In Vitro Maintenance of a Pure-Cone Retina

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The retina of the lizard, *Sceloporus occidentalis,* appears to have only cone photoreceptors. Eyecups from this animal were incubated in media containing Earle's Balanced Salts, supplemented with amino acids and vitamins, and gassed with 5% CO_2/95% O_2. Under these conditions, good morphology, protein synthesis, and normal cyclic AMP and cyclic GMP levels were maintained for 1–2 days. This in vitro preparation is likely to be useful for pharmacological studies of cone photoreceptors. Invest Ophthalmol Vis Sci 27:666–673, 1986

Isolated retinae or eyecups of a variety of animals are now widely used in pharmacological and physiological studies of the retina. Like all in vitro systems, they afford greater pharmacological control than using the whole animal. Furthermore, they have an added advantage over perfused-eye preparations, in that the retina can be exposed to substances that cannot permeate the blood–retina barrier.

To date, however, most retinae (or segments of retinae) used for in vitro studies have been rod-dominated; use of cone-dominated retinae in vitro has been limited to an occasional electrophysiological study (e.g., ref 1). Our aim was to develop a way for maintaining a pure-cone retina in vitro. The western-fence lizard, *Sceloporus occidentalis,* like the majority of lizards and snakes,[2] has a pure-cone retina.[3,4] Consequently, we sought culture conditions in which eyecups from adult *Sceloporus* could be kept viable for 1–2 days. Viability was tested on the grounds of maintenance of morphology, rates of amino acid uptake and incorporation, and levels of adenosine 3′,5′-monophosphate (cyclic AMP) and guanosine 3′,5′-monophosphate (cyclic GMP).

Materials and Methods

Adult *Sceloporus occidentalis* were caught locally and kept in a vivarium for a few weeks until use, on a 12 hr light/12 hr dark cycle that coincided approximately with day and night. To prepare eyecups, firstly lizards were decapitated, double-pithed, and their eyes enucleated. The cornea and lens were then cut out from each eye while the eye was submerged in Hanks' balanced salt solution (GIBCO; Grand Island, NY). The resulting posterior segments (i.e., eyecups) were incubated under axenic conditions for 2, 10, 26, or 50 hr in medium-filled culture dishes on a rotator (30 cycles/min) at 24°C. Incubations began about 6 hr after light onset, and thereafter eyecups were kept under dim cool fluorescent illumination (50 lux). During the last 2 hr of incubation, eyecups were exposed to 100 μCi/ml ^{3}H-L-leucine (specific activity, 61 Ci/mmol; Amersham; Arlington Heights, IL). At the end of incubation, they were rinsed briefly several times in medium without ^{3}H-leucine, and then usually cut in half down the dorso–ventral axis, so that one half could be used for one type of analysis and the other half for another.

The procedures used conform to the ARVO Resolution on the Use of Animals in Research.

Microscopy and Autoradiography

Eyecups to be examined for morphology were fixed in 2% formaldehyde in phosphate buffer. In some cases, they were transferred after 2 hr to fixative that also contained 2% glutaraldehyde. All tissues were postfixed in buffered 1% OsO_4, dehydrated in ethanol, and embedded in Araldite. Semi-thin sections (1 μm) were collected on glass slides. For light microscopic autoradiography, the slides were dipped in 50% Kodak NBT-2 emulsion (Rochester, NY), and, after suitable exposure, the autoradiograms were developed in full strength D-19 and fixed in Kodak fixer. The tissues were then stained with toluidine blue.

Ultrathin sections were cut for electron microscopic autoradiography from eyecups that had been labeled somewhat differently. These eyecups were exposed to 500 μCi/ml ^{3}H-leucine for 30 min, and then maintained through a series of "washes" in unlabeled medium for 5.5 hr before fixation (i.e., pulse-labeled, followed by a "cold chase"). The sections were collected

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Fig. 1. Light micrograph montage of a section from a *Sceloporus* eyecup maintained in vitro for 26 hr. Arrow indicates deep fovea. Scale bar = 1 mm.

on parlodian-coated (1%) glass slides, stained with uranyl acetate and lead citrate, carbon-coated, and then dipped in 20% Ilford L4 emulsion. After exposure, the autoradiograms were developed in Phenidon for 1 min at 15°C, stopped, and fixed in 30% sodium thiosulfate. Next, the parlodian film was floated off the slides on to water, and grids were placed over the sections. The film, sections and grids were retrieved from the water surface with wet filter paper. After drying, the parlodian film was thinned by brief immersion in amyl acetate.

