Verlag.

DENTON, E. J. & WYLLIE, J. H. (1955). Study of the photosensitive pigments in the pink and green rods of the frog. *Journal of Physiology* **127**, 81-89.

DRENTHE, E. (1981). *Topology of Phospholipids in Rod Disk Membrane.* Ph.D. Thesis. Nijmegen, Holland: Catholic University of Nijmegen.

FAIN, G. L. (1976). Sensitivity of toad rods: dependence on wave-length and background illumination. *Journal of Physiology* **261**, 71–101.

FAIN, G. L. (1985). Evidence for a role of internal messengers in phototransduction. In *Molecular Mechanism of Photoreception*, ed. STIEVE, H. Berlin: Dahlem Konferenzen, (in the Press).

FAIN, G. L. & LISMAN, J. E. (1981). Membrane conductances of photoreceptors. *Progress in Biophysics and Molecular Biology* **37**, 91–147.

FAIN, G. L. & SCHRÖDER, W. H. (1985). Calcium content and light-induced release from photoreceptors: Measurements with laser micro-mass analysis. In *Contemporary Sensory Neurobiology*, ed. CORREIA, M. J. & PERACHIO, A. A., pp. 3–20. New York: Alan R. Liss.

FEIN, A. & SZUTS, E. Z. (1982). *Photoreceptors: Their Role in Vision.* Cambridge: Cambridge University Press.

GOLD, G. H. & KORENBROT, J. I. (1981). The regulation of calcium in the intact retinal rod: a study of light-induced calcium release by the outer segment. In *Current Topics in Membranes and Transport, Molecular Mechanisms of Photoreceptor Transduction*, ed. BRONNER, F., KLEINZELLER, A. & MILLER, W. H., pp. 307–330. New York: Academic Press.

HAGINS, W. A., ROBINSON, W. E. & YOSHIKAMI, S. (1975). Ionic aspects of excitation in rod outer segments. In *Energy Transformation in Biological Systems*, pp. 169–189. Amsterdam: Elsevier.

HAGINS, W. A. & YOSHIKAMI, S. (1975). Ionic mechanisms in excitation of photoreceptors. *Annals of the New York Academy of Sciences* **264**, 314–325.

HAROSI, F. I. (1975). Absorption spectra and linear dichroism of some amphibian photoreceptors. *Journal of General Physiology* **66**, 357–382.

HESS, H. H. (1975). The high calcium content of retinal pigmented epithelium. *Experimental Eye Research* **21**, 471–479.

KAUPP, U. B. & SCHNETKAMP, P. P. M. (1982). Calcium metabolism in vertebrate photoreceptors. *Cell Calcium* **3**, 83–112.

KITAZAWA, T., SHUMAN, H. & SOMLYO, A. P. (1983). Quantitative electron probe analysis: Problems and solutions. *Ultramicroscopy* **11**, 251–262.

KORENBROT, J. I. (1985). Signal mechanisms of phototransduction in retinal rods. *Critical Reviews of Biochemistry* **17**, 223–256.

KÜHN, H. (1984). Interactions between photoexcited rhodopsin and light-activated enzymes in rods. *Progress in Retinal Research* **3**, 123–156.

LATIES, A. M. & LIEBMAN, P. A. (1970). Cones of living amphibian eye: selective staining. *Science* **168**, 1475–1477.

LIEBMAN, P. A. (1978). Rod disk calcium movement and transduction: a poorly illuminated story. *Annals of the New York Academy of Sciences* **307**, 642–644.

LUFT, J. H. (1961). Improvements in epoxy resin embedding mixtures. *Journal of Biophysical and Biochemical Cytology* **9**, 409–414.

MULLINS, L. J. (1979). Transport across axon membranes. In *Membrane Transport in Biology*, vol. 2: *Transport Across Single Biological Membranes*, ed. TOSTESON, D.C., pp. 161–210. Berlin: Springer-Verlag.

