Frog Rod Outer Segments with Attached Inner Segment Ellipsoids as an In Vitro Model for Photoreceptors on the Retina

MICHAEL S. BIERNBAUM and M. DERIC BOWNDS*

From the Laboratory of Molecular Biology and the Department of Zoology, University of Wisconsin, Madison, Wisconsin 53706

ABSTRACT Purified suspensions of frog rod outer segments still attached to the mitochondria-rich inner segment portion of the receptor cell (OS-IS) can be obtained in quantities (0.1 mg/retina) sufficient for chemical analysis. In oxygenated glucose-bicarbonate Ringer's medium with added Percoll, they display normal dark currents, light sensitivity, and photocurrent kinetics for several hours. Two millimolar cytoplasmic levels of ATP and GTP are maintained, fivefold higher than in isolated OS. The levels are not altered by abolition of the dark current with ouabain. Nucleoside triphosphates are more effectively buffered than in isolated OS, and their levels remain constant during changes in external calcium levels. $^{32}P_i$ is incorporated into endogenous ATP and GTP pools twice as efficiently as in isolated OS, and is used in the phosphorylation of rhodopsin. OS-IS take up and release $^{45}Ca^{++}$ by Na^+-, Ca^{++}-, and IBMX-sensitive mechanisms. Illumination causes release of $^{45}Ca^{++}$, which confirms retinal studies by other groups using Ca^{++}-sensitive electrodes. Thus, OS-IS suspensions model the behavior of photoreceptors still attached to the living retina. Their availability permits the simultaneous assay and correlation of electrophysiological and chemical changes occurring during excitation and adaptation.

INTRODUCTION

Chemical changes accompanying sensory transduction have been better characterized in rod photoreceptors than in other sensory cells because light-sensitive outer segments can be prepared in quantity and reasonable purity, and their protein complement is devoted mainly to the specialized function of transduction (Bownds, 1981). The sensory input can be quantitated because the photochemistry of the light-receiving pigment, rhodopsin, is adequately understood. A goal of studies in this area has been to determine the biochemical changes that regulate the changes in transmembrane ion fluxes induced by illumination. A

* With the technical assistance of Charles S. Schobert.

Address reprint requests to Dr. M. Deric Bownds, Laboratory of Molecular Biology, University of Wisconsin, 1525 Linden Dr., Madison, WI 53706.

significant problem has been that preparations suitable for electrophysiological analysis, whole retinas or single isolated cells, have been unsuitable for measuring the time course of light-induced chemical changes; conversely, photoreceptor membrane suspensions prepared in sufficient purity and quantity for biochemical analysis have lost both their physiological activity and important controlling molecules (Robinson et al., 1980). The main purpose of this paper is to propose that this problem might be remedied by the study of purified suspensions of physiologically active outer segments still attached to their inner segments ("OS-IS").

Chemical studies in this laboratory thus far have used suspensions of outer segments isolated from the retina to study light-induced changes in protein phosphorylation (Polans et al., 1979; Hermolin et al., 1982), cyclic GMP (Woodruff et al., 1977; Woodruff and Bownds, 1979; Polans et al., 1981), and nucleoside triphosphates (Biernbaum and Bownds, 1979), as well as calcium and ATP regulation of cyclic GMP phosphodiesterase (P. R. Robinson et al., 1980, 1982; Kawamura and Bownds, 1981). The use of preparations in which outer segment plasma membranes were intact has permitted correlations between permeability, cyclic nucleotide levels, and protein phosphorylation (Bownds and Brodie, 1975; Brodie and Bownds, 1976). Measurements using these suspensions have also provided the first evidence that the cyclic GMP decrease triggered by illumination occurs on a time scale of milliseconds (Woodruff et al., 1977).

These studies have had the serious drawback that the outer segment structures have been separated not only from the retina but also from the mitochondria-rich ellipsoid portion of the photoreceptor inner segment. This portion of the inner segment is a source of energy-rich phosphates for the outer segment, and detachment of the outer segment from the inner segment causes a rapid decay in levels of nucleoside triphosphates (Biernbaum and Bownds, 1979) and cyclic GMP (Woodruff and Bownds, 1979). The ellipsoid portion of the inner segment is also the source of a dark current that normally flows into the outer segment (Hagins et al., 1970; Yau et al., 1981). It is the suppression of this dark current by light that causes the voltage change influencing the release of transmitter from the cell. These limitations have raised the question of whether the light-sensitive chemistry observed in isolated outer segments is relevant to the normal physiology of outer segments still attached to the retina.

Recent work in several laboratories has revealed that electrical responses can be obtained from isolated outer segments still attached to their ellipsoids (OS-IS) (see, for example, Baylor et al., 1979; Bader et al., 1979; Yau et al., 1981; Schnapf, 1983; Liebman et al., 1984). Photocurrent recordings using the suction electrode technique introduced by Baylor et al. (1979) appear very similar to those obtained from outer segments still attached to the living retina. This suggests that if these structures could be prepared in sufficient quantity and homogeneity, they could be used for the simultaneous measurement of changes in conductance and biochemistry caused by illumination. Several preliminary accounts have suggested that such preparations can be obtained (Fliesler et al., 1980; Pugh and Liebman, 1981; Kavipurapu et al., 1982).

In this paper, we describe a method for preparing purified suspensions of OS-

IS and characterize the following electrophysiological and biochemical properties: their dark currents and light sensitivity, nucleoside triphosphate levels and synthesis, and light-induced calcium extrusion. The experiments show that by the above criteria, OS-IS are a favorable in vitro model of photoreceptors on the living retina.

The following paper (Biernbaum and Bownds, 1985) examines reversible light-induced changes in nucleoside triphosphate levels in OS-IS and compares them with aspects of the electrophysiological response. Another paper (Cote et al., 1984) examines the kinetics of light-induced changes in levels of cyclic GMP. A preliminary report on this work was presented earlier (Biernbaum and Bownds, 1982).

METHODS

Retinal Dissection

The experiments used bullfrogs (*Rana catesbeiana* or *Rana grylio*) 10–15 cm in length (Central Valley Biologicals, Clovis, CA). The animals were kept in light-tight tanks with continuously flowing water for 2–6 wk before use. A 12-h light-dark cycle was provided, with the light period providing intermittent illumination in the following cycle: 5 min of dim light, 5 min of light flashes at a frequency of 1/s, and 5 min of darkness. This variation during the light period was intended to provide better exercise for the visual system during storage. Each frog was fed ~3 g of Purina dog chow, blended in water with a vitamin A and E supplement, three times a week. Frogs were killed, by pithing of brain and spinal column, 1–6 h before the end of the dark period. Each eye was opened with a shallow circumferential incision just anterior to the edge of the sclera, so that the retina remained attached to the front of the eye. The cornea and lens with attached retina were then lifted away from the eyecup and its associated pigment epithelium, so that the retina had the appearance of a hanging drop. This procedure minimized contamination from the pigment epithelium.

The retina was then cut from the front half of the eye and gently rinsed in a Ringer's solution containing 6–11% Percoll (Pharmacia Fine Chemicals, Piscataway, NJ), n_D^{20} = 1.3355–1.3361, to remove any remaining contamination from the vitreous humor and pigment epithelium. The Ringer's solution consisted of 105 mM NaCl, 2.5 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM glucose, 5 mM $NaHCO_3$, pH 7.5–7.6, with 10 mM HEPES as the buffer. The solution was chilled and oxygenated (100% O_2) for at least 10 min before use. The pH of the medium remained constant during the experiments. Addition of the Percoll colloid helps maintain intactness of the outer segments as measured by their ability to exclude the fluorescent dye didansylcysteine (DDC) (Yoshikami et al., 1974). This replaces the calf serum used for this purpose in earlier work (cf. Woodruff and Bownds, 1979). Different Percoll suspensions obtained from Pharmacia Fine Chemicals were found to have different levels of osmotically active solute and different gradient-forming characteristics. For this reason, the Percoll-Ringer's solutions were dialyzed against Ringer's solution before use and confirmed to be iso-osmotic with the Ringer's solution. Measurements of osmotic activity were made with an Osmette osmometer (Precision Systems, Inc., Sudbury, MA).

Any variation in the composition of the Ringer's solution is noted in the text. Unless noted otherwise, all operations were carried out with infrared illumination, using a head-mounted infrared converter (FJW Industries, Mount Prospect, IL). All glassware was

treated (200°C, 45 min) with tri-*n*-butylchlorosilane (Pfaltz and Bauer, Stamford, CT) to minimize cellular adhesion to the glass surface (Lamb et al., 1981).

Preparation of Suspensions of Outer Segments with Attached Ellipsoids (OS-IS)

A procedure involving gentle suction applied to the retina and shredding of the retina was used to obtain outer segments still attached to their ellipsoids (OS-IS). This procedure minimized rupturing of the cilium connecting the outer segment of the photoreceptor to the inner segment and enhanced separation from the retina below the mitochondria-rich ellipsoid region of the inner segment. From each retina, a final purified suspension containing ~0.1 mg of OS-IS could be obtained.

Freshly dissected and minimally disturbed retinas were rinsed gently in Ringer's solution and then transferred, photoreceptor side up, onto a wax block covered with a sheet of Parafilm. The retina was pinned at three or four points along the edges, covered with ~0.7 ml of Ringer's solution, and mounted on a dissecting microscope stage. Infrared optics were used to view the preparation and observe the suction action of a 100-μl pipette applied to the surface of the pinned retina. This pipette, with its tip cut to an orifice diameter of 1.5–2 mm, was applied to the retina with suction just sufficient to draw the retina up to the tip along with <50 μl of medium. Upon contact with the tip, the retina and the fluid drawn into the pipette were released in a short burst of positive pressure, a technique freeing small surface patches of the retina and leaving a creased and puckered retinal surface. Direct sustained suction applied to the retina was not effective in causing release of OS-IS.