Biochemical Analysis of Amino Acid Uptake and Incorporation

Each retina (retinal pigment epithelium [RPE] plus neural retina) was separated from the choroid and removed as one piece from its eyecup, and then immediately frozen in liquid nitrogen. After storage at −80°C, retinae were rapidly homogenized as they thawed in cold phosphate-buffered saline. Aliquots were: (1) added to cold 20% (final w/v) trichloroacetic acid (TCA) for determination of the radioactivity of the protein and nonprotein fractions; (2) added to sample buffer for sodium dodecyl sulfate, polyacrylamide gel electrophoresis (SDS-PAGE); (3) used to measure protein concentration.

After centrifugation at 2000 g for 30 min at 4°C, the supernatant was removed and saved. The pellet was washed twice with 1 ml 10% TCA and redissolved in 1 N NaOH. Aliquots of the supernatant, redissolved pellet, and the homogente before the addition of TCA were added to scintillation vials and their radioactivity determined by scintillation counting in 5 ml Aquamix (Westchem; San Diego, CA). The radioactivity of the pellet was taken as a measure of ^{3}H-leucine incorporated into protein, and that of the supernatant as a measure of ^{3}H-leucine taken up by cells but not incorporated into protein. As a check, the sum of these two was compared to the radioactivity of the homogenate.

Samples were kept at least overnight in sample buffer, containing SDS and β-mercaptoethanol. Eighty micrograms of tissue protein was added to each well of slab gels, consisting of a stacking gel and a 10% SDS-polyacrylamide running gel. Electrophoresis was carried out at 15 mA for the first hour, and then 20 mA. Gels were stained for detection of protein with Coomassie brilliant blue R. The incorporation of radioactivity into retinal proteins resolved by gel electrophoresis was assessed by fluorography: Gels were infiltrated with Autofluor (National Diagnostics; Somerville, NJ), dried on to filter paper, and then

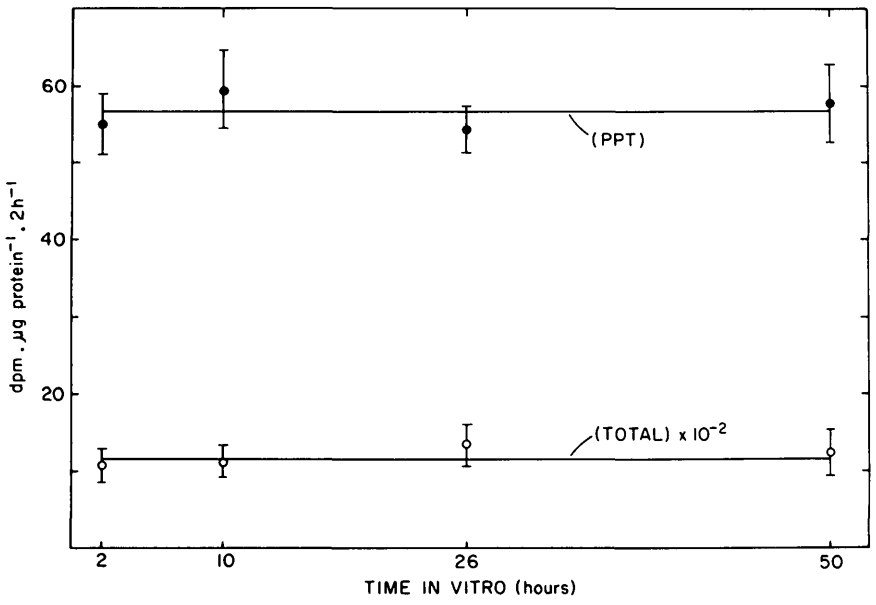

Fig. 2. Rate of ^{3}H-leucine uptake and incorporation into retinae (dpm. μg protein^{-1} · 2 h^{-1}), as a function of total time incubated in RMPI 1640. Eyecups were exposed to 100 μCi/ml during the last 2 hr in vitro. Specific activities of the retinal homogenates (TOTAL), representing total uptake, and the TCA-precipitable fractions (PPT) of the homogenates, representing that incorporated into protein, were determined. Data were aggregated from three separate experiments, with each point representing the mean (±1 SEM) of six to eight samples.

placed in contact with x-ray film (Kodak SB-5). After suitable exposure, the film was developed and fixed.