NÖLL, G., STIEVE, H. & WINTERHAGER, J. (1979). Interaction of bovine rhodopsin with calcium ion. II. Calcium release in bovine rod outer segments upon bleaching. *Biophysics of Structure and Mechanism* **5**, 43–53.

ORNBERG, R. L. & REESE, T. S. (1980). A freeze-substitution method for localizing divalent cations: examples from secretory systems. *Federation Proceedings* **39**, 2802.

REBHUHN, L. I. (1972). Freeze substitution and freeze drying. In *Principles and Techniques of Electron Microscopy – Biological Applications*, vol. 2, ed. HAYAT, M. A., pp. 3–42. New York: Van Nostrand Reinhold Co.

REQUENA, J. (1983). Calcium transport and regulation in nerve fibers. *Annual Reviews of Biophysics and Bioengineering* **12**, 237–257.

SCHNETKAMP, P. P. M. (1979). Calcium translocation and storage of isolated intact cattle rod outer segments in darkness. *Biochimica et biophysica acta* **554**, 441–459.

SCHNETKAMP, P. P. M. (1980). Ion selectivity of the cation transport system of isolated intact cattle rod outer segments. Evidence for a direct communication between the rod plasma membrane and the rod disk membrane. *Biochimica et biophysica acta* **598**, 66–90.

SCHRÖDER, W. H. (1981). Quantitative LAMMA analysis of biological specimens I. Standards II. Isotope Labeling. *Fresenius Zeitschrift für Analytische Chemie* **308**, 212–217.

SCHRÖDER, W. H. & FAIN, G. L. (1983). Calcium content and calcium exchange in intact vertebrate photoreceptors. *Biophysical Journal* **41**, 126a.

SCHRÖDER, W. H. & FAIN, G. L. (1984*a*). Light-dependent release of Ca from rods measured by laser micro mass analysis. *Biophysical Journal* **45**, 341a.

SCHRÖDER, W. H. & FAIN, G. L. (1984*b*). Light-dependent calcium release from photoreceptors measured by laser micro-mass analysis. *Nature* **309**, 268–270.

SCHRÖDER, W. H., FRINGS, D. & STIEVE, H. (1980). Measuring calcium uptake and release by invertebrate photoreceptor cells by laser microprobe mass spectroscopy. In *Scanning Electron Microscopy*, vol. 2, ed. O'HARE, A. M. F., pp. 606 & 647–654. Chicago: SEM, Inc.

SEAMON, K. B. & KRETSINGER, R. H. (1983). Calcium-modulated proteins. In *Calcium in Biology*, vol. 6 of *Metal Ions in Biology*, ed. SPIRO, T.G., pp. 1–51. New York: John Wiley & Sons.

SHUMAN, H., SOMLYO, A. V. & SOMLYO, A. P. (1976). Quantitative electron probe microanalysis of biological thin sections: Methods and validity. *Ultramicroscopy* **1**, 317–339.

SOMLYO, A. P. & WALZ, B. (1985). Elemental distribution in *Rana pipiens* retinal rods: quantitative electron probe analysis. *Journal of Physiology* **358**, 183–195.

SZUTS, E. Z. (1980). Calcium flux across disk membranes. Studies with intact rod photoreceptors and purified disks. *Journal of General Physiology* **76**, 253–286.

SZUTS, E. Z. & CONE, R. A. (1977). Calcium content of frog rod outer segments and discs. *Biochimica et biophysica acta* **468**, 194–208.

UNGAR, F., PISCOPO, I., LETIZIA, J. & HOLTZMAN, E. (1984). Uptake of calcium by the endoplasmic reticulum of the frog photoreceptor. *Journal of Cell Biology* **98**, 1645–1655.

VOGT, H., HEINEN, H. J., MEIER, S. & WECHSUNG, R. (1981). LAMMA 500. Principle and technical description of the instrument. *Fresenius Zeitschrift für Analytische Chemie* **308**, 195–200.