This procedure was applied over the entire surface of the retina, particularly the peripheral rod-rich areas. The Percoll-Ringer's fluid, now containing the detached OS-IS, was removed and replaced with fresh medium. Suction was then applied to the retina with slightly increased vigor, sufficient to cause some tearing and shredding of the retinal surface. (Approximately one-third of the retinas used were comparatively resistant to shredding, requiring more forceful suction or direct shredding with forceps to generate particles, and yielding higher populations of OS and OS fragments. Retinas that responded readily to gentle suction with the generation of abundant particles gave the highest yields of OS-IS.) The resulting suspension of particles was removed, and the combined suspensions were agitated by mixing gently with a pipette (5-mm orifice). This further dissociated OS-IS from the small patches of retina present and preferentially disrupted any isolated OS. The retinas were then gently shaken in fresh medium or cut into small pieces and gently tumbled.

After the heavier retinal particles in each of the fractions had settled, the supernatant suspensions were combined and subjected to mixing by pipette, followed by low-speed vortex mixing (Vortex-Genie, Springfield, MA). OS-IS were much more resistant to rupture by this procedure than OS, and thus the relative content of intact OS-IS was enhanced. Suspensions were then layered on a discontinuous Percoll gradient (an upper layer of 1 ml of 42–45% Percoll, n_D^{20} = 1.3413–1.3418; an intermediate layer of 0.5 ml of 48–52% Percoll, n_D^{20} = 1.3423–1.3430; a lower layer of 1.0 ml of 68–72% Percoll, n_D^{20} = 1.3457–1.3464 in a transparent polycarbonate 11- × 77-mm centrifuge tube) and centrifuged for 1 min at 18,000 *g* (SS-3 centrifuge with SS-34 rotor, Sorvall Inc., Norwalk, CT) or for 4 min at 2,500 *g* (HN-SII centrifuge, International Equipment Co., Needham Heights, MA). (At calcium concentrations of <1 mM, a lower Percoll density is required for the intermediate layer to obtain separation of OS from OS-IS.)

The appearance of the gradient bands after centrifugation is shown in Fig. 1. The top band at the first interface contains disrupted OS and OS-IS leaky to DDC, as well as retinal fragments. The middle band is enriched in intact OS (i.e., OS that exclude DDC).

Light-microscopic observation of suspensions obtained from this band showed that the OS content usually exceeded 80%. The bottom band is enriched in intact OS-IS. Suspensions containing >50% OS-IS obtained from this lower band were used for the electrophysiological and biochemical measurements described below. Further enrichment of the OS-IS content could be obtained by repeating the vortex mixing and Percoll gradient steps, but at the expense of yield and nucleotide content.

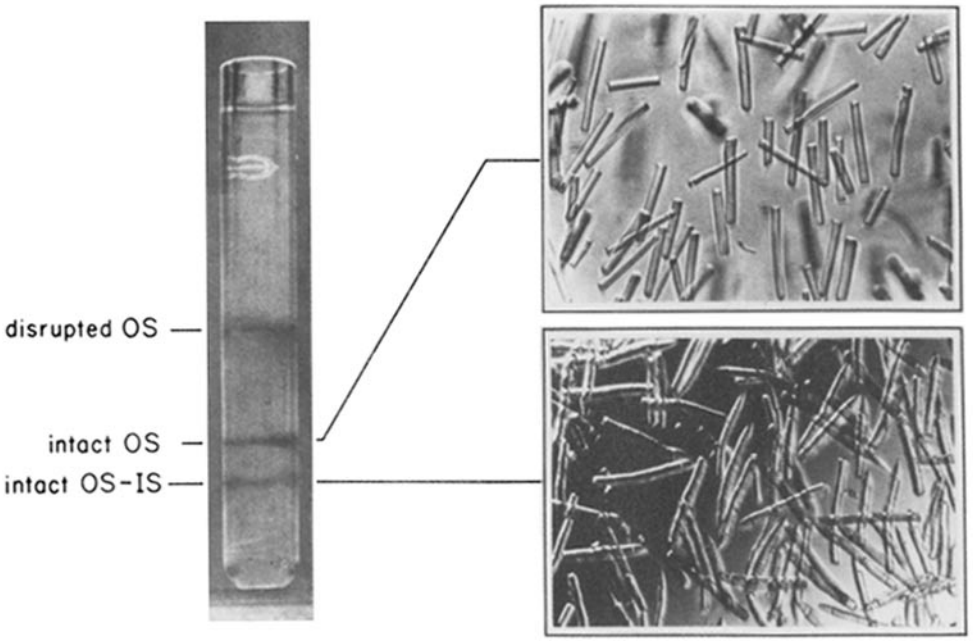

FIGURE 1. Separation of intact OS-IS from OS by discontinuous Percoll gradient sedimentation. The three bands shown in the centrifuge tube result from sedimentation of material obtained by suction/shredding treatment of the retina. The top band contains broken OS and retinal debris. The middle band contains intact OS, shown in the upper right micrograph (× 160), and the lower band is enriched in intact OS-IS, shown in the lower right micrograph.

Measurement of Dark Currents and Light Responses in OS-IS

Individual OS-IS in the suspensions obtained from the above gradient were assayed for electrophysiological activity using the suction electrode technique of Baylor et al. (1979). Electrodes were prepared according to Lamb et al. (1981). The inner segment portion of the OS-IS was drawn up into a tightly fitting suction pipette to just past the junction between inner and outer segments, with a seal resistance of 10–12 MΩ. The dark current that normally flows out of the inner segment to the outer segment was deflected through a current-measuring circuit and then to the outer segment.

Measurements were accomplished by transferring OS-IS suspensions, using dim red illumination for ease of manipulation, into a glass recording chamber mounted on the stage of an inverted microscope (Olympus Optical Co., Tokyo, Japan). The chamber was silanized as described above and contained 0.6 ml of freshly oxygenated Ringer's solution with 6–30% Percoll and an Ag/AgCl reference electrode. The recording chamber was

separated from the dim red illumination in the room by a black cloth, and the OS-IS population was viewed using infrared illumination with a TV monitor (RCA, Lancaster, PA) connected to an infrared-sensitive camera (Cohu Inc., San Diego, CA). Individual OS-IS suspended in the chamber were sampled at random by sucking them into a recording electrode connected to a hydraulic system and an Ag/AgCl electrode. Dark current was measured with a current-voltage converter circuit (515J, Analog Devices, Norwood, MA, with a 500-MΩ feedback resistor). The recording apparatus was interfaced with an Apple II Plus computer (Cupertino, CA), which triggered the light stimulus and was used for data storage and retrieval. Current responses were also recorded on a chart recorder (Gould, Inc., Elk Grove, IL). Illumination was delivered either by the continuous source described by Brodie and Bownds (1976) or by a flash source (Vivitar Corp., Santa Monica, CA), using orange and heat filters. The number of isomerizations was determined by bleaching a suspension of rods in the recording chamber with a diffuse beam from the unattenuated lamp, directly measuring the bleach by difference spectroscopy (Bownds et al., 1971), and then attenuating the intensity with calibrated neutral density filters (Oriel Corp., Stamford, CT) interposed between the light source and the chamber. A photodiode (United Detector Technology, Culver City, CA), whose response was calibrated against this and inserted at the position normally occupied by the preparation, was sometimes used to measure light intensity. All experiments were performed at room temperature.

Measurement of Nucleotides

Adenine and guanine nucleotide levels in suspensions of OS-IS and OS were measured by high-pressure liquid chromatography (HPLC) as described previously (Biernbaum and Bownds, 1979). Suspensions of OS-IS obtained from the gradient purification described above were diluted as required with fresh Ringer's medium containing Percoll. The final rhodopsin concentration was 2–10 μM, determined by difference spectroscopy as described previously (Bownds et al., 1971). Portions (100 μl) of this suspension were withdrawn and, at times appropriate to each experiment, quenched with 10% trichloroacetic acid (TCA; 200 μl). Samples were then immediately chilled, sedimented (for 15 min at 3,000 *g*), and analyzed by HPLC. In experiments involving changes in the external calcium concentration, the Ringer's solution contained 0.1 mM $CaCl_2$ and aliquots were diluted with EGTA- or Ca^{++}-containing solutions to give the final desired Ca^{++} concentration (Polans et al., 1981).

Measurement of $^{45}Ca^{++}$ Uptake and Release

Loading of OS-IS and OS with $^{45}Ca^{++}$ was accomplished in the following manner. Suspensions of rods in Na-Ringer's medium containing Percoll were diluted with 3 vol of K-Ringer's solution (in which the NaCl was replaced with KCl), and the OS-IS were gently sedimented (16 s at 600 *g*, then 5 s at 3,000 *g*). The supernatant was removed and the pellet was gently resuspended in K-Ringer's medium (747 μl) containing Percoll. (Isethionate [Eastman Kodak Co., Rochester, NY], an impermeant anion, replaced chloride in many experiments to suppress any volume changes that might be caused by osmotic fluxes accompanying changes in the external calcium concentration [cf. Hagins and Yoshikami, 1977].) $^{45}Ca^{++}$ (48 μl, 67 μg Ca/ml [Amersham Corp., Arlington Heights, IL] made isoosmotic with 5.3 μl of 10× K-Ringer's medium) was added to the suspension to give a final Ca^{++} concentration of 0.1 mM (3.3×10^7 cpm). In other experiments, a portion was incubated in the same medium containing 50 μM isobutylmethylxanthine (IBMX; Aldrich Chemical Co., Milwaukee, WI); in these experiments, both control and IBMX suspensions contained 1% ethanol.