Protein concentrations were measured by the method of Lowry et al,[5] using bovine serum albumin as a standard.

Measurements of Cyclic AMP and GMP

Each retina was quickly removed and homogenized in 0.1 N HCl. After boiling and centrifugation, the acid supernatant was diluted in 50 mM sodium acetate buffer (pH 6.2), and its cyclic AMP and GMP content was determined by the radioimmunoassay described by Farber and Lolley.[6] To increase sensitivity, the cyclic nucleotides were acetylated.[7] The protein content of the HCl homogenate was determined as above.[5]

Results

Retinal Organization

The retina of *Sceloporus* has been reported to contain no rod photoreceptors.[3,4] We have also never seen a rod in this retina during the present study and in studies (manuscript in preparation) on outer segment disc shedding. As in the projects of Young[3] and Bernstein et al,[4] our study on disc shedding has involved extensive examination of the RPE-photoreceptor layer by light and electron microscopy.

There are three types of cone photoreceptors: single cones, and the chief and accessory members of the double cones.[3] The spacing of the cones varies widely across the retina. At the periphery, they form a coarse mosaic, with the cone inner segments about 12 μm in width and the outer nuclear layer (ONL) containing only a single row of nuclei. Towards the center, the cone outer segments (COSs) are longer, the cone mosaic becomes finer, and the retina becomes thicker, as the ONL consists of several layers of nuclei (Fig. 1). The cones are packed most densely in the region of a deep fovea, or retinal pit (Fig. 1). Here COSs extend over 30 μm in length and have a center-to-center spacing of 1–2 μm.

Amino Acid Uptake and Incorporation

First, we tested a variety of culture media for their ability to sustain amino acid uptake and incorporation. The media tested contained Earle's Balanced Salts (EBS), which is buffered largely by HCO_3^-/CO_2, plus varieties of amino acids and vitamins. Previous reports have indicated that retinae require significant amounts of bicarbonate[8,9] and amino acids[10] for the normal functioning of some processes in vitro. Retinal metabolism is severely reduced in phosphate buffer (see discussion in ref 11). Tested media were (1) EBS plus 1 g/l casamino acids (Difco; Detroit, MI); (2) Medium 199; (3) Dulbecco's modified Eagle medium; (4) Dulbecco's modified Eagle medium plus 10% fetal calf serum; (5) RMPI 1640; (6) RMPI 1640 plus 10% fetal calf serum. Antibiotics (100 μg/ml Gentamicin sulfate (Sigma; St. Louis, MO) and 5 μg/ml Amphotericin B (Sigma)) were added to each medium. Medium 199, Dulbecco's modified Eagle medium, and RMPI 1640 are commercially available (GIBCO), mainly for use with mammalian systems, but equally useful for reptilian tissues since mammalian and reptilian plasmas have similar osmolarities. They were developed for use with different cells and tissues, so that, although they have the HCO_3^-/CO_2 buffer in common, they vary in their precise salt, amino acid, and vitamin content (see