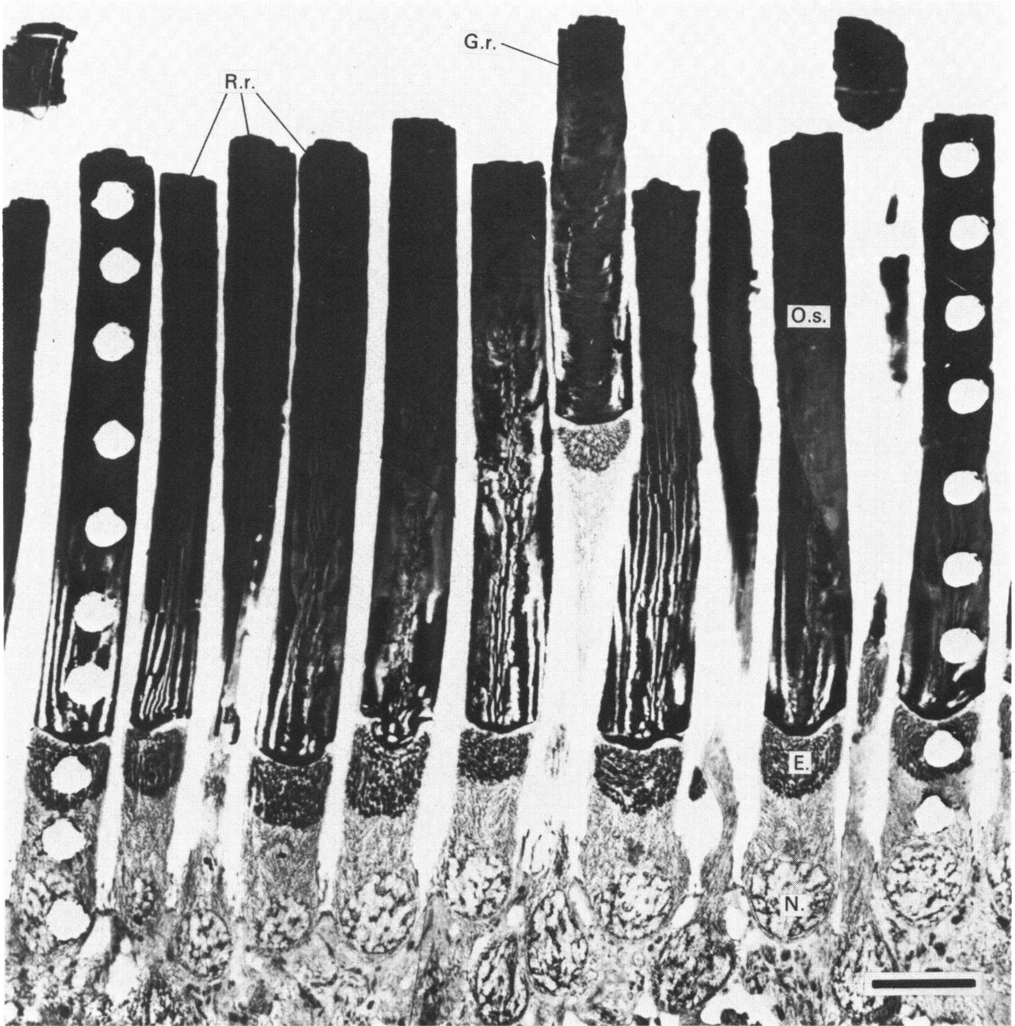
WALZ, B. & SOMLYO, A. P. (1984). Quantitative electron probe analysis (EPMA) of frog retinal rods. *Abstracts of the Eighth International Biophysics Congress*. p. 226.