Incubation was terminated after 3.5–7 min by twofold dilution with K-Ringer's solution containing 10^{-6} M Ca^{++} (1 mM $CaCl_2$ plus 1.028 mM EGTA) and gentle sedimentation of the OS-IS (16 s at 600 *g*, then 5 s at 3,000 *g*). The supernatant was removed and the incubation tube was rinsed with additional K-Ringer's medium (600 μl), with minimal disturbance of the pellet. After gentle sedimentation, the rinse supernatant was removed. (This reduced carry-over of radioactivity from the incubation medium to a negligible 300 cpm/μl of medium.) The pellet was resuspended in K-Ringer's medium containing Percoll (800 μl) adjusted to 10^{-6} M Ca^{++} as described above, and portions (100 μl) were withdrawn as required.

$^{45}Ca^{++}$ uptake in individual samples was determined at various times thereafter by dilution with K-Ringer's medium (500 μl) containing 10^{-6} M Ca^{++} and no Percoll, followed by gentle sedimentation (16 s at 600 *g*, then 10 s at 3,000 *g*). The supernatant (~550 μl, containing <0.02% of the total rhodopsin in both dark and illuminated samples) was removed. The pellet was resuspended in K-Ringer's medium, extracted (200 μl 10% TCA), and sedimented (15 min at 3,000 *g*). Portions of the original suspension supernatant and the TCA extract of the pellet were withdrawn, and their ^{45}Ca content was determined by liquid scintillation counting (Searle Radiographics, Inc., Des Plaines, IL).

In parallel experiments examining the effect of illumination on ^{45}Ca in purified OS-IS and OS, samples were exposed in one experiment to 1 s of illumination, bleaching 0.1% of the rhodopsin, and in two other experiments to 3 min of illumination, bleaching 1% of the rhodopsin. At various times between 0.25–4.5 min after the onset of illumination, the suspension was diluted and sedimented and the supernatants were removed as described above. In experiments examining the effect of Na^+, Ca^{++}, or other agents on incorporated $^{45}Ca^{++}$, the same protocol was followed, except for the following modification: experimental or control samples were withdrawn from separate portions of the final suspension, which had been treated with an equal volume of Na-Ringer's (115 mM) or K-Ringer's solution, respectively, or were withdrawn after addition of solutions containing appropriate amounts of Ca^{++}, EGTA, A23187 (Calbiochem-Behring Corp., La Jolla, CA), or other agents.

Measurement of $^{32}P_i$ Incorporation into Nucleotides and Proteins

In experiments examining the effect of illumination on $^{32}P_i$ incorporation into nucleotides and proteins by OS-IS, carrier-free $^{32}P_i$ (3.3 mCi, 70 μl, New England Nuclear, Boston, MA) in Na-Ringer's solution was added to a suspension (900 μl) containing >80% OS-IS in Na-Ringer's medium containing 49% Percoll. After incubation in the presence of $^{32}P_i$ for periods up to 43 min, portions (100 μl) were removed and quenched with 10% TCA (200 μl) for determination of levels of incorporation in the dark, and a portion (100 μl) was removed for determination of rhodopsin content. Other samples were exposed to dim or bright illumination and quenched. The membranes were pelleted, washed, and analyzed by sodium dodecyl sulfate gradient polyacrylamide gel electrophoresis (Polans et al., 1979).

To determine the [$^{32}P_i$]-phosphoprotein content, specific protein bands from the gel were excised and solubilized and their radioactivity was measured by liquid scintillation counting. The procedure used was that described previously (Biernbaum and Bownds, 1979), modified by the addition of 0.2 ml perchloric acid (60%) for each 0.4 ml of hydrogen peroxide (30%). The radionucleotide content of the supernatants in these samples was determined by HPLC and liquid scintillation counting (Biernbaum and Bownds, 1979).

RESULTS

Structure of Intact OS-IS

Fig. 2 shows the appearance of purified and intact OS-IS using Nomarski optics. The outer segments are attached to cusp-shaped portions of the inner segment containing the mitochondria (cf. Biernbaum and Bownds, 1979). The OS-IS are in a Ringer's medium containing 50% Percoll. At this density, the OS-IS sediment very slowly, cell-to-cell contact is minimized, and structural and osmotic intactness is maintained for several hours. Intactness of the structures is demonstrated by their failure to take up DDC (Yoshikami et al., 1974), their inability to incorpo-

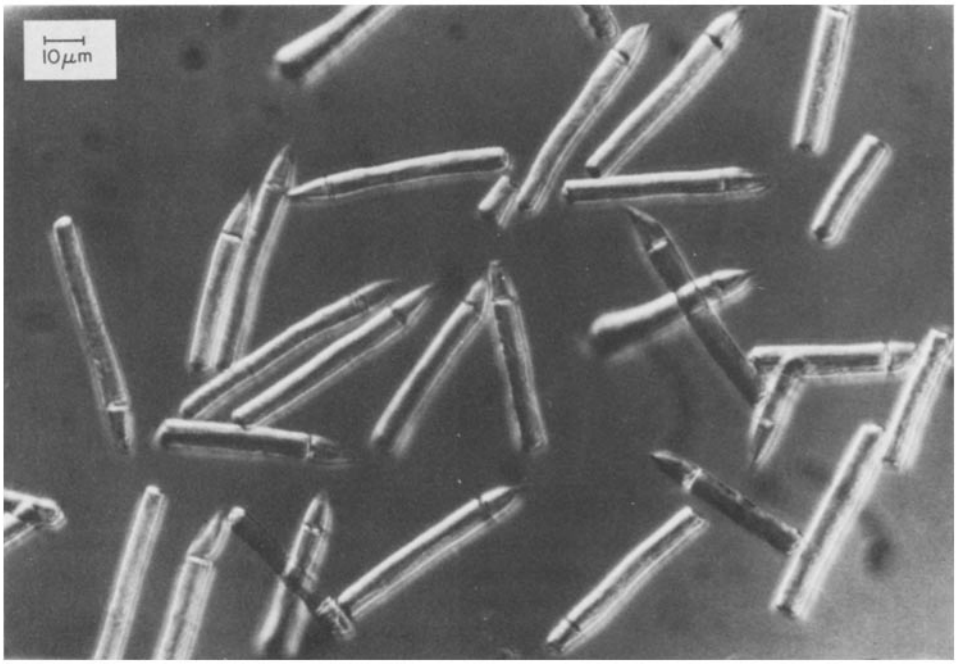

FIGURE 2. A suspension of purified OS-IS in 50% Percoll medium is shown under Nomarski optics (a 10-μm bar indicates the scale). Short cusp-shaped portions of the inner segment contain the mitochondria-rich ellipsoid and are attached via a ciliary connection to the outer segment. Normal anatomy and electrophysiological responses are observed for 2–3 h if the structures are maintained in an oxygenated Ringer's medium containing glucose, bicarbonate, calcium, and Percoll.

rate the terminal phosphate of exogenous [γ^{32}P]ATP into endogenous proteins, and their different response to hypoosmotic shocks when compared with leaky structures. Most important, they maintain normal electrophysiological light responses (see below).

The OS-IS content of purified suspensions used in the experiments reported in this paper varied from 50 to 99%. Light-microscopic examination of these suspensions showed the remainder to be OS, generally with <1% contamination by cones, spherical cells, and cell fragments. Biochemical experiments described

in this and the following paper (Biernbaum and Bownds, 1985; see also Cote et al., 1984) were performed with purified preparations highly enriched in OS-IS so that any contribution from OS would be small. In the case of nucleoside triphosphates, levels were so much lower in OS than in OS-IS that any contribution to the data from OS could usually be discounted (see below).

OS-IS Maintain Dark Currents Suppressible by Light

The suppression of the dark current in a single OS-IS caused by flashes of increasing intensity is shown in Fig. 3. The numbers adjacent to the traces are the number of rhodopsin molecules bleached per outer segment. The responses are similar to those described by Baylor et al. (1979) for rod receptors still attached to the living retina, and could be obtained for 2–3 h after isolation of OS-IS from the retina.

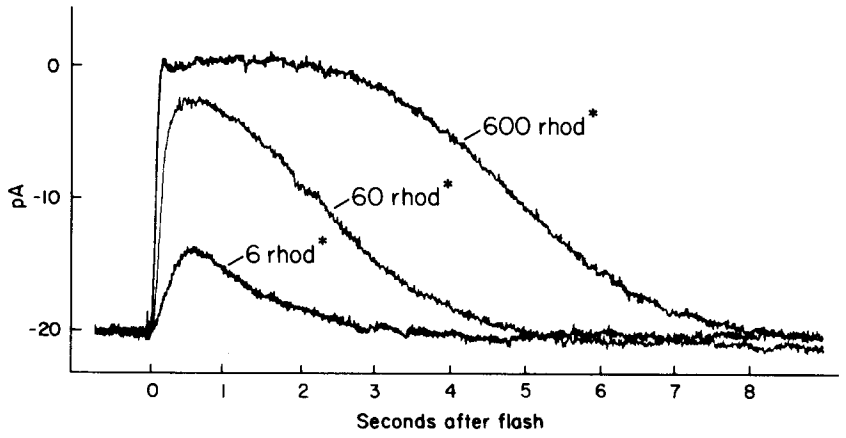

FIGURE 3. Light suppression of dark current is observed in OS-IS suspensions. The inner segment end of an OS-IS is drawn up into a suction pipette to just past the junction of inner and outer segment, and flashes bleaching the indicated number of rhodopsin molecules are presented. The dark current in picoamperes is shown as a function of time in seconds following the flash. By convention, outward current across the outer segment membrane is drawn upward. Each trace is the average of 7–13 responses. The suspending medium was a 20% Percoll-Ringer's solution containing 1 mM Ca^{++}.