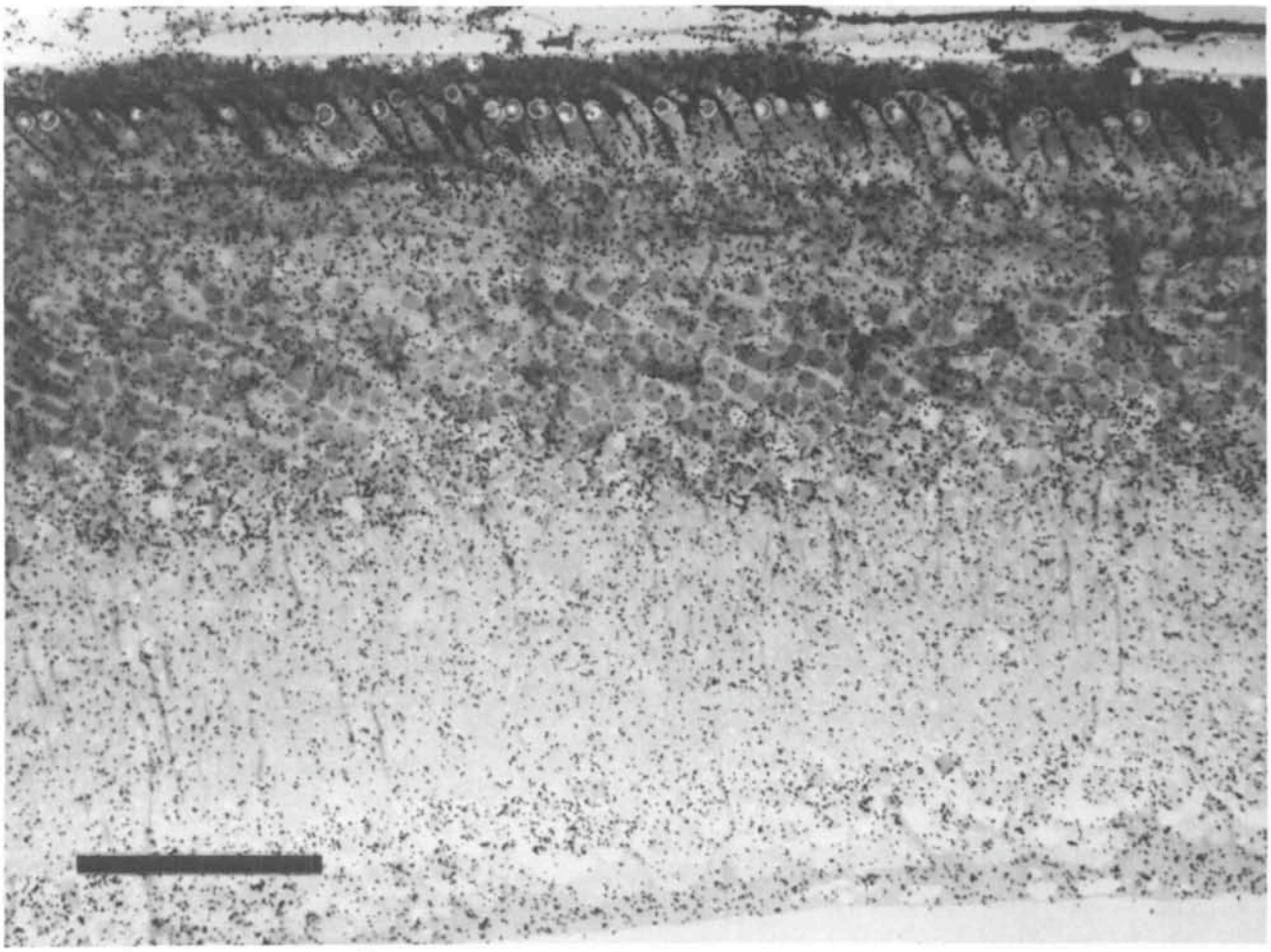

Fig. 3. Light microscopic autoradiograph of a retina maintained in eyecup culture for 50 hr, and exposed to ^{3}H-leucine during the last 2 hr. (Note that the darkness between the cones is due mostly to pigment [and not radiolabel] in the apical processes of the RPE—a normal characteristic of this retina.) Scale bar = 50 μm.

ref 12 for details). The six tested media thus differed according to (1) whether or not they contained fetal calf serum; (2) minor modifications of EBS; and (3) the types and amounts of amino acids and vitamins in them (with EBS + casamino acids containing the simplest mixture).

Overall uptake and incorporation of ^{3}H-leucine by retinae was measured by determining the specific activities of retinal homogenates and of the precipitable and soluble fractions of the homogenates after protein had been precipitated by TCA. The radioactivities of the TCA-soluble and TCA-precipitable fractions represent, respectively, label taken up but not incorporated into protein, and label that was incorporated into protein. Their sum represents the total amount of label taken up by cells and was found to be in good agreement with the radioactivity of the homogenate before TCA precipitation.

Radioactivities of both the soluble and precipitable fractions were found not to vary with time in vitro for a given culture medium. When the amount of unlabeled L-leucine was brought to the same concentration (105 mg/l) in all media, no significant difference was found among the radioactivities of samples from the different media. Thus, all these EBS-based media, with or without 10% fetal calf serum, appeared to support a constant and similar rate of ^{3}H-leucine uptake and incorporation into protein for up to 2 days in vitro. Data from eyecups incubated in RMPI 1640 without fetal calf serum (50 mg/l L-leucine) are shown in Figure 2. This medium, which contains all amino acids except alanine, was used in obtaining the results that follow.