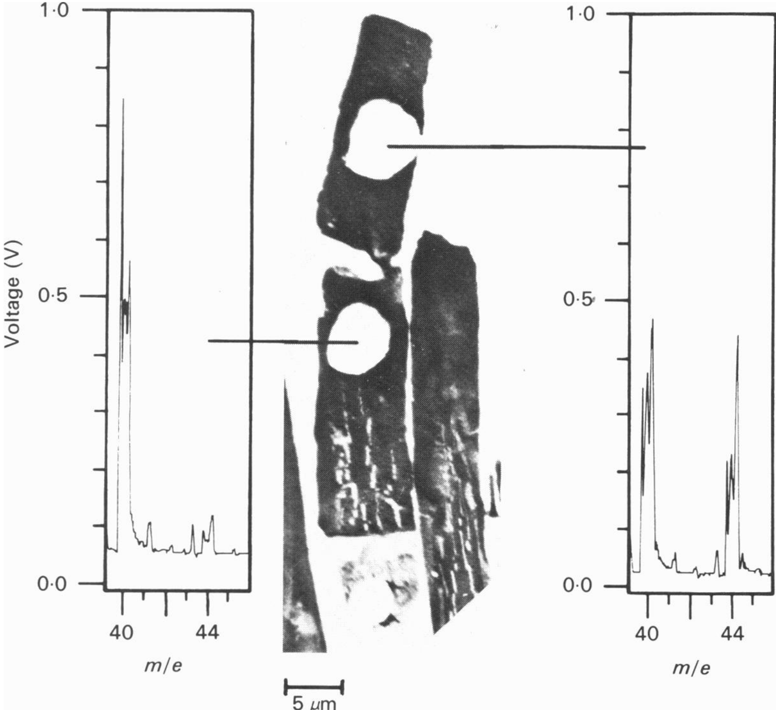
WECHSUNG, R., HILLENKAMP, F., KAUFMANN, R., NITSCHE, R. & VOGT, H. (1979). LAMMA – a new laser microprobe. In *Scanning Electron Microscopy*, vol. 1, ed. O'HARE, A. M. F., pp. 611–620. Chicago: SEM, Inc.

WESTON, R. E. & SCHWARTZ, H. A. (1972). *Chemical Kinetics*. Englewood Cliffs, NJ: Prentice-Hall.

YAU, K. W. & NAKATANI, K. (1984). Electrogenic Na–Ca exchange in retinal rod outer segment. *Nature* **311**, 661–663.

YOSHIKAMI, S. & HAGINS, W. A. (1971). Light, calcium, and the photocurrent of rods and cones. *American Biophysics Society Abstracts* **15**, 47a.


G.r.
R.r.
O.s.
E.
N.



G. L. FAIN AND W. H. SCHRÖDER

EXPLANATION OF PLATES

PLATE 1

Low power electron micrograph of section of retina from which l.a.m.m.a. measurements were made. Retina was from dark-adapted toad dissected in dim red light, frozen directly after the dissection, and dehydrated by freeze substitution in acetone containing 0·5 % OsO_4. Holes in photoreceptors were made by Nd:YAG laser of the LAMMA-500, and each hole represents a separate measurement. Calibration bar, 10 μm. Symbols are as follows: r.r., red rod; g.r., green rod; o.s., outer segment; e., ellipsoid; and n., nucleus.

PLATE 2

L.a.m.m.a. measurements from rod with partially broken-off outer segment. Dark-adapted retina was incubated for 5 min in darkness in Ringer containing the normal Ca concentration but with the ^{40}Ca substituted with ^{44}Ca. Spectrum on left is from the proximal half of the outer segment and is typical of spectra from intact outer segments, having a large ^{40}Ca peak and a small ^{44}Ca peak (see Fig. 6). Spectrum on right is from distal half of outer segment which is partially detached from the rest of receptor. Spectrum from this part of rod shows less ^{40}Ca and more ^{44}Ca and is typical of spectra from broken-off outer segments (see Fig. 7).