The averaged responses of OS-IS from >10 different preparations to a 2-ms flash illumination are shown in Fig. 4. The average dark current of 15 pA is half-suppressed by a flash bleaching ~60 rhodopsin molecules per outer segment, a value about twice that obtained by recordings from photoreceptors on the retina (Baylor et al., 1979). Average dark currents and light sensitivities were found to decline as the OS-IS preparations aged more than several hours. For this reason, biochemical and electrophysiological experiments were performed between 45 min and 2 h after detachment of OS-IS from the retina.

One important feature distinguishes these electrophysiological measurements from the chemical measurements reported in this and the following paper (see

also Cote et al., 1984) on the OS-IS preparation. The chemistry measured is an average of all the cells in the suspension ($>10^6$), while the electrophysiology normally provides a sample of only 10–20 OS-IS. To reduce sampling bias, electrophysiological responses were recorded not only from obviously intact and well-formed outer segments, but also from the smaller number of bent, crinkled, or distorted ones. A surprising observation has been that there is little correlation between the physical appearance of an OS-IS and its light responses (excluding cells obviously damaged by the recording chamber or electrode). Some sampling bias was introduced by the presence of OS-IS that were either too small or too large to form an appropriate seal with the recording pipette. Electrophysiological

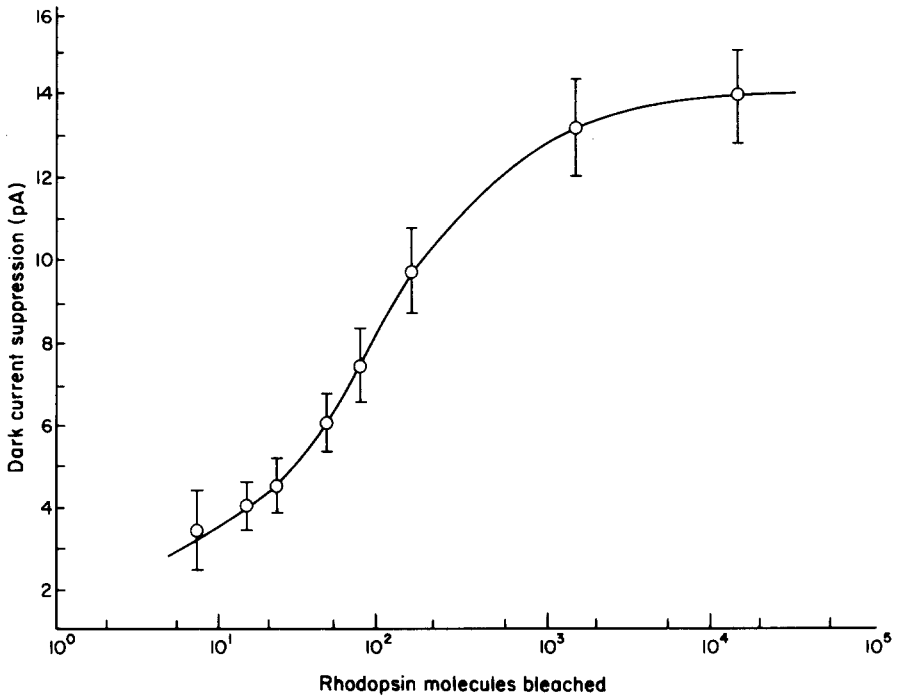

FIGURE 4. Sensitivity of dark current to flash illumination. Suppression of dark current (picoamperes) is plotted as a function of the number of rhodopsin molecules bleached by a 2-ms flash (110 cells). Error bars indicate the 95% confidence limit.

measurements were made on most of the OS-IS suspensions used in the biochemical experiments described below to ensure that the preparations were viable and had comparable physiological responses.

OS-IS Maintain Millimolar ATP and GTP Levels, Independent of the Dark Current

The data in Fig. 5 show that millimolar ATP and GTP levels are maintained for 2 h by OS-IS in glucose-bicarbonate Ringer's medium containing Percoll. Approximately 0.3 mol of GTP per mole rhodopsin and 0.25 mol of ATP per mole rhodopsin remain after 1–2 h in isolation. Given a rhodopsin concentration of 3

mM (assuming its uniform distribution throughout the outer segment) (Liebman and Entine, 1968) and a cytoplasmic volume equal to 50% of the total volume of the rod, these levels correspond to cytosolic ATP and GTP concentrations of 1.5 and 1.8 mM, respectively. Crude suspensions of OS have levels comparable to OS-IS immediately upon detachment from the retina, but decay within minutes to residual levels of 0.06–0.08 mol per mole rhodopsin, even when the structures are maintained intact in the same medium as the OS-IS. These structures lack the mitochondria-rich portion of the inner segment that presumably assists in maintaining nucleoside triphosphate levels.

ATP and GTP levels in OS-IS are almost fivefold higher than in OS obtained

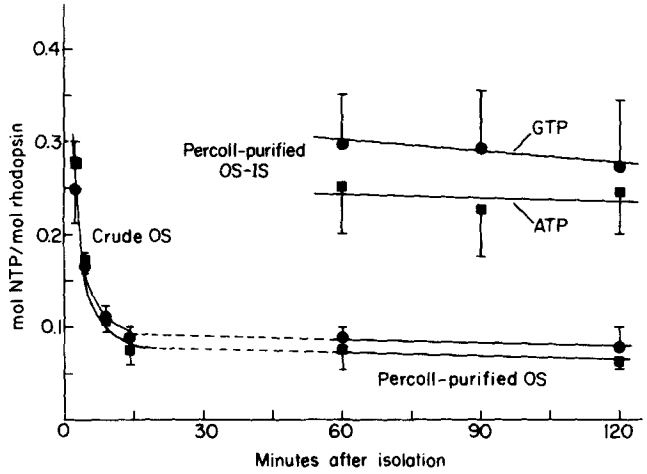

FIGURE 5. OS-IS maintain higher ATP and GTP levels than OS. Levels of GTP (circles) and ATP (squares) are shown as moles of each nucleotide per mole of rhodopsin present, in Percoll-purified OS-IS (upper curves) and in crude and Percoll-purified OS (lower curves). Because of the time required for purification by Percoll gradient sedimentation, values for the rapid initial decay of nucleotides in OS can be obtained only in crude preparations, and these values were taken from Biernbaum and Bownds (1979). Levels are plotted as a function of time in minutes after the start of isolation from the retina. Means are shown ± SEM for OS-IS (n = 3 at 60 min, n = 12 at 90 min, and n = 9 at 120 min) and for purified OS, (n = 3 at 60 min; a range of two values is shown at 120 min).

in the same Percoll gradients (for GTP, 4.7 ± 1.4 [SEM, 5]; for ATP, 4.6 ± 1.9 [SEM, 5]). GTP levels in OS-IS are also slightly higher than ATP levels. In 30 separate OS-IS preparations, the ratio of the GTP level to that of ATP was 1.2 ± 0.06 (SEM). This differs from most other cell types, in which the ATP concentration exceeds GTP by 10-fold (Soling, 1982). Retinal contaminants present in the crude suspensions and routinely separated by Percoll gradients from intact OS or OS-IS show high levels of ATP compared with GTP.

Attempts to enhance substantially the levels of ATP and GTP in OS-IS by altering the composition of the suspending medium were unsuccessful. Adding

fresh medium, introducing more oxygen, and increasing the bicarbonate level from 5 mM to as high as 35 mM were without effect. In three experiments, replacement of glucose by 5 mM glucose-6-phosphate caused a rise in ATP and GTP levels of 20% (measured after 20 min), but isolation of OS-IS in either Eagle's Dulbecco's minimal essential medium nutrient medium or Ringer's medium with glucose replaced by 5 mM glucose-6-phosphate resulted in normal nucleotide levels. When perturbations caused either slight increases or decreases in nucleotide levels, both ATP and GTP were affected similarly, and the ratio of ATP to GTP remained constant. This suggests an active buffering connection between the two, most likely mediated by the transphosphorylation discussed below.

Levels of ATP and GTP show little dependence on the maintenance of the dark current. In four experiments, the addition of sufficient ouabain (0.1 mM) to completely abolish the dark current caused no significant change in ATP levels ($-9 \pm 10\%$ SEM) and decreased GTP levels only slightly ($-19 \pm 6\%$ SEM) 3–12 min after addition. Conversely, over the range of 0.1–0.5 mol of ATP (and GTP) per mole of rhodopsin, there is no correlation between dark current and nucleotide content. In five different OS-IS preparations in which nucleotide levels varied over this range, comparable dark currents and kinetics were measured.