Microscopical Autoradiography

Light microscopic autoradiography showed that ^{3}H-leucine was incorporated fairly evenly into cells of all retinal layers (Fig. 3). Electron microscopic autoradiography confirmed that the COSs were radiolabeled (Fig. 4), indicating that newly synthesized protein was

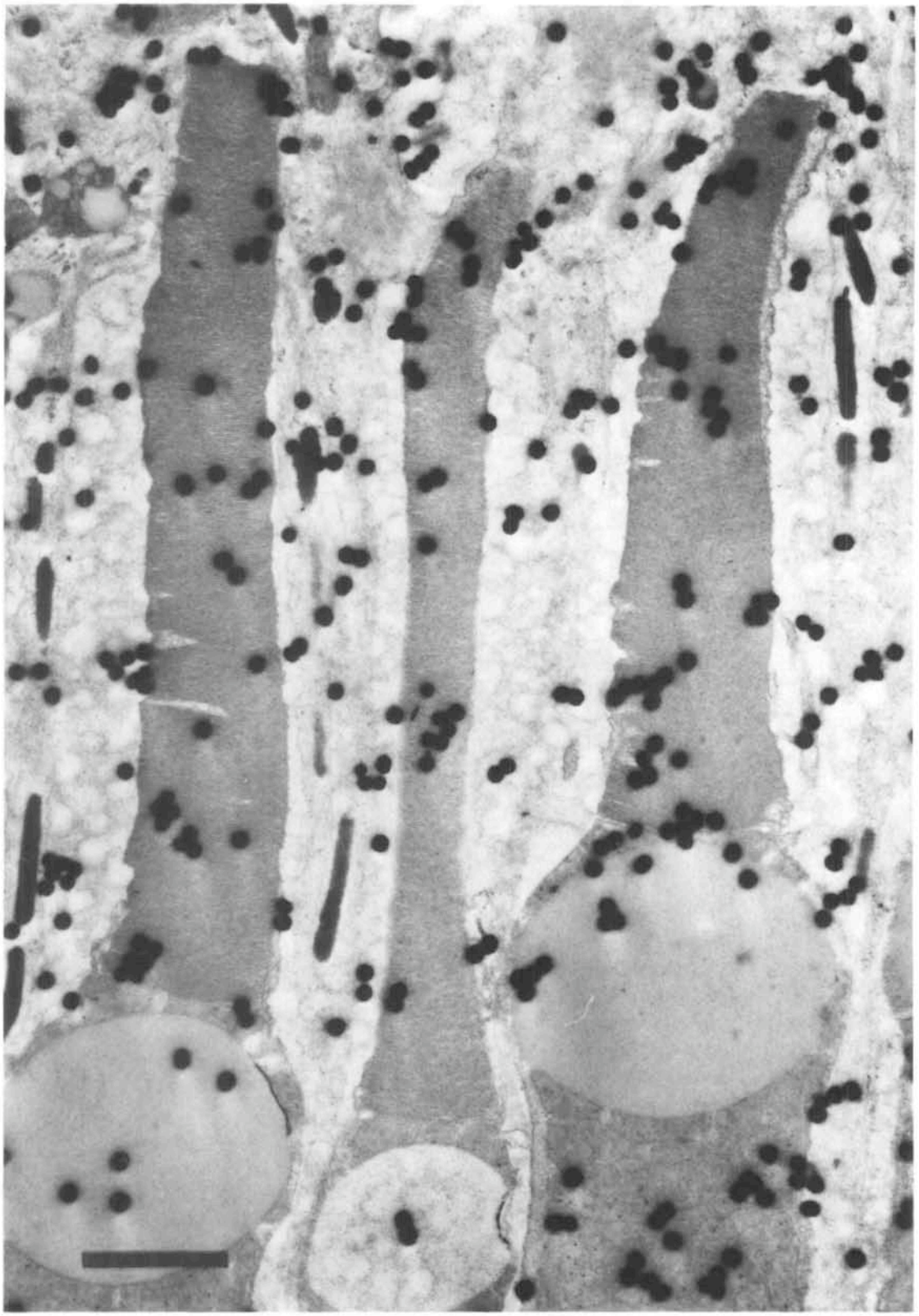

Fig. 4. Electron microscopic autoradiograph of cone outer segments in an eyecup maintained in culture for 6 hr (0.5 hr in labeled medium, followed by 5.5 hr in unlabeled medium). All three are from single cones about midway between the fovea and periphery. Scale bar = 2 μm.

incorporated into the COSs, probably as a result of the synthesis and assembly of new disc membrane.

Morphology

Deterioration of morphology was not evident until the second day in vitro. At this time, the ganglion cells appeared somewhat vacuolated (e.g., Fig. 3), and the inner limiting membrane was less well-defined.

During dissection, considerable care was required to avoid detaching the retinae from the RPE or disrupting the fragile connections between cone inner and outer segments. On the occasions when retinae were detached—either with the COSs intact or broken off—the inner retinal cells showed considerable vacuolation and degeneration in the region of the damage within 1 day in vitro.

Gel Electrophoresis and Fluorography

The distribution of radiolabel as a function of molecular weight of retinal proteins was examined by SDS-PAGE and fluorography. In Coomassie blue-stained gels there was no discernible difference in the distribution of total protein among the 2, 10, 16, and 50 hr samples. From fluorography of these gels, it was evident that there was also no difference in the distribution of labeled proteins which had been synthesized during the last 2 hr of culture (i.e., the period of exposure to ^{3}H-leucine) (Fig. 5).

Cyclic Nucleotide Levels

Retinal cyclic AMP and cyclic GMP levels remained constant from 2–50 hr in vitro (Fig. 6) and were similar to those found in vivo.[13]

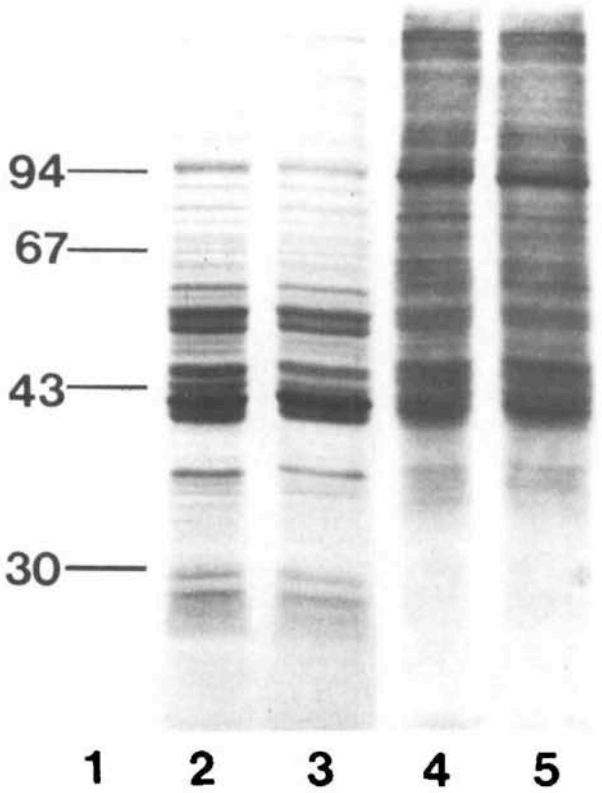

Fig. 5. SDS-PAGE patterns of Coomassie blue-stained proteins and corresponding fluorograms of retinal homogenates. Lane 1, molecular weights (kD) determined from the following standards (Pharmacia; Uppsala, Sweden): phosphorylase B (94 kD); bovine serum albumin (67 kD); ovalbumin (43 kD); carbonic anhydrase (30 kD). Lanes 2 and 3, Coomassie blue-stained proteins after 2 hr and 50 hr in vitro, respectively (total of 80 μg protein in each). Lanes 4 and 5, fluorograms of lanes 2 and 3, respectively.

Discussion

Retinal Organization

The retina of *Sceloporus* contains only cone photoreceptors. Nevertheless, it is characterized by regional specializations. In particular, the foveal region is clearly designed to optimize visual acuity. Underlying the fovea, the cones are packed as densely as the finest of all—practical and theoretical—photoreceptor mosaics[14,15]; if they were any closer together, excessive crosstalk between neighboring cones would degrade resolution.[14] In addition, it is likely that one function of the deep fovea is to magnify the retinal image in this region. This function has been proposed for similar foveae in birds of prey[16] and jumping spiders.[17] In essence, the base of the deep fovea acts as a negative lens, which, together with the cornea and lens, provides a telephoto lens system.[16,17]

Maintenance In Vitro

Our tests with different EBS-based culture media showed that overall ^{3}H-leucine uptake and incorporation into retinae was sustained for up to 2 days. The particular type of medium, or whether or not it was supplemented with 10% fetal calf serum, made no difference, so that none of the media appeared to lack any essential component for this term of culture. In subsequent tests, we found that the viability of this pure-cone retina was maintained during 1–2 days of eyecup culture in RMPI 1640, according to the following parameters: amino acid uptake and incorporation into all retinal layers and major proteins, morphology, and cyclic AMP and cyclic GMP content.