GTP and ATP Levels in OS-IS Are Not Altered by Changes in External Calcium

Lowering external calcium from millimolar to nanomolar levels enhances the rod dark current (Yau et al., 1981) and depresses light sensitivity (Bastian and Fain, 1982). In isolated outer segments, it causes a decrease in GTP and ATP levels and abolishes the sensitivity of GTP to light (Biernbaum and Bownds, 1979). The data of Fig. 6 demonstrate that OS-IS behave differently, maintaining constant ATP and GTP levels as the external calcium concentration is lowered. The sensitivity of GTP and ATP levels to light is also preserved (see Biernbaum and Bownds, 1985). This suggests the presence in OS-IS of a mechanism, absent in isolated OS, for maintaining constant nucleoside triphosphate levels. While maintenance of ATP levels ultimately depends on ATP synthesis in the mitochondria in the inner segment, this buffering of GTP and ATP levels may involve synthetic pathways present in the outer segment (Dontsov et al., 1978; de Azeredo et al., 1981; Schnetkamp and Daemen, 1981; Berger et al., 1980). These data make the further point that the physiological changes noted in low calcium (Yau et al., 1981) cannot be explained by changes in nucleoside triphosphate levels.

Fig. 6 indicates the levels of GMP, GDP, and GTP (upper panel) and of AMP, ADP, and ATP (lower panel) observed 5 min after the external Ca^{++} concentration was either raised or lowered from the 0.1 mM Ca^{++} concentration in the isolation medium to the value indicated on the abscissa. (The medium contained 0.1 mM Ca^{++} so that lowering the calcium concentration to nanomolar levels would require only 2.78 mM EGTA, avoiding the possible artifacts and osmotic complications introduced by using the 23.95 mM EGTA that would be required starting with 1 mM Ca^{++}.) ATP, GTP, GMP, and AMP levels remained close to

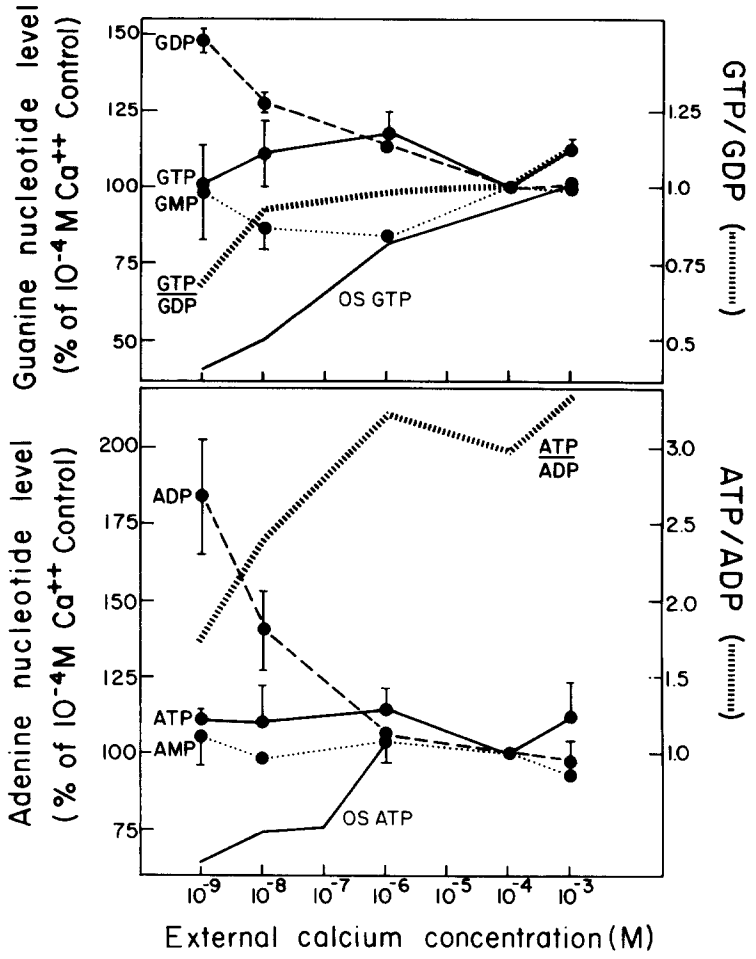

FIGURE 6. OS-IS nucleotide pools are stable to changes in external calcium concentration. Guanine and adenine nucleoside triphosphate (solid lines), diphosphate (dashed lines), and monophosphate (dotted lines) levels are plotted as a function of external calcium concentration. After isolation of OS-IS in 10^{-4} M Ca^{++}, the Ca^{++} concentration was changed to the levels indicated by the addition of EGTA. (OS-IS were isolated in 10^{-4} M Ca^{++} to reduce the amount of EGTA required for buffering.) After 5 min, samples were acid-quenched and analyzed by HPLC. Levels are expressed as percent of initial control level (left ordinate). The range of experimental values obtained in two similar experiments (using suspensions containing 90 and 75% OS-IS) is shown, except where only a single value is available. ATP and GTP values for OS are taken from Biernbaum and Bownds (1979). Values of the GTP/GDP and ATP/ADP ratios (cross-hatched lines) are indicated on the right ordinate.

their initial values, but significant changes were noted in ADP and GDP levels (dashed lines), which rose sharply as Ca^{++} was lowered below 10^{-6} M. The diphosphate increases were partially reversible: 5 min after restoration of Ca^{++}

(to 1 mM), ADP and GDP recovered to 70 and 50%, respectively, of their initial levels (data not shown). HPLC analysis indicated that these increases in diphosphate levels resulted from synthesis of 10–25% additional nucleotide mass upon lowering the external Ca^{++} concentration.

Monophosphate levels in OS-IS were high (twice that of diphosphates and half that of triphosphates); this pattern is characteristic of membrane-rich preparations such as mitochondria, which maintain large monophosphate pools as substrate for nucleotide synthesis (Soling, 1982). It is unlikely that the high monophosphate levels reflect an energy-depleted state caused by degradation of triphosphates, since the ATP/ADP and GTP/GDP ratios (with values of 7 and 1.2, respectively, in OS-IS maintained in 1 mM Ca^{++}) are similar to those reported earlier for living retinas and freshly prepared outer segments (de Azeredo et al., 1981; Robinson and Hagins, 1979). From the above ratios and the measured amounts of ATP and GTP (Fig. 5), absolute levels of all the nucleotides shown in Fig. 6 can be determined.

It is interesting that as external calcium levels are lowered (and presumably also internal calcium levels), triphosphate levels remain constant, while diphosphate levels increase. This results in a twofold decrease in the internal ATP/ADP and GTP/GDP ratios. This might have the effect of altering some enzyme reactions that are regulated by the triphosphate/diphosphate ratio (Atkinson, 1977). P. R. Robinson et al. (1980, 1982) and Kawamura and Bownds (1981) have shown that lowering calcium and decreasing the GTP/GDP ratio desensitizes the pathway linking rhodopsin bleaching to cyclic GMP phosphodiesterase activation via a GTP binding protein.

OS-IS Synthesize More ATP and GTP Than OS

The capacity of OS-IS to support nucleoside triphosphate synthesis is demonstrated by the incorporation of added $^{32}P_i$ into endogenous ATP and GTP (Fig. 7). During a 43-min incubation with $^{32}P_i$ in the dark (closed circles), the specific activities of ATP and GTP continuously increased. (Different OS-IS preparations vary in the extent of $^{32}P_i$ incorporation.) The ATP and GTP specific activities in OS-IS preparations were twice those measured in identically prepared and treated OS suspensions; this, taken together with the fact that ATP and GTP masses are fivefold higher in OS-IS than in OS, is a further indication that OS-IS maintain a much more active nucleotide metabolism than OS. It should be emphasized that the appearance of radioactive ATP and GTP does not give information on the rates at which these compounds are synthesized and degraded. All estimates of ATP turnover (cf. Witkovsky and Yang, 1982) indicate a lifetime for outer segment ATP of only a few minutes at most, and the more extended time course in Fig. 7 probably reflects the rate at which $^{32}P_i$ becomes available to participate in the synthesis of ATP and GTP.

Incorporation of external phosphate into ATP and GTP can be observed very soon after addition of the isotope to the medium. In one experiment, sampling the suspension 10, 30, and 60 s after addition of $^{32}P_i$ revealed ATP and GTP pools containing several hundred counts per minute. On the extended time scale of Fig. 7, the specific activities of ATP and GTP are equal and rise together. Thus, synthesis of GTP appears to be closely linked to synthesis of ATP, probably

by transphosphorylation from ATP. Transphosphorylation enzymes have been observed in outer segments (Berger et al., 1980; Schnetkamp and Daemen, 1981), and it seems likely that they underlie the parallel behavior of ATP and GTP levels here and in response to small perturbations (see above).

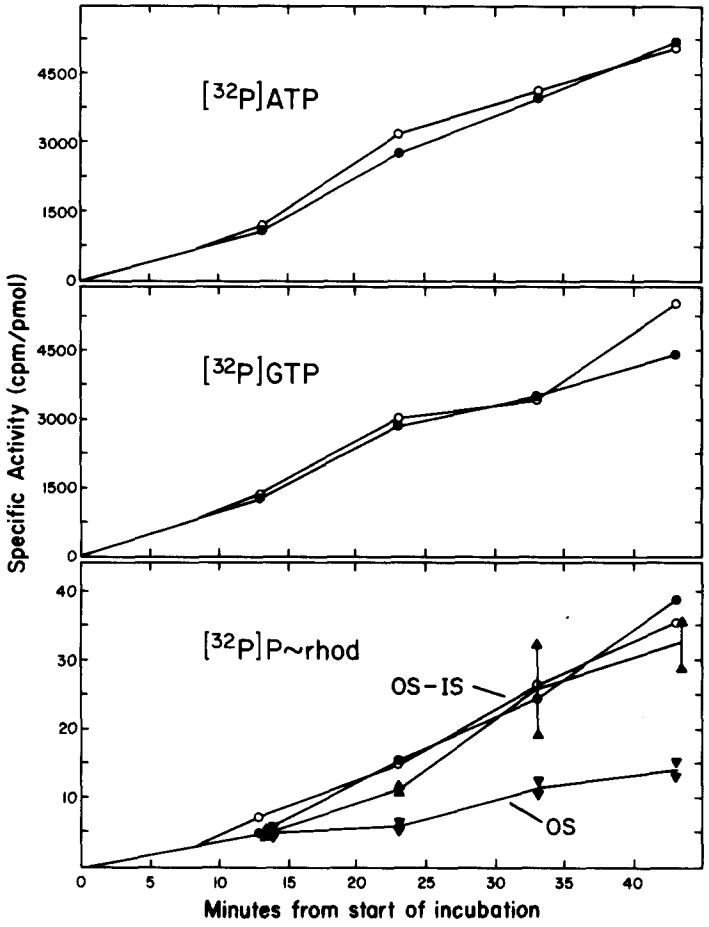

FIGURE 7. OS-IS synthesize ATP and GTP used for protein phosphorylations. Specific activities of ATP (upper panel) and of GTP (middle panel), expressed as cpm per picomole of nucleotide, are plotted as a function of incubation time (minutes) in the presence of $^{32}P_i$, as described in Methods. Data are from one of three similar experiments, showing uptake in the dark (closed circles) and with dim flash illumination (open circles). The flash bleached 115 rhodopsin molecules and was repeated every 10 s, starting 8 min after the addition of $^{32}P_i$. In the lower panel, levels of rhodopsin-bound [^{32}P]phosphate in the same dark and illuminated OS-IS samples are shown (circles). Additional data from comparable OS-IS (triangles) and OS (inverted triangles) preparations are included. Protein-bound phosphate increases continuously in the dark, paralleling the incorporation of $^{32}P_i$ into ATP and GTP pools. Radioactivity (cpm per picomole of rhodopsin) measured is that associated with the solubilized rhodopsin monomer and dimer bands after polyacrylamide gel electrophoretic separations.

Dim illumination sufficient to saturate the photoresponse—a flash bleaching 115 rhodopsin molecules per OS, presented every 10 s—does not alter either the mass or the specific activity of ATP and GTP (Fig. 7, open circles) until a cumulative bleach of $\sim 2 \times 10^4$ rhodopsin molecules per OS has occurred (middle panel, right-hand points). HPLC analysis demonstrates that the 24% increase in GTP specific activity observed at this point reflects a decrease in GTP mass, while total radioactivity remains constant. This suggests compartmentalization of the GTP pool, with light-activated GTPase activity drawing mainly on GTP that has not yet become labeled. This might occur if radioactive GTP is first synthesized by transphosphorylase activity from ATP and subsequently diffuses to the site in the outer segment, where its degradation by light-activated GTPase occurs. A further experiment using brighter illumination (a 2% rhodopsin bleach performed 33 min after the start of incubation with $^{32}P_i$) has both confirmed this result and shown further that, in contrast to GTP, ATP specific activity decreases upon illumination. Stated most directly, illumination reduces the mass more than the radioactivity associated with GTP (178,000 cpm/134 pmol GTP + light → 157,000 cpm/87 pmol GTP), while the radioactivity associated with ATP is reduced more than its mass (210,000 cpm/130 pmol ATP + light → 151,000 cpm/105 pmol ATP). This could be taken to suggest that most of the light-induced depletion of ATP occurs close to its site of synthesis.

Newly synthesized nucleoside triphosphate containing $^{32}P_i$ is used for phosphorylation of rhodopsin, which accounts for ~70% of the total phosphorylated protein in the outer segment. Fig. 7 (lower panel) shows that the increase in protein-bound [^{32}P]phosphate in the same preparation described in the upper panels parallels the increase in the specific activity of the triphosphate pools. This indicates that turnover of protein-bound phosphate, which is probably mediated by kinase-phosphatase activities, is ongoing in the dark, and that this turnover uses freshly synthesized triphosphate. Further, just as the specific activities of the triphosphates in OS-IS grow to exceed that in OS by a factor of 2 during the incubation period, so the protein phosphorylations drawing on them are twofold more efficient in OS-IS than in isolated OS. The amount of protein-bound phosphate, however, is <4% of the total radioactivity available in the donor triphosphate pool. The dim illumination used caused no change in the amount of rhodopsin-bound phosphate (open circles), which indicates a lower sensitivity to illumination than the kinase activity previously measured in crude disrupted OS preparations using added [^{32}P]ATP (Hermolin et al., 1982).

Calcium Movements in OS and OS-IS

Since light is known to increase the calcium concentration in the medium immediately surrounding outer segments on the retina (Gold and Korenbrot, 1980; Yoshikami et al., 1980), a further test of OS-IS viability was whether such an increase occurs in OS-IS suspensions. Thus, we examined the uptake and release of $^{45}Ca^{++}$ by OS and OS-IS in the dark and after illumination (Table I). This provided an opportunity to confirm reports that a sodium-calcium exchange mechanism operates in outer segment membranes (Schnetkamp, 1980) and that calcium movements are sensitive to cyclic GMP (Caretta and Cavaggioni, 1983; George and Hagins, 1983).

The following procedure, described more fully in the Methods section, was developed to maximize initial uptake of $^{45}Ca^{++}$, and then to retard its efflux so that light- or ion-stimulated changes might be more easily monitored. Briefly, OS and OS-IS were prepared in Na-Ringer's solution and then transferred for several minutes to a Ringer's solution in which KCl (115 mM) replaced all NaCl, and 0.1 mM Ca^{++} (with trace amounts of $^{45}Ca^{++}$) was present. This provided sodium and calcium gradients optimal for uptake (high sodium inside, high calcium outside) and resulted in uptake of as much as 20% of the $^{45}Ca^{++}$ added. Release of $^{45}Ca^{++}$ was then examined under the conditions listed in Table I. Much of the data in Table I were obtained with suspensions of intact OS. The

TABLE I

Uptake and Release of $^{45}Ca^{++}$ in OS-IS and OS

	Ca^{++} uptake* (moles exchangeable Ca per mole rhodopsin)	Stimulus	$^{45}Ca^{++}$ released*,‡
			%
Purified intact OS-IS§	0.14±0.02 (4)‖	Illumination (0.1–1%)	8±2 (3)
Purified intact OS	0.13±0.01 (2)	Illumination	−3±2 (3)
Crude OS	0.27±0.02 (20)¶	Illumination	−1±6 (12)
"		Fragmentation**	75±2 (5)
"		+ Na^+ (57 mM)	54±3 (6)
"		+ Ca^{++} (0.1 mM)	35±1 (2)
"		− Ca^{++} (≤0.1 μm)	34±6 (5)
Fragmented crude OS**	0.26±0.01 (5)	+ Na^+ (57 mM)	49±12 (3)
"		+ Ca^{++} (0.5 mM)	29±6 (2)
"		Refragmentation**	41

* Error is expressed as s for $n > 2$; for $n = 2$, the range of values about means is shown.

‡ Release from OS-IS was measured 0.25–4.5 min after start of stimulus; from OS, after 0.5–4, 0.25–3, 1–2, 8–11, 10, and 8–15 min, respectively; from fragmented OS, after 6–11 min.

§ Purified OS-IS and OS were prepared in Na-Ringer's medium and then incubated with $^{45}Ca^{++}$ in K-Ringer's medium, as described in Methods. OS-IS content ranged from 50 to 85%.

‖ In Percoll-purified OS and OS-IS, calcium uptake was lower than in crude suspensions because the outer segments had aged for >1 h before calcium uptake was examined. Miki et al. (1980) and Schnetkamp et al. (1977) have reported a similar effect.

¶ Replacement of Na^+ by K^+ during isolation of OS decreased $^{45}Ca^{++}$ uptake to 0.03 ± 0.02 (s, $n = 9$).

** Outer segment structures were broken into fragments 10–30 μm in length by pipetting with shearing force against glass for 1 min.

technically more difficult OS-IS preparation was used only to confirm the same basic loading and release behavior and then to examine the effects of illumination.

The most striking difference between OS-IS and isolated OS is in their response to light. In 15 separate experiments on OS, no reproducible change in accumulated $^{45}Ca^{++}$ occurred in response to illumination (Table I). Neither the addition of the calcium ionophore A23187 nor disruption of the outer segments before or after illumination revealed any light-induced response. Illumination of OS-IS preparations, on the other hand, caused in each of three separate experiments a release of $^{45}Ca^{++}$ into the extracellular medium (~14,000 cpm, measured 15 s

after illumination). If one assumes that the calcium released has the same specific activity as the calcium added to the medium, this corresponds to ~15 calcium ions per molecule of rhodopsin bleached. This is lower than the actual value, since isotope dilution occurs as entering $^{45}Ca^{++}$ mixes with nonlabeled Ca^{++} present in the outer segment (Schnetkamp, 1979; Schroder and Fain, 1983).

It is interesting that incubation of OS-IS in the presence of 50 μM IBMX, a phosphodiesterase inhibitor known to cause elevation of internal cyclic GMP levels (Woodruff et al., 1977), increased the $^{45}Ca^{++}$ uptake by ~50% (48% ± 6% [n = 2]). The data of Capovilla et al. (1983) are compatible with the action of the inhibitor being relatively specific when added at these low concentrations, and this reinforces the suggestion that cyclic GMP may play a role in regulating internal calcium levels (Cervetto and McNaughton, 1983).