Morphology appeared to be most sensitive to deterioration with time in vitro; minor changes were found after 1 day in vitro. The ganglion cell layer was the first

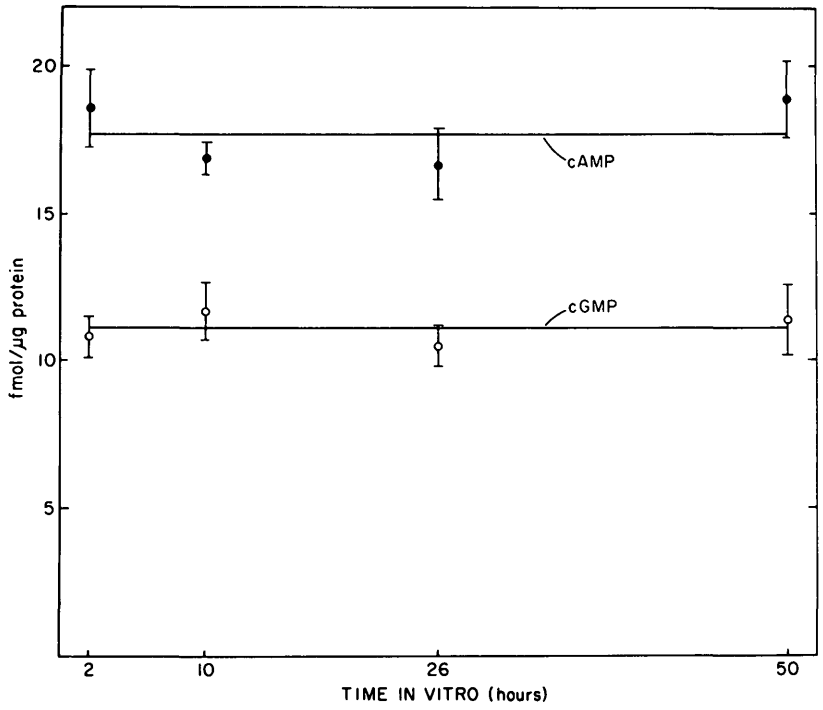

Fig. 6. Retinal cyclic AMP and cyclic GMP levels (fmol/μg protein) as a function of time eyecups were maintained in vitro. Data were aggregated from three separate experiments, with each point representing the mean (±1 SEM) of six to nine samples. Each sample was assayed at least twice.

region to deteriorate, perhaps because the optic nerve was cut.

The similarity of fluorographs of different samples suggests that synthesis of the same types of at least the major proteins is continued throughout 2 days of culture.

That cyclic nucleotide levels remained constant indicates that retinal cells did not become anoxic or unduly affected by ischemia. In retinae[18,19] and other neuronal tissue,[20] ischemia and anoxia alter cyclic GMP and especially cyclic AMP levels. Mitzel et al[19] found that detached mice retinae (i.e., without their RPE) underwent transient changes in cyclic AMP and cyclic GMP levels during incubation in EBS. However, these mouse retinae did not appear to have fared well in vitro, for after 60 min they exhibited pronounced morphological deterioration. The aim of the present study was to establish a basis for culture of lizard eyecups for periods significantly longer than 60 min. Thus, our first sample time was 120 min. It is possible that retinal cyclic nucleotide levels might have temporarily fluctuated during the first hour of our incubations. But, if such fluctuations did occur, we found that (1) they did not interfere with amino acid uptake or protein synthesis; and (2) by 2 hr, normal levels had been restored—with morphology intact. We have also found (unpublished observations) that the form of the electroretinogram response to a flash of light was comparable between eyecups that had been kept in vitro for 1 day and eyecups that had been freshly dissected.

In conclusion, the present findings suggest that incubation of lizard eyecups in RMPI 1640 (and probably other EBS-based media) is a useful preparation for short-term (ca 1–2 days) pharmacological studies of a pure-cone retina. Historically, advances in studies of cone photoreceptors have lagged far behind those of rods. In part, this difference is due to the absence of adequate experimental systems for studying cones in isolation from rods. The introduction of the pure-cone in vitro preparation, described here, should prove valuable in developing a better understanding of cone photoreceptors.

Key words: retina, cone photoreceptors, in vitro, protein synthesis, cyclic nucleotides

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