The calcium movements observed are associated with outer segment membranes rather than with inner segments or other contaminating retinal material (cf. George and Hagins, 1983). In the 30 separate experiments that contributed the data shown in Table I, using both crude and purified outer segments, the inner segment content varied more than 20-fold (from 4 to 85%), but the amount of $^{45}Ca^{++}$ taken up and released correlated only with the amount of rhodopsin present. Intentional contamination with pigment epithelium did not alter this result. The experiments of Schroder and Fain (1984) also show a relatively low calcium content in the inner segment compared with the outer segment and no significant involvement of the mitochondria in calcium uptake and release.

In basic agreement with the data obtained by Schnetkamp (1980) for bovine rod outer segments, calcium release from the frog outer segments was facilitated by the addition of external sodium or calcium ions (Table I). Further, while mechanical disruption of outer segments by shearing also released calcium (cf. Schnetkamp et al., 1977), fragmented outer segments prepared by shearing could accumulate and release calcium under these conditions (Table I). These data are consistent with the presence of a sodium-calcium exchange process in both disk and plasma membrane (cf. Schnetkamp, 1980; Bastian and Fain, 1982; Yau et al., 1981). (The sensitivity of the system to mechanical shock leads us to suspect that the failure of Szuts [1980] to observe significant calcium uptake or sodium-calcium exchange by frog disk suspensions may have been caused by the constant stirring employed during incubation.).

DISCUSSION

Outer Segments with Attached Ellipsoids as an In Vitro Model of Photoreceptors on the Retina

A primary goal of this work has been to determine whether purified suspensions of OS-IS (Fig. 2) might be a favorable preparation for correlating chemical and electrophysiological changes following light absorption. To evaluate this point, we have compared the properties of the OS-IS preparation with previous measurements on intact isolated OS, as well as with photoreceptors still attached to the retina. Such a comparison shows that OS-IS differ from isolated OS in many respects and more closely reflect the in vivo situation than OS.

Much of the similarity between OS-IS and photoreceptors on the retina can

be attributed to the presence of the mitochondria-rich ellipsoid portion of the inner segment (Fig. 3). The ellipsoid region is the source of the light-modulated dark current, and 70–90% of the OS-IS have dark currents and a light sensitivity similar to those recorded from outer segments still attached to the living retina. Further, this electrophysiological activity is sustained for several hours. However, separation from the retina does cause some impairment, since OS-IS lose viability over a period of many hours. Isolated OS, on the other hand, show no dark current, and indirect osmotic assays of the light-induced decrease in sodium conductance (Korenbrot and Cone, 1972; Bownds and Brodie, 1975) indicate that the conductance change, if it occurs at all, is much smaller than in vivo.

The dark current measured by the suction electrode technique is presumed to be accounted for mainly by efflux of potassium from the inner segment and uptake of sodium by the outer segment (Hagins et al., 1970). This movement is supported by a ouabain-sensitive Na^+,K^+-ATPase located in the plasma membrane of the inner segment (Stirling and Lee, 1980), and indeed the addition of ouabain to OS-IS completely abolishes the dark current as it does on the retina. It seems likely that detaching the outer segment from this current source causes internal sodium and calcium levels to rise, thus poisoning the conductance mechanism (Yau et al., 1981; Bastian and Fain, 1982).

Attachment of the inner and outer segments is also required for the observation of a light-induced release of calcium into the suspending medium (Table I). The rapidity and magnitude of the increase in $^{45}Ca^{++}$ in the external medium are consistent with an active extrusion of calcium from OS-IS, which was not seen with the OS because they are deficient in both nucleotide levels and the normal dark current. The observation of light-stimulated $^{45}Ca^{++}$ release provides a useful supplement to the calcium electrode measurements (Gold and Korenbrot, 1980; Yoshikami et al., 1980), which did not determine whether the rise in extracellular calcium resulted from a reduction of calcium influx or an efflux. The demonstration of light-induced $^{45}Ca^{++}$ release in OS-IS preparations provides evidence that some of the rise in extracellular calcium can be accounted for by efflux.

The finding that calcium accumulation can be enhanced by a phosphodiesterase inhibitor known to increase cyclic GMP levels suggests that the OS-IS preparation may be a useful one for further studies of detailed interactions between the calcium and cyclic GMP regulatory mechanisms (cf. Hermolin et al., 1982).

Compartmentalization of Nucleoside Triphosphates

The presence of the mitochondria-rich ellipsoid region of the inner segment helps maintain not only ion gradients, but also fivefold higher levels of ATP and GTP than in isolated OS and ATP/ADP ratios of 4–7 (in Ringer's medium containing 1 mM Ca^{++}). These values are similar to those obtained in vivo by direct freeze-sectioning of the photoreceptor layer of living retinas (de Azeredo et al., 1981; Berger et al., 1980) in isolated frog OS immediately upon detachment from the retina (Biernbaum and Bownds, 1979; W. E. Robinson and Hagins, 1979), and in other tissues (Mandel, 1964; Henderson and Paterson, 1973; Klingenberg and Heldt, 1982).

The data in this paper do not address the question of how the ATP and GTP

pools are partitioned between inner and outer segment portions of the OS-IS. The earlier analysis of Biernbaum and Bownds (1979) indicated that 65–75% of measured ATP was associated with inner segment ellipsoids, while inner segment GTP accounted for ~30% of the total. The freeze-sectioning studies of de Azeredo et al. (1981) indicate a similar partitioning. This distribution might underlie the functional inhomogeneity of the ATP and GTP pools shown by the tracer experiments described in connection with Fig. 7. The tracer data could be explained if both ATP and GTP are synthesized in the inner segment, with the light-induced decrease in ATP mainly occurring there, while the GTP decrease mainly occurs in the outer segment. It seems likely that ATP and GTP levels in both the inner and outer segment are maintained between 0.2 and 1 mM, well above the micromolar K_m values of the enzymes known to use them. Because there are numerous intracellular binding sites for nucleotides, however, the actual cytoplasmic activities probably are lower.

Significance of Comparisons Between Chemistry and Physiology

OS-IS appear to be a more favorable preparation than isolated OS for correlating the chemistry and electrophysiology occurring in the functioning photoreceptor. A significant issue in interpreting such experiments, however, is whether the chemical measurements are reporting on the same population of outer segments as the physiological measurements. To take the most extreme example, one could imagine that the light-induced chemical changes reported in the following paper (Biernbaum and Bownds, 1985) are contributed mainly by physiologically inactive OS or disrupted OS-IS in the population, while the electrophysiology reflects only what happens in the intact viable cells in which the chemical changes are smaller. With respect to ATP and GTP measurements reported here and their light-sensitive responses analyzed in the following paper, the issue is not a serious one, for overall levels are fivefold higher in OS-IS than in isolated OS (Fig. 6). Given that all of the data are obtained on preparations containing 50–90% OS-IS, this sets an upper limit of 10% as the OS contribution to total nucleoside triphosphate. Random sampling of these OS-IS populations demonstrates that 7–9 out of every 10 cells have normal dark currents. This represents a lower limit, since some of the nonresponding cells have probably been damaged by the electrode. Thus, it seems likely that with respect to the nucleoside triphosphates, physiological and chemical measurements monitor properties of the same population of cells. Similar criteria will need to be applied to each biochemical property studied.

We are grateful to Drs. Heidi Hamm, Rick Cote, Grant Nicol, and Patricia Witt for critical comments on this manuscript.
This work was supported by National Institutes of Health grant EY-00463.

Original version received 19 December 1983 and accepted version received 24 September 1984.

REFERENCES

Atkinson, D. E. 1977. Adenylate control and the adenylate energy charge. *In* Cellular Energy Metabolism and Its Regulation. Academic Press, Inc., New York. 85–107.

Bader, C. R., P. R. MacLeish, and E. A. Schwartz. 1979. A voltage-clamp study of the light response in solitary rods of the tiger salamander. *J. Physiol. (Lond.).* 296:1–26.

Bastian, B. L., and G. F. Fain. 1982. The effects of sodium replacement on the responses of toad rods. *J. Physiol. (Lond.).* 330:331–347.

Baylor, D. A., T. D. Lamb, and K.-W. Yau. 1979. The membrane current of single rod outer segments. *J. Physiol. (Lond.).* 288:589–611.

Berger, S. J., G. W. DeVries, J. G. Carter, D. W. Schulz, P. N. Passonneau, O. H. Lowry, and J. A. Ferrendelli. 1980. The distribution of components of the cyclic GMP cycle in retina. *J. Biol. Chem.* 255:3128–3133.

Biernbaum, M. S., and M. D. Bownds. 1979. Influence of light and calcium on guanosine 5′-triphosphate in isolated frog rod outer segments. *J. Gen. Physiol.* 74:649–669.

Biernbaum, M. S., and M. D. Bownds. 1982. Studies on purified suspensions of frog rod outer segments with attached ellipsoids. *Invest. Ophthalmol. Visual Sci.* 22:187. (Abstr.)

Biernbaum, M. S., and M. D. Bownds. 1985. Light-induced changes in GTP and ATP in frog rod photoreceptors. Comparison with recovery of dark current and light sensitivity during dark adaptation. *J. Gen. Physiol.* 85:107–121.

Bownds, M. D. 1981. Molecular mechanisms of visual transduction. *Trends Neurosci.* 4:214–217.

Bownds, D., and A. E. Brodie. 1975. Light-sensitive swelling of isolated frog rod outer segments as an in vitro assay for visual transduction and dark adaptation. *J. Gen. Physiol.* 66:407–425.

Bownds, D., A. Gordon-Walker, A. C. Gaide-Huguenin, and W. Robinson. 1971. Characterization and analysis of frog photoreceptor membranes. *J. Gen. Physiol.* 58:225–237.

Brodie, A. E., and D. Bownds. 1976. Biochemical correlates of adaptation processes in isolated frog photoreceptor membranes. *J. Gen. Physiol.* 68:1–11.

Capovilla, M., L. Cervetto, and V. Torre. 1983. The effect of phosphodiesterase inhibitors on the electrical activity of toad rods. *J. Physiol. (Lond.).* 343:277–294.

Caretta, A., and A. Cavaggioni. 1983. Fast ionic flux activated by cyclic GMP in the membrane of cattle rod outer segments. *Eur. J. Biochem.* 132:1–8.

Cervetto, L., and P. A. McNaughton. 1983. Inhibition of the light-sensitive current in vertebrate rods by La^{3+} ions. *J. Physiol. (Lond.).* 343:75P–76P.

Cote, R. C., M. S. Biernbaum, G. D. Nicol, and M. D. Bownds. 1984. Light-induced decreases in cGMP concentration precede changes in membrane permeability in frog rod photoreceptors. *J. Biol. Chem.* 259:9635–9641.

de Azeredo, F. A. M., W. D. Lust, and J. V. Passoneau. 1981. Light-induced changes in energy metabolites, guanine nucleotides, and guanylate cyclase within frog retinal layers. *J. Biol. Chem.* 256:2731–2735.

Dontsov, A. E., P. A. Zak, and M. A. Ostrovskii. 1978. Regeneration of ATP in outer segments of frog photoreceptors. *Biochem. USSR.* 43:471–474.

Fliesler, S. J., R. E. Anderson, and D. M. K. Lam. 1980. Isolation of metabolically active truncated rod photoreceptors. *ARVO Suppl. Invest. Ophthalmol. Visual Sci.* 178.

George, J. S., and W. A. Hagins. 1983. Control of Ca^{++} in rod outer segment disks by light and cyclic GMP. *Nature (Lond.).* 303:344–348.

Gold, G. H., and J. I. Korenbrot. 1980. Light-induced calcium release by intact retinal rods. *Proc. Natl. Acad. Sci. USA.* 77:5557–5561.

Hagins, W. A., R. D. Penn, and S. Yoshikami. 1970. Dark current and photocurrent in retinal rods. *Biophys. J.* 10:380–412.

Hagins, W. A., and S. Yoshikami. 1977. Intracellular transmission of visual excitation in

photoreceptors: electrical effects of chelating agents introduced into rods by vesicle fusion. *In* Vertebrate Photoreception. H. B. Barlow and P. Fatt, editors. Academic Press, Inc., New York. 97–139.

Henderson, J. F., and A. R. P. Paterson. 1973. Free nucleotides of cells and tissues. *In* Nucleotide Metabolism. Academic Press, Inc., New York. 9–21.

Hermolin, J., M. A. Karell, H. E. Hamm, and M. D. Bownds. 1982. Calcium and cyclic GMP regulation of light-sensitive protein phosphorylation in frog photoreceptor membranes. *J. Gen. Physiol.* 79: 633–655.

Kavipurapu, P. R., D. B. Farber, and R. N. Lolley. 1982. Degradation and resynthesis of cyclic 3′,5′-guanosine monophosphate in truncated rod photoreceptors from bovine retina. *Exp. Eye Res.* 34:181–189.

Kawamura, S., and M. D. Bownds. 1981. Light adaptation of the cyclic GMP phosphodiesterase of frog photoreceptor membranes mediated by ATP and calcium ions. *J. Gen. Physiol.* 77:571–591.

Klingenberg, M., and H. W. Heldt. 1982. The ADP/ATP translocation in mitochondria and its role in intracellular compartmentation. *In* Metabolic Compartmentation. H. Sies, editor. Academic Press, Inc., New York. 101–122.

Korenbrot, J. I., and R. A. Cone. 1972. Dark ionic flux and the effects of light in isolated outer segments. *J. Gen. Physiol.* 60:20–45.

Lamb, T. D., P. A. McNaughton, and K.-W. Yau. 1981. Spatial spread of activation and background desensitization in toad rod outer segments. *J. Physiol. (Lond.).* 319:463–496.

Liebman, P. A., and G. Entine. 1968. Visual pigments of frog and tadpole (*Rana pipiens*). *Vision Res.* 8:761–775.

Liebman, P. A., P. Mueller, and E. N. Pugh, Jr. 1984. Protons suppress the dark current of frog retinal rods. *J. Physiol. (Lond.).* 347:85–110.

Mandel, P. 1964. Free nucleotides in animal tissues. *Prog. Nucleic Acid Res. Mol. Biol.* 3:299–334.

Miki, N., C.-H. Kuo, Y. Hayashi, and M. Akiyama. 1980. Functional role of calcium in photoreceptor cells. *Photochem. Photobiol.* 32:503–508.

Polans, A. S., J. Hermolin, and M. D. Bownds. 1979. Light-induced dephosphorylation of two proteins in frog rod outer segments. Influence of cyclic nucleotides and calcium. *J. Gen. Physiol.* 74:595–613.

Polans, A. S., S. Kawamura, and M. D. Bownds. 1981. Influence of calcium on guanosine 3′,5′-cyclic monophosphate levels in frog rod outer segments. *J. Gen. Physiol.* 77:41–48.

Pugh, E. N., and P. A. Liebman. 1981. Photocurrents of solitary rod outer segments with ellipsoids. *Invest. Ophthalmol. Visual Sci.* 20:182. (Abstr.)

Robinson, P. R., S. Kawamura, B. Abramson, and M. D. Bownds. 1980. Control of the cyclic GMP phosphodiesterase of frog photoreceptor membranes. *J. Gen. Physiol.* 76:631–645.

Robinson, P. R., M. J. Radeke, and M. D. Bownds. 1982. Regulation of the flash responses of frog photoreceptor cyclic GMP phosphodiesterase and GTP binding protein. *Invest. Ophthalmol. Visual Sci.* 22:186. (Abstr.)

Robinson, W. E., and W. A. Hagins. 1979. GTP hydrolysis in intact rod outer segments and the transmitter cycle in visual transduction. *Nature (Lond.).* 280:398–400.

Schnapf, J. L. 1983. Dependence of the single photon response on longitudinal position of absorption in toad rod outer segments. *J. Physiol. (Lond.).* 343:147–159.

Schnetkamp, P. P. M. 1979. Calcium translocation and storage of isolated intact cattle rod outer segments in darkness. *Biochim. Biophys. 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 membranes. *Biochim. Biophys. Acta.* 598:66–90.

Schnetkamp, P. P. M., and F. J. M. Daemen. 1981. Transfer of high-energy phosphate in bovine rod outer segments. A nucleotide buffer system. *Biochim. Biophys. Acta.* 672:307–312.

Schnetkamp, P. P. M., F. J. M. Daemen, and S. L. Bonting. 1977. Biochemical aspects of the visual process. XXXVI. Calcium accumulation in cattle rod outer segments: evidence for a calcium-sodium exchange carrier in the rod sac membrane. *Biochim. Biophys. Acta.* 468:259–270.

Schroder, W. H., and G. L. Fain. 1983. Calcium content and calcium exchange in intact vertebrate photoreceptors. *Biophys. J.* 41:126*a*. (Abstr.)

Schroder, W. H., and G. L. Fain. 1984. Light-dependent calcium release from photoreceptors measured by laser micro-mass analysis. *Nature (Lond.).* 309:268–270.

Soling, H. D. 1982. Species dependent compartmentation of guanine nucleotide metabolism in the vertebrate liver cell. *In* Metabolic Compartmentation. Sies, H., editor. Academic Press, Inc., New York. 123–146.

Stirling, C. E., and A. Lee. 1980. ^{3}H Ouabain autoradiography of frog retina. *J. Cell Biol.* 85:313–324.

Szuts, E. Z. 1980. Calcium flux across disk membranes. Studies with intact rod photoreceptors and purified disks. *J. Gen. Physiol.* 76:253–286.

Witkovsky, P., and C.-Y. Yang. 1982. Transport and phosphorylation of 2-deoxy-D-glucose by amphibian retina. Effects of light and darkness. *J. Gen. Physiol.* 80:173–190.

Woodruff, M. L., and M. D. Bownds. 1979. Amplitude, kinetics and reversibility of a light-induced decrease in guanosine 3′,5′-cyclic monophosphate in frog photoreceptor membranes. *J. Gen. Physiol.* 73:629–653.

Woodruff, M. L., D. Bownds, S. H. Green, F. L. Morrisey, and A. Shedlovsky. 1977. Guanosine 3′,5′-cyclic monophosphate and the in vitro physiology of frog photoreceptor membranes. *J. Gen. Physiol.* 69:667–679.

Yau, K.-W., P. A. McNaughton, and A. L. Hodgkin. 1981. Effect of ions on the light-sensitive current in retinal rods. *Nature (Lond.).* 292:502–505.

Yoshikami, S., J. S. George, and W. A. Hagins. 1980. Light-induced calcium fluxes from outer segment layer of vertebrate retinas. *Nature (Lond.).* 286:395–398.

Yoshikami, S., W. E. Robinson, and W. A. Hagins. 1974. Topology of the outer segment membranes of retinal rods and cones revealed by a fluorescent probe. *Science (Wash. DC).* 185:1176–1179.